Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.


Supplemental Information

# A Chloromethyl-triazole Fluorescent Chemosensor for $O^6$-Methylguanine DNA Methyltransferase

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## 1. General Information

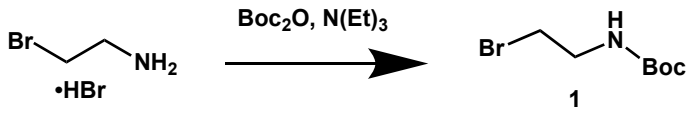
All reagents and solvents were purchased from commercial suppliers and used without further purification. Reactions were monitored by thin layer chromatography (TLC) using silica gel 60 $F_{254}$ aluminum sheets and visualized under UV-C (254 nm) or UV-A (365 nm) light. Flash column chromatography was performed with Silicycle® Siliaflash® P60, 40-63 μM silica gel.

The $^{1}H$ and $^{13}C$ NMR spectra were recorded using a Bruker AV-III-400 (at an operating frequency of 400 MHz for $^{1}H$ and 100 MHz for $^{13}C$ NMR) and a 500 MHz Agilent DD2 NMR spectrometer (at an operating frequency of 500 MHz for $^{1}H$ and 125.7 MHz for $^{13}C$ NMR) at 298 K using deuterated solvents with tetramethylsilane (TMS) as internal standard. Chemical shifts were reported in parts per million (ppm) and coupling constants (J values) are given in Hz. Splitting patterns are indicated as follows: s, singlet; d, doublet; t, triplet; q, quartet; p, pentet; m, multiplet. HRMS were acquired in the AIMS Mass Spectrometry Laboratory at the Department of Chemistry, University of Toronto, and low-resolution mass spectra were acquired using a Waters Quattro Ultima LC-MS with electrospray ionization (ESI). UV-VIS absorption spectra were recorded with a 1.0 cm path length cuvette on a Shimadzu UV-1800 UV-VIS spectrophotometer. The fluorescence experiments were performed with a Shimadzu RF-6000 spectrophotometer with a 1.0 cm path length cuvette. Fluorescence images and of the gels were taken with Invitrogen iBright ™ 1500 or BioRad Gel Doc XR+ imager.

## 2. Experimental Procedures and Characterization Data

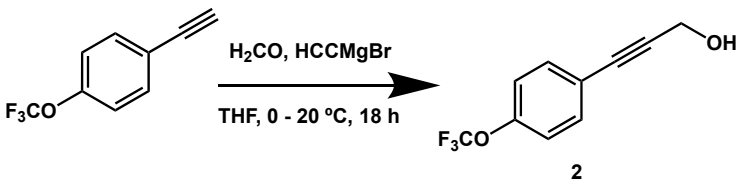
### 2.1. Procedures to Synthesize AACW-CCVJ

**Synthesis of 1. N-Boc-2-bromoethylamine**




Synthesis of **1** was carried out as previously described in the literature, with some modifications.[1] In brief, 533 mg $Boc_2O$ (2.44 mmol) was dissolved in 13 mL of ice cold DCM at 0 ℃, then 556 mg 2-bromoethylamine hydrobromide (2.71 mmol) was added in one portion. 508 µL triethylamine (3.66 mmol) was then added dropwise over 10 minutes at 0 ℃, then the reaction was slowly allowed to warm up to RT and stirred for 18 hours in total. The reaction mixture was washed with water 2x and brine 2x, then the organic layer was dried with sodium sulphate, filtered, concentrated by rotovap, and the product was purified by column chromatography (1:1 DCM:Hexanes → DCM) to obtain a slightly yellow oil (55%). $^1$H NMR (400 MHz, DMSO-$d_6$): δ 7.08 (t, $J$ = 5.9 Hz, 1H), 3.42 (t, $J$ = 6.6 Hz, 2H), 3.29 (q, $J$ = 6.3 Hz, 2H), 1.38 (s, 9H). $^{13}$C NMR (100 MHz, DMSO-$d_6$): δ 155.93, 78.51, 42.40, 32.81, 28.62. HRMS (ESI): m/z calc. for $C_7H_{14}BrNO_2$ $[M+H]^+$ 224.02, found 270.31 $[M+CH_2O_2+H]^+$.

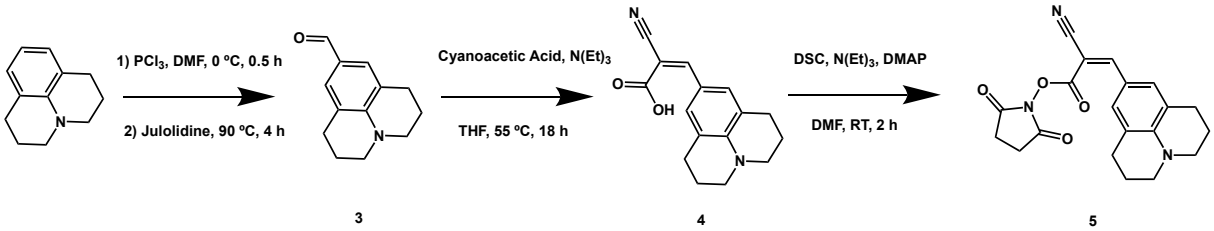
**Synthesis of 2. (3-(4-(trifluoromethoxy)phenyl)prop-2-yn-1-ol**



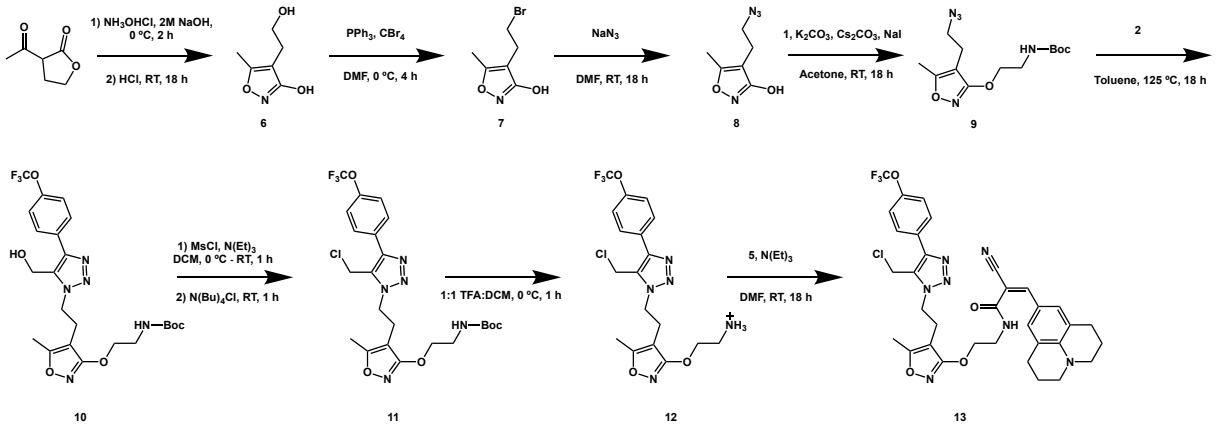

Synthesis of **2** was carried out as previously described in literature.[2] In brief, 365 mg 1-ethynyl-4-(trifluoromethoxy) benzene (1.96 mmol), was added to 4.7 mL of 0.5 M ethynyl magnesium bromide solution in THF (2.35 mmol), and stirred for 30 minutes at RT. 70.6 mg paraformaldehyde (2.5 mmol) was then added, and the solution was

stirred for 24 hours at RT. Next, the solution was quenched with saturated ammonium chloride, the aqueous layer was then extracted with EtOAc, the organic layers were pooled together and concentrated by rotary evaporation. The product was isolated by column chromatography (Hexanes → 1:1 Hex:EtOAc) as a viscous yellow oil (95%). $^{1}$H NMR (400 MHz, DMSO-$d_6$): δ 7.53 (d, $J$ = 8.7 Hz, 2H), 7.31 (d, $J$ = 7.9 Hz, 2H), 5.42 (t, $J$ = 5.9 Hz, 1H), 4.32 (d, $J$ = 5.9 Hz, 2H). $^{13}$C NMR (100 MHz, DMSO-$d_6$): δ 148.49 (d, $J$ = 1.8 Hz), 133.67, 122.23, 121.58, 120.42 (q, $J$ = 256.9 Hz), 91.29, 82.60, 49.84.

**Synthesis of 5. (CCVJ-NHS)**




Synthesis of **3** and **4** (9-(2-carboxy-2-cyanovinyl)julolidine (CCVJ)) were carried out as previously described in literature.[3] 10 mg CCVJ (0.037mmol) and 20 mg N,N′-Disuccinimidyl carbonate (0.078 mmol) were added to 2 mL DMF. 28 µL DIPEA and ~0.1 mg DMAP were then added, and the solution was stirred at RT for 1 h. The solvent was removed by speedvac, and the product was separated by column chromatography (1:1 Hex:EtOAc to EtOAc), to yield an orange solid (90%). $^{1}$H NMR (500 MHz, DMSO-$d_6$): δ 7.96 (s, 1H), 7.62 (s, 2H), 3.41 (t, $J$ = 5.8 Hz, 4H), 2.84 (s, 4H), 2.68 (t, $J$ = 6.1 Hz, 4H), 1.88 (p, $J$ = 5.8 Hz, 4H). $^{13}$C NMR (400 MHz, DMSO-$d_6$): δ 170.35, 160.96, 149.71, 121.20, 117.60, 117.16, 80.58, 49.89, 26.88, 25.47, 25.23, 20.24. HRMS (ESI): m/z calc. for $C_{20}H_{19}N_3O_4$ $[M+H]^+$ 366.14, found 366.14 $[M+H]^+$.




**Scheme S1**. Scheme for the synthesis of AACW-CCVJ.

**Synthesis of 6. (4-(2-hydroxyethyl)-5-methylisoxazol-3-ol)**

Synthesis of **6** was carried out as previously described in literature.[4] In brief, 1.1 g of hydroxylammonium chloride (15.8 mmol) was dissolved in 7.8 mL of 2 M NaOH, to achieve a solution of pH ~10, then cooled to 0 - 5 ℃ on ice. While stirring vigorously, 2 g of $\alpha$-acetylbutyrolactone (15.6 mmol) was slowly added over 30 minutes and 2 M NaOH was simultaneously added to maintain pH ~10 (monitored by pH paper). An additional ~7.8 mL 2 M NaOH were added. Stirring was continued for another 30 minutes, then the mixture was poured into 11.7 mL of ice cold concentrated HCl and allowed to sit overnight at RT. The mixture was extracted 3 times with DCM, then upon cooling the DCM overnight, the product was obtained as white crystals (24%). $^{1}$H NMR (400 MHz, DMSO-$d_6$): δ 11.01 (s, 1H), 4.77 (s, 1H), 3.45 (t, $J$ = 6.9 Hz, 2H), 2.34 (t, $J$ = 6.9 Hz, 2H), 2.19 (s, 3H). $^{13}$C NMR (100 MHz, DMSO-$d_6$): δ 170.12, 166.36, 102.61, 60.40, 25.08, 11.77. HRMS (ESI): m/z calc. for $C_6H_9NO_3$ $[M+H]^+$ 144.06, found 144.07 $[M+H]^+$.

**Synthesis of 7. (4-(2-bromoethyl)-5-methylisoxazol-3-ol)**

1.1 g triphenylphosphine (4.2 mmol) and 500 mg compound **6** (3.5 mmol) were added to 20 mL dry DMF at 0 ℃. 1.4 g $CBr_4$ (4.2 mmol) was dissolved in 4 mL dry DMF separately then added dropwise to the first mixture and stirred at 0 ℃ for 4 hours. The resulting solution was added to DCM, and extracted 5 times with water. The organic layer was then dried with sodium sulphate, filtered, and concentrated using a rotary evaporator. The product was purified by column chromatography (1:1 Hex:EtOAc to EtOAc) to obtain an off-white powder (63%). $^1$H NMR (400 MHz, DMSO-$d_6$): δ 11.28 (s, 1H), 3.56 (t, $J$ = 7.0 Hz, 2H), 2.78 (t, $J$ = 7.0 Hz, 2H), 2.24 (s, 3H). $^{13}$C NMR (100 MHz, DMSO-$d_6$): δ 169.81, 166.96, 102.82, 33.22, 25.04, 11.86. HRMS (ESI): m/z calc. for $C_6H_8BrNO_2$ $[M+H]^+$ 205.97, found 205.98 $[M+H]^+$.

**Synthesis of 8. (4-(2-azidoethyl)-5-methylisoxazol-3-ol)**

380 mg compound **7** (1.84 mmol) and 190 mg sodium azide (2.92 mmol) were added to 5.7 mL of dry DMF, then stirred at 80 ℃ for 18 h. The mixture was then taken up in EtOAc and washed 3 times with brine. The organic layer was then dried with sodium sulphate, filtered, and dried by rotovap. The product was purified by column chromatography (1:1 Hex:EtOAc to EtOAc) to obtain a yellow solid (65%). $^1$H NMR (400 MHz, $CDCl_3$): δ 11.63 (s, 1H), 3.47 (t, $J$ = 6.8 Hz, 2H), 2.56 (t, $J$ = 6.8 Hz, 2H), 2.30 (s, 3H).$^{13}$C NMR (100 MHz, $CDCl_3$): δ 169.51, 166.43, 101.00, 49.33, 20.78, 11.20. HRMS (ESI): m/z calc. for $C_6H_8N_4O_2$ $[2M–N_3]$ 294.12, found 294.12 $[2M–N_3]$.

**Synthesis of 9. (tert-butyl (2-((4-(2-azidoethyl)-5-methylisoxazol-3-yl)oxy)ethyl)carbamate)**

200 mg of compound **8** (1.2 mmol), 320 mg of $Cs_2CO_3$ (1 mmol) and 320 mg of $K_2CO_3$ (2.3 mmol) were added to 4 mL of dry acetone, then stirred vigorously at RT for 15 minutes. 275 mg of compound **1** (1.2 mmol) and 108 mg of sodium iodide (0.7 mmol) were then added, and the solution was stirred vigorously at RT for 18 h. The mixture was then concentrated by rotovap, and the product was purified by column chromatography (EtOAc) to obtain the product as a viscous yellow oil (29%). $^1$H NMR (400 MHz, DMSO-$d_6$): δ 6.89 (t, *J* = 6.0 Hz, 1H), 3.77 (t, *J* = 6.3 Hz, 2H), 3.43 (t, *J* = 6.9 Hz, 2H), 3.15 (q, *J* = 6.2 Hz, 2H), 2.43 (t, *J* = 6.9 Hz, 2H), 2.22 (s, 3H), 1.36 (s, 9H). $^{13}$C NMR (100 MHz, DMSO-$d_6$): δ 167.94, 166.60, 155.42, 104.77, 77.82, 48.82, 45.64, 37.58, 28.16, 21.10, 11.42. HRMS (ESI): m/z calc. for $C_{13}H_{21}N_5O_4$ [M–N+H] 298.16, found 298.35 [M–N+H].

**Synthesis of 10. (tert-butyl (2-((4-(2-(5-(hydroxymethyl)-4-(4-(trifluoromethoxy)phenyl)-1H-1,2,3-triazol-1-yl)ethyl)-5-methylisoxazol-3-yl)oxy)ethyl)carbamate)**

73 mg compound **9** (0.23 mmol) and 95 mg compound **2** (0.46 mmol) were dissolved in 0.5 mL dry toluene then added to a pressure vessel. The mixture was stirred at 125 ℃ for 18 h. The mixture was then concentrated by rotovap and the product was separated by column chromatography (1:4 → 4:1 acetone:hexanes) as a thick, yellow oil (45%). The cycloaddition reaction yielded two isomers at the newly formed triazole group – a 1,4-triazole and a 3,4-triazole isomer. The desired 1,4-triazole isomer was identified using $^1$H-$^{13}$C HMBC. (see Figure S16 and S17) $^1$H NMR (400 MHz, DMSO-$d_6$): δ 7.87 (d, *J* = 8.7 Hz, 2H), 7.49 (d, *J* = 8.3 Hz, 2H), 6.90 (t, *J* = 5.9 Hz, 1H), 5.70

(t, *J* = 5.2 Hz, 1H), 4.65 (d, *J* = 5.2 Hz, 2H), 4.54 (t, *J* = 6.8 Hz, 2H), 3.78 (t, *J* = 6.3 Hz, 2H), 3.14 (q, *J* = 6.2 Hz, 2H), 2.78 (t, *J* = 6.8 Hz, 2H), 1.89 (s, 3H), 1.34 (s, 9H). $^{13}$C NMR (100 MHz, DMSO-$d_6$): δ 168.05, 166.76, 155.42, 147.92, 143.17, 133.41, 130.50, 128.89, 121.45, 119.68 (q, *J* = 251.0), 104.30, 77.83, 50.81, 46.09, 45.76, 37.58, 28.15, 22.39, 10.85. HRMS (ESI): m/z calc. for $C_{23}H_{28}F_3N_5O_6$ $[M+H]^+$ 528.21, found 528.21 $[M+H]^+$.

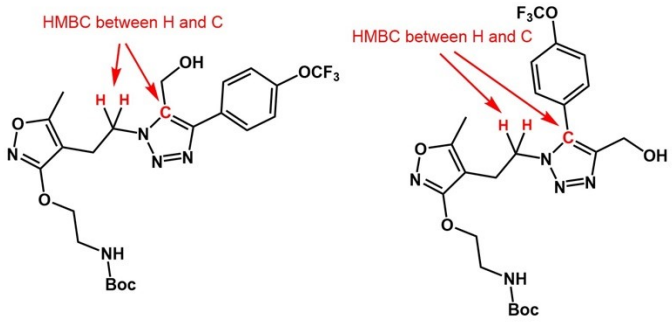



**Figure S1.** Structure of both isomers of compound **11** are shown (1,4-triazole left, 3,4-triazole right), with arrows indicating HMBC coupling between C and H for each isomer, which was used to distinguish them.

**Synthesis of 11. (tert-butyl (2-((4-(2-(5-(chloromethyl)-4-(4-(trifluoromethoxy)phenyl)-1H-1,2,3-triazol-1-yl)ethyl)-5-methylisoxazol-3-yl)oxy)ethyl)carbamate)**

25 mg of compound **10** (0.047 mmol) was dissolved in 1.5 mL of DCM at 0 ℃. 5.5 µL methanesulfonyl chloride and 10 µL triethylamine were then added and the mixture was stirred for 30 minutes on ice, followed by 30 minutes at RT. 20 mg tetrabutylammonium chloride was then added and the mixture was stirred at RT for 1 h. The mixture was then concentrated by rotovap, and the product was separated by column chromatography (1:1 Hex:EtOAc to EtOAc) to obtain the product as a thick yellow oil (91%). $^1$H NMR (400 MHz, DMSO-$d_6$): δ 7.91-7.83 (m, 2H), 7.54 (d, *J* = 8.3 Hz, 2H), 6.90 (t, *J* = 6.0 Hz, 1H), 5.12 (s, 2H), 4.59 (t, *J* = 6.9 Hz, 2H), 3.79 (t, *J* = 6.3

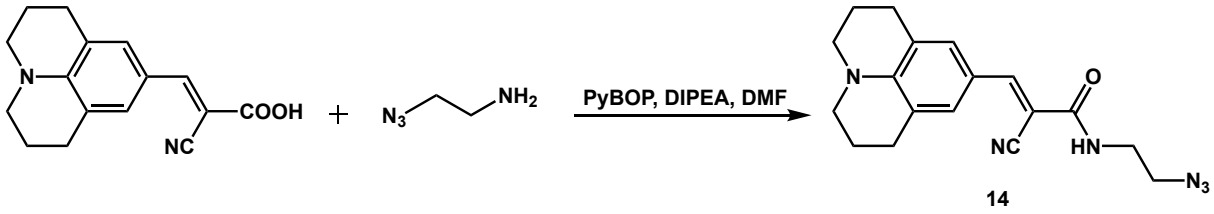
Hz, 2H), 3.15 (q, *J* = 6.1 Hz, 2H), 2.82 (t, *J* = 6.9 Hz, 2H), 1.95 (s, 3H), 1.35 (s, 9H). $^{13}$C NMR (100 MHz, DMSO-$d_6$): δ 168.35, 167.11, 155.88, 148.71 (d, *J* = 1.9 Hz), 143.92, 130.54, 130.00 (d, *J* = 1.9 Hz), 129.26, 122.08, 120.53 (q, *J* = 256.6 Hz), 104.61, 78.27, 46.42, 46.17, 38.06, 33.19 (d, *J* = 6.8 Hz), 28.57, 22.73, 11.46. HRMS (ESI): m/z calc. for $C_{23}H_{27}ClF_3N_5O_5$ $[M+H]^+$ 546.17, found 546.17 $[M+H]^+$.

**Synthesis of 13. AACW-CCVJ**

200 µL of 1:2 TFA:DCM at 0 ℃ was added to 10 mg compound **11**, and the mixture was stirred on ice for 1 h. The solvent (and most of the TFA) was then removed by co-evaporation with ethyl ether, then the mixture was left on a speedvac overnight to remove the remaining TFA. Compound **12** was obtained as a black oil and was used in the next reaction without further purification. Compound **12** (5 mg) was then dissolved in 150 µM DMF, along with 4 mg compound **5**, and 2 µL DIPEA. The mixture was stirred at RT for 2 h, then DMF was removed by speedvac, and the product (compound **13**) was purified by C18-RP HPLC to obtain a dark orange solid (28%). $^1$H NMR (500 MHz, DMSO-$d_6$): δ 8.05 (t, *J* = 5.8 Hz, 1H), 7.86 (d, *J* = 8.3 Hz, 2H), 7.75 (s, 1H), 7.52 (d, *J* = 8.2 Hz, 2H), 7.38 (s, 2H), 5.08 (s, 2H), 4.55 (t, *J* = 7.0 Hz, 2H), 3.92 (t, *J* = 6.0 Hz, 2H), 3.28 (dd, *J* = 6.7, 4.8 Hz, 4H), 2.80 (t, *J* = 7.1 Hz, 2H), 2.64 (dd, *J* = 7.1, 5.4 Hz, 4H), 1.96 (s, 3H), 1.84 (p, *J* = 6.3 Hz, 4H). $^{13}$C NMR (100 MHz, DMSO-$d_6$): δ 168.22, 166.95, 162.64, 150.48, 148.27, 146.71, 143.45, 130.24, 130.12 (d, *J* = 4.4 Hz), 129.55, 128.84, 121.70, 120.43, 120.11 (q, *J* = 257.5 Hz), 118.47, 117.54, 104.23, 99.50, 94.21, 49.32, 46.02, 45.46, 37.44, 32.77, 27.07, 22.29, 20.63, 11.15. HRMS (ESI): m/z calc. for $C_{34}H_{33}ClF_3N_7O_4$ $[M+H]^+$ 696.22, found 696.23 $[M+H]^+$.

## 2.2. Procedures to Synthesize Chemosensors

### Synthesis of 14. CCVJ- 2-azidoethan-1-amine linker

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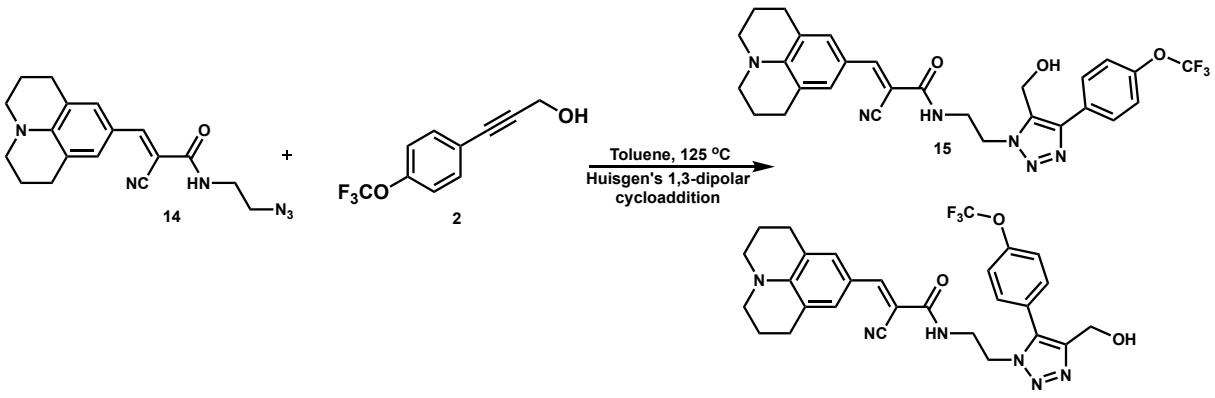
Synthesis of the 2-azidoethan-1-amine linker was carried out as previously described in the literature.[5] 150 mg CCVJ (0.56 mmol) and 30 mg 2-azidoethan-1-amine (0.36 mmol) were dissolved in 2 mL dry DMF. 370 mg PyBOP (0.72 mmol) and 310 µL DIPEA (1.78 mmol) were then added, and the solution was stirred at RT for 2 h. The reaction was diluted with 10 mL EtOAc, then quenched by 3 extractions with 10 mL saturated brine. After drying with sodium sulphate, the solvent was evaporated, and the product was separated by flash column chromatography (2:1 Hex:EtOAc to EtOAc) as a yellow solid (80%). $^{1}$H NMR (400 MHz, $CDCl_3$): δ 8.01 (s, 1H), 7.45 (s, 2H), 6.46 (s, 1H), 3.55 (dq, *J* = 17.8, 5.8 Hz, 4H), 3.31 (t, *J* = 5.8 Hz, 4H), 2.75 (t, *J* = 6.3 Hz, 4H), 1.96 (p, *J* = 6.2 Hz, 4H). $^{13}$C NMR (100 MHz, $CDCl_3$): δ 163.25, 151.03, 147.21, 130.76, 120.91, 118.94, 118.03, 94.61, 50.14, 49.81, 27.55, 21.11. HRMS (ESI): m/z calc. for $C_{18}H_{20}N_6O$ $[M+H]^+$ 337.17, found 337.16 $[M+H]^+$.

### Synthesis of 15. CCVJ- €-2-cyano-*N*-(2-(5-(hydroxymethyl)-4-(4-(trifluoromethoxy)phenyl)-1*H*-1,2,3-triazol-1-yl)ethyl)-3-(2,3,6,7-tetrahydro-1*H*,5*H*-pyrido[3,2,1-*XXXuinoline*lin-9-yl)acrylamide




100 mg compound **14** (0.3 mmol) and 100 mg compound **2** (0.46 mmol) were dissolved in 0.5 mL dry toluene then added to a pressure vessel. The mixture was stirred at 125 ℃ for 18 h. The mixture was then concentrated by rotary evaporation and the product was separated by column chromatography (3:1 Hex:EtOAc to EtOAc) as a thick yellow oil (60%). The cycloaddition reaction yielded two isomers at the newly formed triazole group – a 1,4-triazole and a 3,4-triazole isomer. The isomer was identified using $^{1}$H-$^{13}$C HMBC. (Figure S28) $^{1}$H NMR (400 MHz, DMSO-$d_6$): δ 8.19 (t, J = 5.8 Hz, 1H), 7.90 (d, J = 9.0 Hz, 2H), 7.80 (s, 1H), 7.49 (d, J = 9.0 Hz, 2H), 7.42 (s, 2H), 5.67 (t, J = 5.4 Hz, 1H), 4.70 (d, J = 5.4 Hz, 2H), 4.63-4.56 (m, 2H), 3.68 (q, J = 6.3 Hz, 2H), 3.30 (q, 4H), 2.67 (t, J = 6.2 Hz, 4H), 1.94-1.82 (m, 4H). $^{13}$C NMR (100 MHz, DMSO-$d_6$): δ 163.35, 151.13, 147.27, 143.44, 134.17, 131.04, 130.81, 129.29, 121.88, 120.92, 118.94, 118.00, 94.30, 51.32, 49.82, 27.54, 21.10.

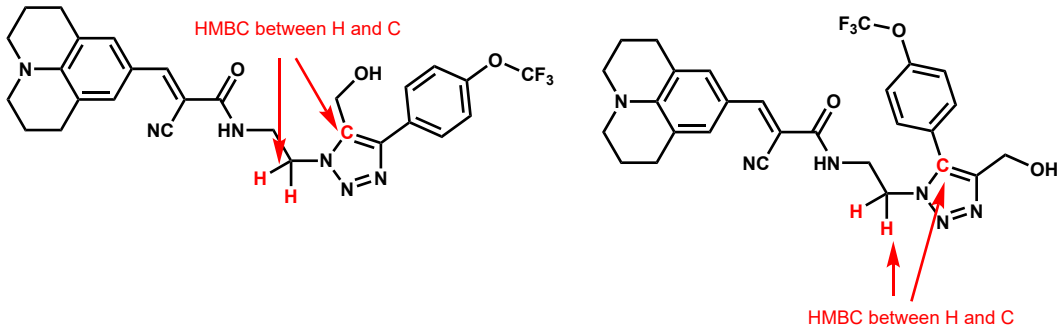
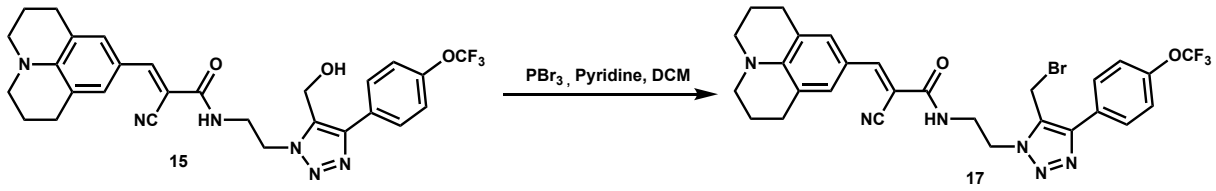


**Figure S2.** Structure of both isomers of compound **15** are shown (1,4-triazole left, 3,4-triazole right), with arrows indicating HMBC coupling between C and H for each isomer, which was used to distinguish them.

**Synthesis of 16. (*E*)-*N*-(2-(5-(chloromethyl)-4-(4-(trifluoromethoxy)phenyl)-1*H*-1,2,3-triazol-1-yl)ethyl)-2-cyano-3-(2,3,6,7-tetrahydro-1*H*,5*H*-pyrido[3,2,1-*ij*]quinolin-9-yl)acrylamide**

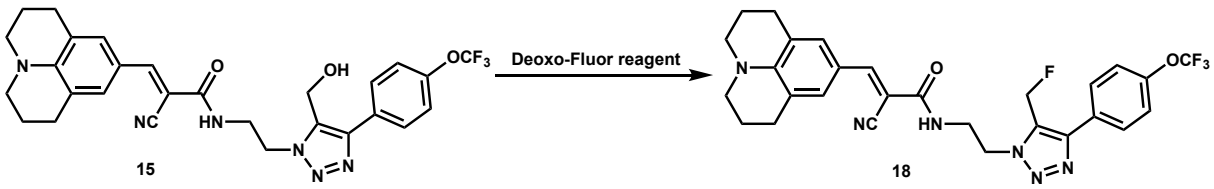

5.5 mg of compound **15** (0.01 mmol) was dissolved in 0.15 mL DCM at 0°C. 0.55 µL methanesulfonyl chloride (MsCl) (0.007 mmol) and 1 µL triethylamine (0.007 mmol) were then added. Note, 20x diluted stocks of MsCl and triethylamine were prepared with DCM to increase the accuracy of MsCl and triethylamine volumes added. The solution was stirred for 30 minutes on ice, followed by 30 minutes at RT. 2 mg tetrabutylammonium chloride (0.07 mmol) was then added and the mixture was stirred at RT for 1 h. The mixture was then concentrated by rotary evaporation. The product was purified by column chromatography (EtOAc) to obtain a pale-yellow powder (60%). $^1$H NMR (400 MHz, DMSO-$d_6$): δ 8.22 (t, J = 5.8 Hz, 1H), 7.94 – 7.88 (m, 2H), 7.82 (s, 1H), 7.55 (d, J = 8.3 Hz, 2H), 7.44 (s, 2H), 5.16 (s, 2H), 4.63 (t, J = 6.4 Hz, 2H), 3.73 (q, J = 6.1 Hz, 2H), 3.31 (s, 2H), 2.69 (t, J = 6.2 Hz, 4H), 1.92 – 1.85 (m, 4H). HRMS (ESI): m/z calc. for $C_{28}H_{26}ClF_3N_6O_2$ $[M+H]^+$ 571.18, found 571.23 $[M+H]^+$.

**Synthesis of 17. (*E*)-*N*-(2-(5-(bromomethyl)-4-(4-(trifluoromethoxy)phenyl)-1*H*-1,2,3-triazol-1-yl)ethyl)-2-cyano-3-(2,3,6,7-tetrahydro-1*H*,5*H*-pyrido[3,2,1-*ij*]quinolin-9-yl)acrylamide**



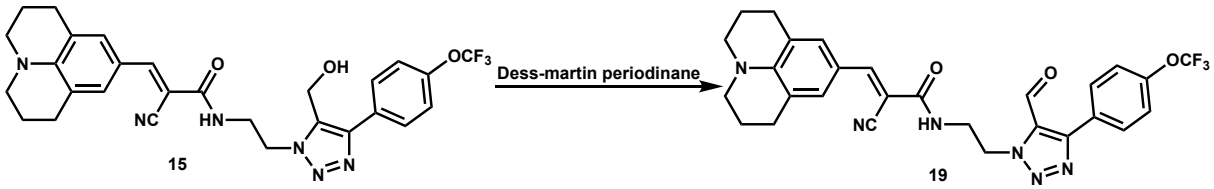

27 mg compound **15** (0.049 mmol) and 20 mg phosphorus tribromide (0.049 mmol) were added to 1 mL anhydrous DCM. 8 µL pyridine (0.098 mmol) was then added and the solution was stirred at RT for 2 h. The resulting solution was added to DCM, and extracted 3 times with saturated ammonium chloride. The organic layer was then dried with sodium sulphate, filtered, and concentrated by rotary evaporation. The product was purified by column chromatography (1:1 Hex:EtOAc to EtOAc) to obtain a pale-yellow powder (58%). $^{1}$H NMR (400 MHz, DMSO-$d_6$): δ 8.19 (t, J = 5.9 Hz, 1H), 7.90 (d, J = 8.9 Hz, 2H), 7.81 (s, 1H), 7.54 (d, J = 8.0 Hz, 2H), 7.42 (s, 2H), 5.03 (s, 2H), 4.58 (t, J = 6.4 Hz, 2H), 3.72 (q, J = 6.5 Hz, 2H), 3.33-3.27 (m, 4H), 2.67 (t, J = 6.3 Hz, 4H), 1.85 (p, J = 5.8 Hz, 4H). $^{13}$C NMR (500 MHz, DMSO-$d_6$): δ 162.88, 150.66, 146.81, 142.97, 133.70, 130.56, 130.34, 128.82, 128.70, 121.65, 121.43, 120.45, 118.48, 117.51, 93.79, 50.83, 49.34, 46.92, 27.07, 20.62. HRMS (ESI): m/z calc. for $C_{28}H_{26}BrF_3N_6O_2$ $[M+H]^+$ 615.13, found 615.20 $[M+H]^+$.

**Synthesis of 17. (*E*)-2-cyano-*N*-(2-(5-(fluoromethyl)-4-(4-(trifluoromethoxy)phenyl)-1*H*-1,2,3-triazol-1-yl)ethyl)-3-(2,3,6,7-tetrahydro-1*H*,5*H*-pyrido[3,2,1-*ij*]quinolin-9-yl)acrylamide**



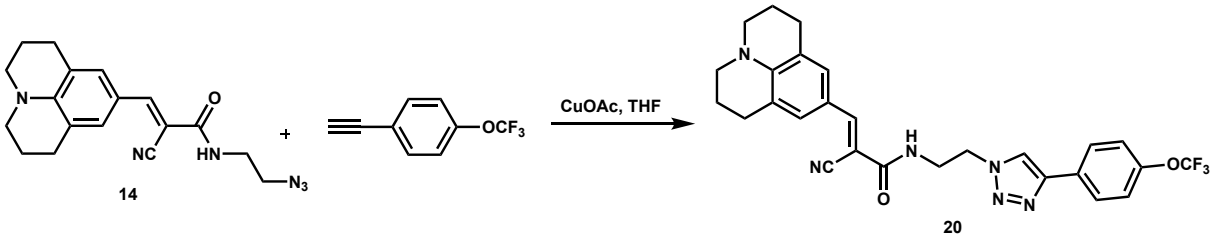

15 mg compound **14** (0.027 mmol) dried into 5 mL round-bottom flask and then dissolved in 300 µL anhydrous DCM. 17 µL Deoxo-fluor (0.046 mmol) was added to round-bottom flask dropwise. The solution instantly became red-orange and was stirred at RT for 2 h. The resulting solution was added to DCM, and extracted 3 times with water. The organic layer was then dried with sodium sulphate, filtered, and concentrated by rotary evaporation. The product was purified by column chromatography (2:1 Hex:EtOAc to EtOAc) to obtain a yellow solid (20 %). $^{1}$H NMR (400 MHz, DMSO-$d_6$): δ 8.19 (t, J = 5.9 Hz, 1H), 7.85 (d, J = 9.0 Hz, 2H), 7.80 (s, 1H), 7.54 (d, J = 7.9 Hz, 2H), 7.43 (s, 2H), 5.78 (s, 1H), 5.66 (s, 1H),4.66 (t, J = 6.1 Hz, 2H), 3.67 (q, J = 6.5 Hz, 2H), 2.69-2.65 (m, 4H), 1.89-1.84 (m, 4H). $^{13}$C NMR (500 MHz, DMSO-$d_6$): δ 163.34, 151.12, 148.81, 147.25, 145.59, 130.80, 129.40, 122.11, 120.90, 118.93, 117.98, 94.26, 69.75, 69.61, 58.49, 57.46, 57.12, 49.80, 27.53, 21.09. HRMS (ESI): m/z calc. for $C_{28}H_{26}F_4N_6O_2$ $[M+H]^+$ 555.21, found 555.19 $[M+H]^+$.

**Synthesis of 18. €-2-cyano-*N*-(2-(5-formyl-4-(4-(trifluoromethoxy)phenyl)-1*H*-1,2,3-triazol-1-yl)ethyl)-3-(2,3,6,7-tetrahydro-1*H*,5*H*-pyrido[3,2,1-*XXXuinoline*lin-9-yl)acrylamide**




85 mg compound **15** (0.15 mmol) was dissolved in 1.5 mL ice cold DCM. Then, 85 mg Dess–Martin periodinane (0.2 mmol) was added to the reaction mixture. The reaction was stirred at RT for half an hour on ice. The resulting solution was concentrated by rotary evaporation. The product was purified by column chromatography (1:1 Hex:EtOAc) to obtain a yellow powder (35 %). $^{1}$H NMR (400 MHz, DMSO-$d_6$): δ 10.09 (s, 1H), 8.13 (t, J = 5.9 Hz, 1H), 8.03 (d, J = 8.9 Hz, 2H), 7.75 (s, 1H), 7.55 (d, J = 8.5 Hz, 2H), 7.41 (s, 1H), 4.88 (t, J = 5.8 Hz, 2H), 3.70 (q, J = 6.0 Hz, 2H), 3.30 (m, 4H), 2.68 (t, J = 6.2 Hz, 4H), 1.88 (p, J = 6.6 Hz, 4H). $^{13}$C NMR (500 MHz, DMSO-$d_6$): δ 181.30, 163.28, 151.10, 149.82, 149.53, 147.26, 131.12, 130.79, 130.64, 121.85, 120.90, 117.94, 94.13, 49.99, 49.80, 27.52, 21.08. HRMS (ESI): m/z calc. for $C_{28}H_{25}F_3N_6O_3$ $[M+H]^+$ 551.19, found 551.20 $[M+H]^+$.

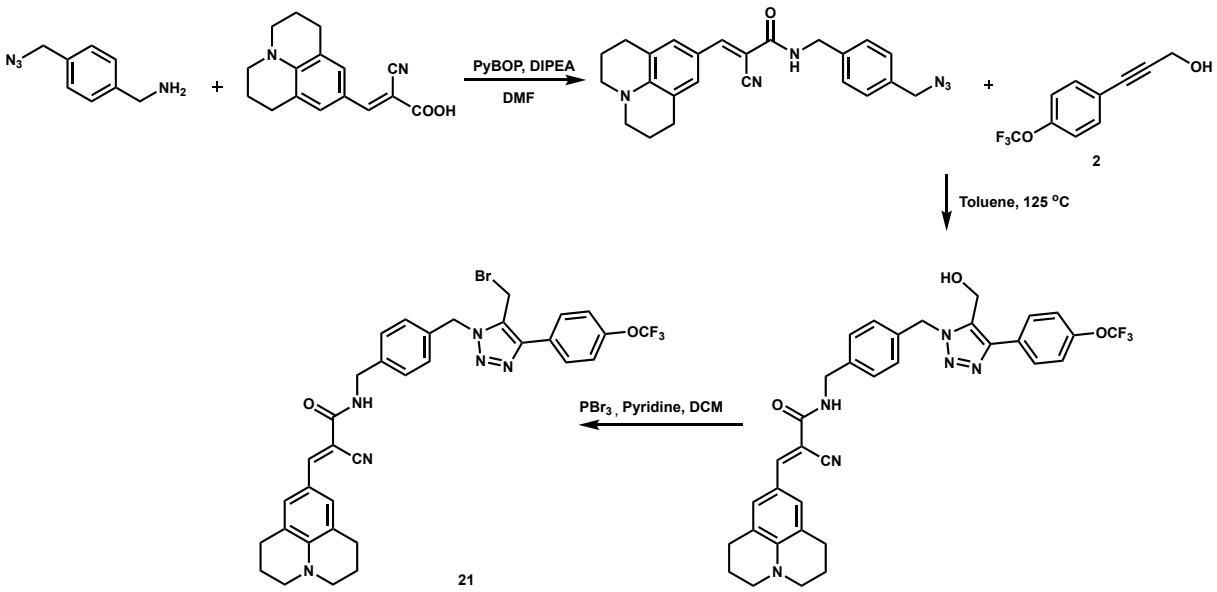
**Synthesis of 19. (*E*)-2-cyano-3-(2,3,6,7-tetrahydro-1*H*,5*H*-pyrido[3,2,1-*ij*]quinolin-9-yl)-*N*-(2-(4-(4-(trifluoromethoxy)phenyl)-1*H*-1,2,3-triazol-1-yl)ethyl)acrylamide**




18 mg compound **14** (0.05 mmol) was dried into 5 mL round-bottom flask. Copper(I) acetate (2% eq) and sodium ascorbate (4% eq) were added to flask. 300 µL of dry THF then added to dissolved reagents. 200 µL of THF+10 µL of acetylene were mixed, then added to the reaction mixture. The reaction was stirred at RT for 3 hours. The resulting solution was concentrated over rotovap. The product was purified by column

chromatography (1:1 Hex:EtoAc) to obtain a pale-yellow powder (67%). $^{1}$H NMR (400 MHz, DMSO-$d_6$): δ 8.66 (s, 1H), 8.21 (t, J = 5.5 Hz, 1H), 7.99 (d, J = 8.8 Hz, 2H), 7.79 (s, 1H), 7.48 (d, J = 8.1 Hz, 2H), 7.41 (s, 1H), 4.59 (t, J = 5.8 Hz, 2H), 3.70 (q, J = 6.5 Hz, 2H), 3.33 (m, 4H), 2.68 (t, J = 6.2 Hz, 4H), 1.92-1.86 (m, 4H). $^{13}$C NMR (500 MHz, DMSO-$d_6$): δ 163.36, 151.03, 148.20, 147.22, 145.42, 130.76, 127.34, 122.77, 122.06, 120.89, 118.92, 117.96, 94.49, 49.78, 49.60, 49.39, 27.52, 21.08. HRMS (ESI): m/z calc. for $C_{27}H_{25}F_3N_6O_2$ $[M+H]^+$ 523.20, found 523.19 $[M+H]^+$.

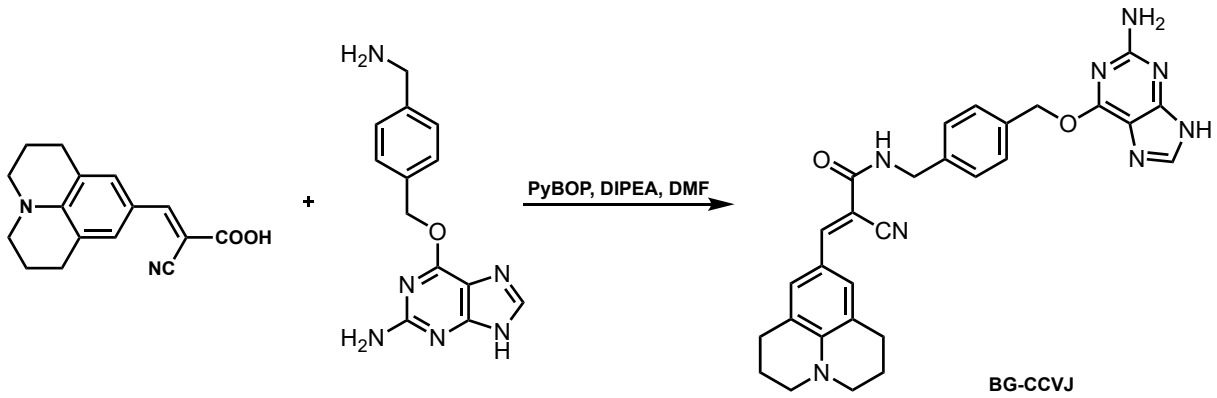
**Synthesis of 20. ((*E*)-*N*-(4-((5-(bromomethyl)-4-(4-(trifluoromethoxy)phenyl)-1*H*-1,2,3-triazol-1-yl)methyl)benzyl)-2-cyano-3-(2,3,6,7-tetrahydro-1*H*,5*H*-pyrido[3,2,1-*ij*]quinolin-9-yl)acrylamide**




Synthesis of the 3-(aminomethyl)benzylazide linker was carried out as previously described in literature.[6] 78 mg CCVJ (0.29 mmol) and 30 mg 3-(aminomethyl)benzylazide (0.19 mmol) were dissolved in 1 mL dry DMF. 198 mg PyBOP (0.38 mmol) and 165 µL DIPEA (0.95 mmol) were then added, and the solution was stirred at RT for 2 h. The reaction was diluted with 5 mL DCM, then quenched by 3 extractions with 5 mL distilled water. After drying with sodium sulphate, the solvent

was evaporated, then the product was purified by column chromatography (1:1 Hex:EtOAc) to obtain an yellow solid (51%). The obtained compound (40 mg, 0.097 mmol) and 33 mg compound **2** (0.15 mmol) were dissolved in 0.5 mL dry toluene then added to a pressure vessel. The mixture was stirred at 125 ℃ for 18 h. The mixture was then concentrated by rotovap and the product was separated by column chromatography (1:1 Hex:EtOAc to EtOAc) as a thick yellow oil (26%). 16 mg of the obtained compound (0.026 mmol) and 4 µL phosphorus tribromide (0.038 mmol) were added to 400 µL anhydrous DCM. 4 µL pyridine (0.052 mmol) was then added and the solution was stirred at RT for 2 hours. The resulting solution was added to DCM, and extracted 3 times with saturated ammonium chloride. The organic layer was then dried with sodium sulphate, filtered, and concentrated by rotary evaporation. The product was purified by column chromatography (2:1 Hex:EtOAc to EtOAc) to obtain a pale-yellow powder (50%). $^{1}$H NMR (500 MHz, DMSO-$d_6$): δ 8.19 (t, J = 5.9 Hz, 1H), 7.90 (d, J = 8.9 Hz, 2H), 7.81 (s, 1H), 7.54 (d, J = 8.0 Hz, 2H), 7.42 (s, 2H), 7.39-7.24 m (4H), 5.67 (s, 2H), 4.65 (s, 2H), 4.35 (d, J = 5.9 Hz, 2H), 3.30-3.27 (m, 4H), 2.67-2.64 (m, 4H), 1.87-1.83 (m, 4H). HRMS (ESI): m/z calc. for $C_{34}H_{30}BrF_3N_6O_2$ $[M+H]^+$ 691.16, found 691.21 $[M+H]^+$.

**Synthesis of BG-CCVJ**




CCVJ[3] and BG-$NH_2$[7] and BG-CCVJ[8] were prepared as previously reported. $^1$H NMR (400 MHz, DMSO-$d_6$): δ 8.57 (t, J = 6.0 Hz, 1H), 7.86(s, 1H), 7.84 (s, 1H), 7.46 (d, J = 8.0 Hz, 2H), 7.43(s, 2H), 7.31 (d, J = 8.2 Hz, 2H), 6.31 (s, 2H), 5.46 (s, 2H), 4.38 (d, J = 5.9 Hz, 2H), 3.29 (t, J = 5.8 Hz, 7H), 2.66 (t, J = 6.3 Hz, 4H), 1.86 (p, J = 6.5 Hz, 4H). $^{13}$C NMR (100 MHz, DMSO-$d_6$): δ 163.00, 160.08, 150.87, 147.13, 139.81, 135.74, 130.72, 128.96, 127.90, 120.88, 119.02, 118.13, 95.01, 67.04, 49.79, 46.35, 43.35, 27.55, 26.42, 21.13.

## 2.3. Characterization Data

### 2.3.1. NMR Spectra

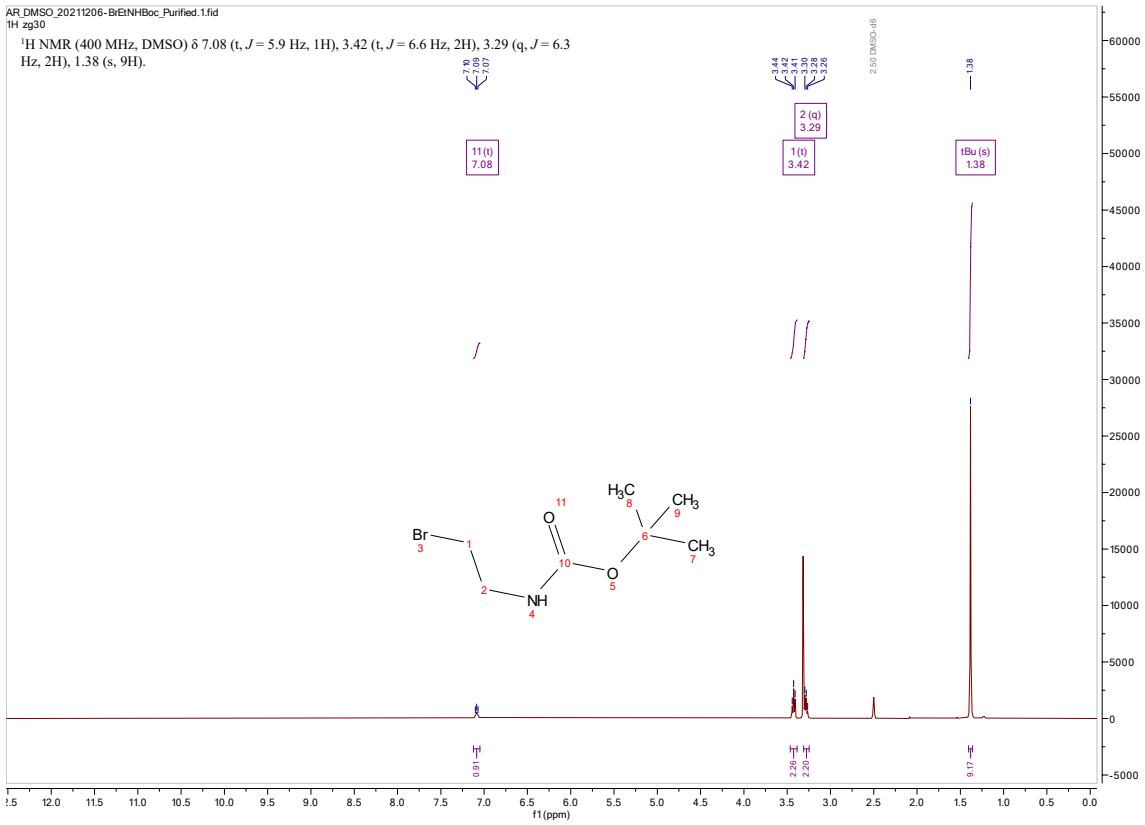



**Figure S3.** $^{1}$H NMR Spectrum of Compound **1**

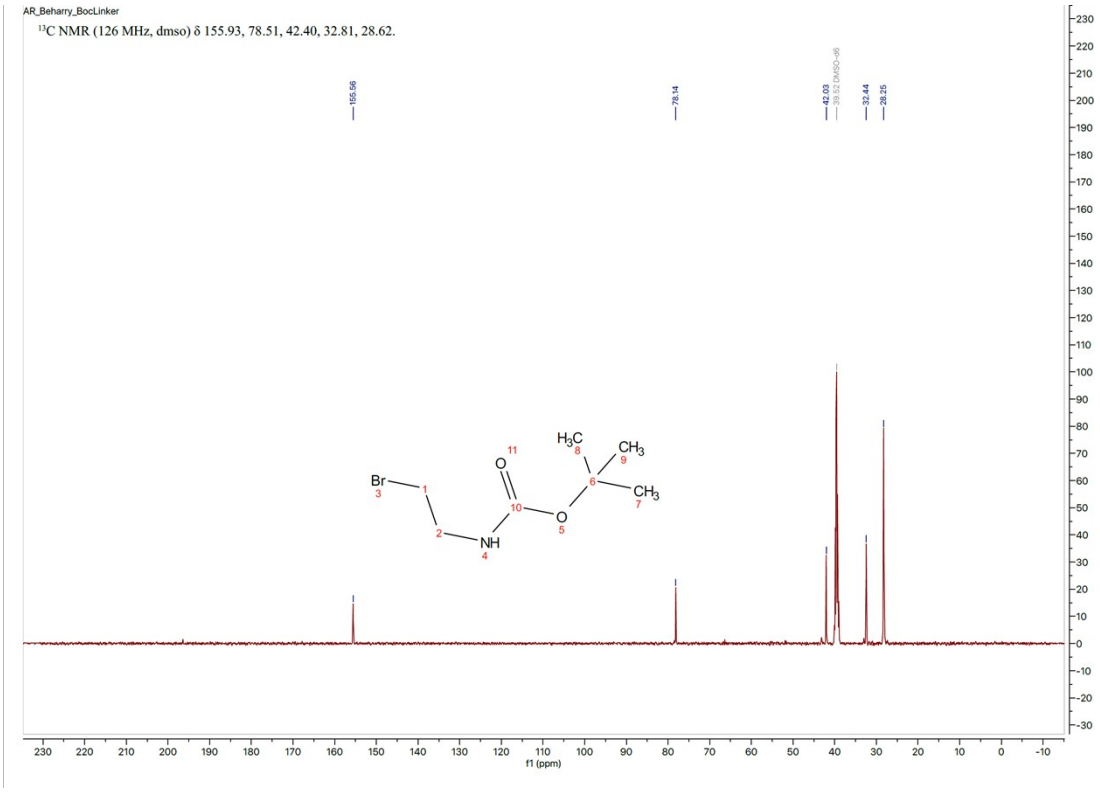



**Figure S4.** $^{13}$C NMR Spectrum of Compound **1**

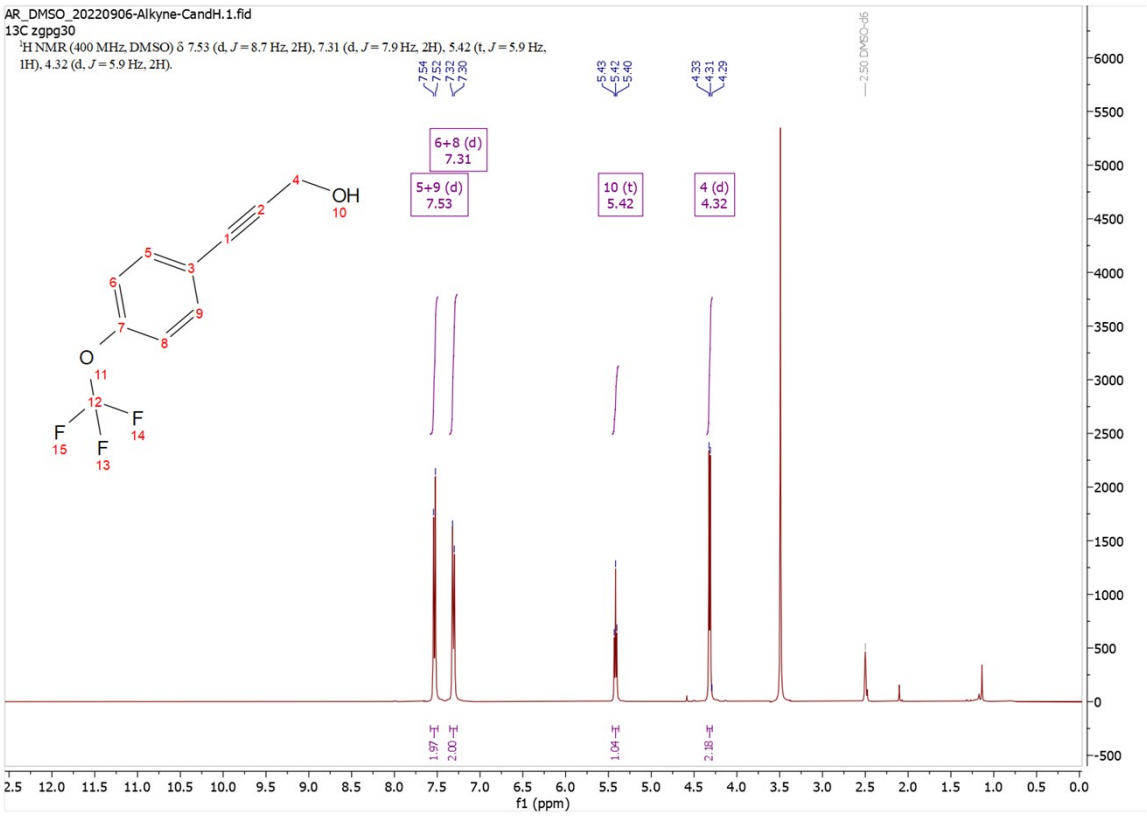



**Figure S5**. $^{1}$H NMR Spectrum of Compound **2**

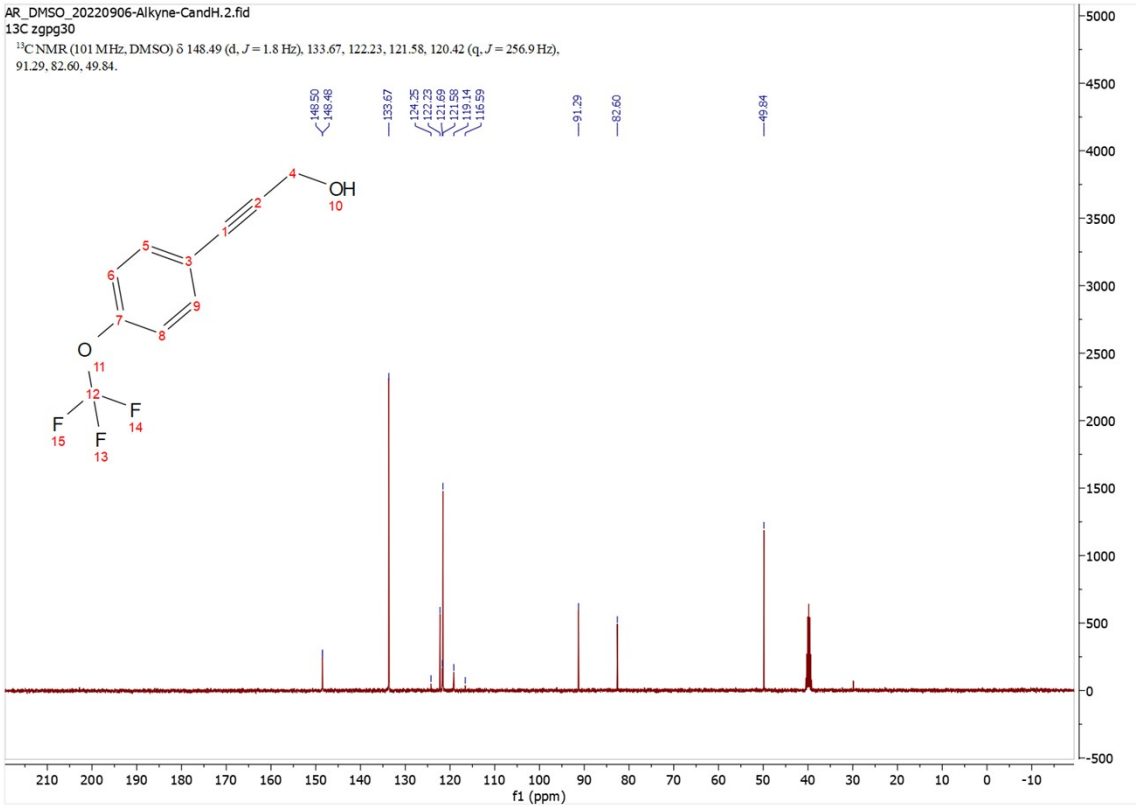



**Figure S6**. $^{13}C$ NMR Spectrum of Compound **2**

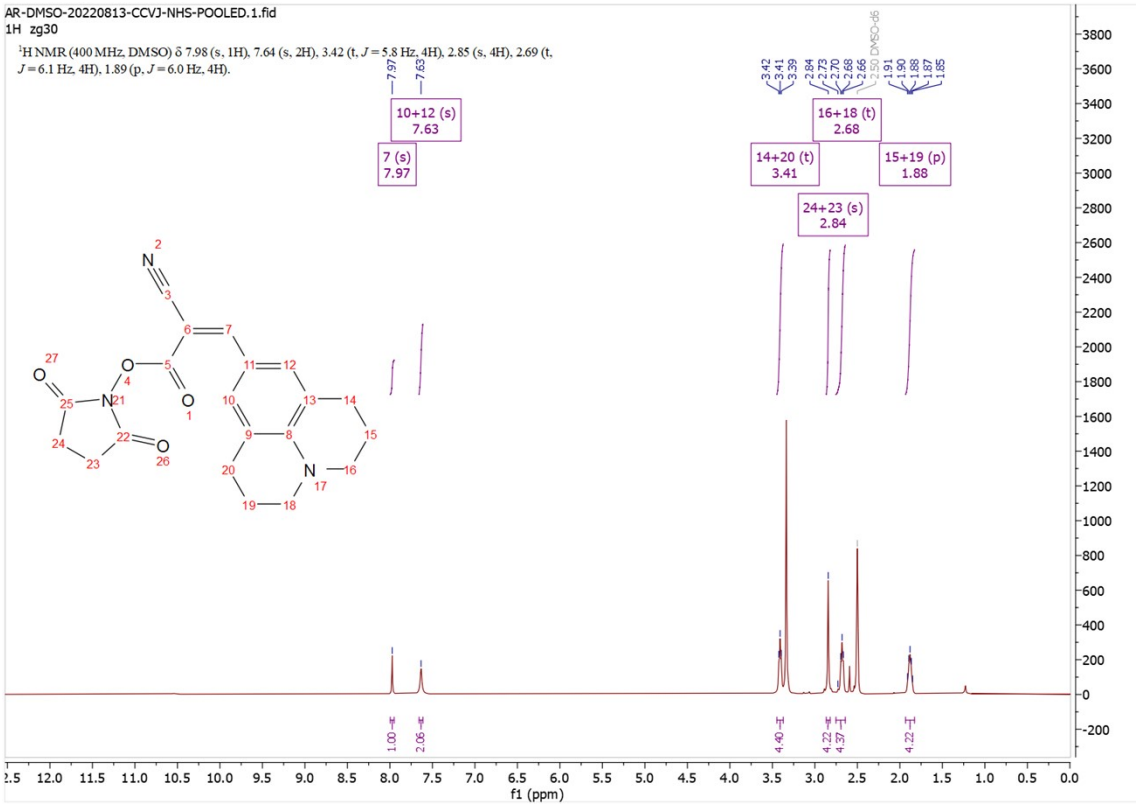



**Figure S7**. $^{1}H$ NMR Spectrum of Compound **5**

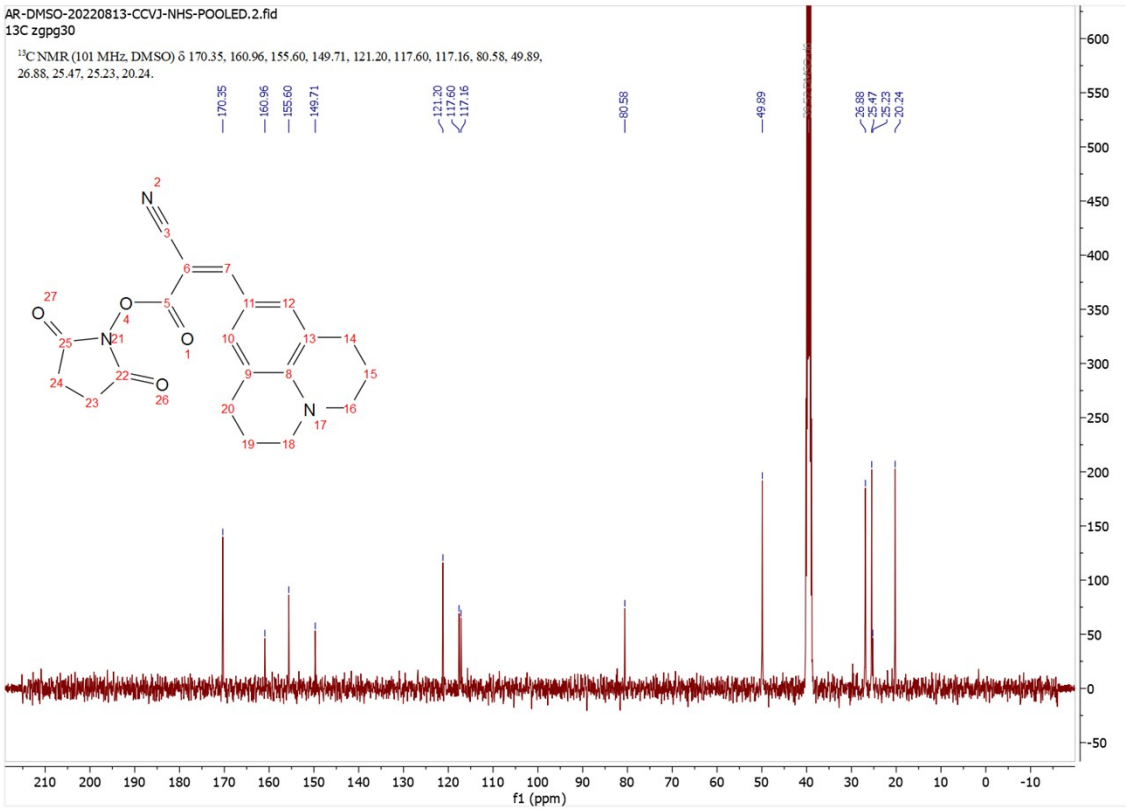



**Figure S8**. $^{13}$C NMR Spectrum of Compound **5**

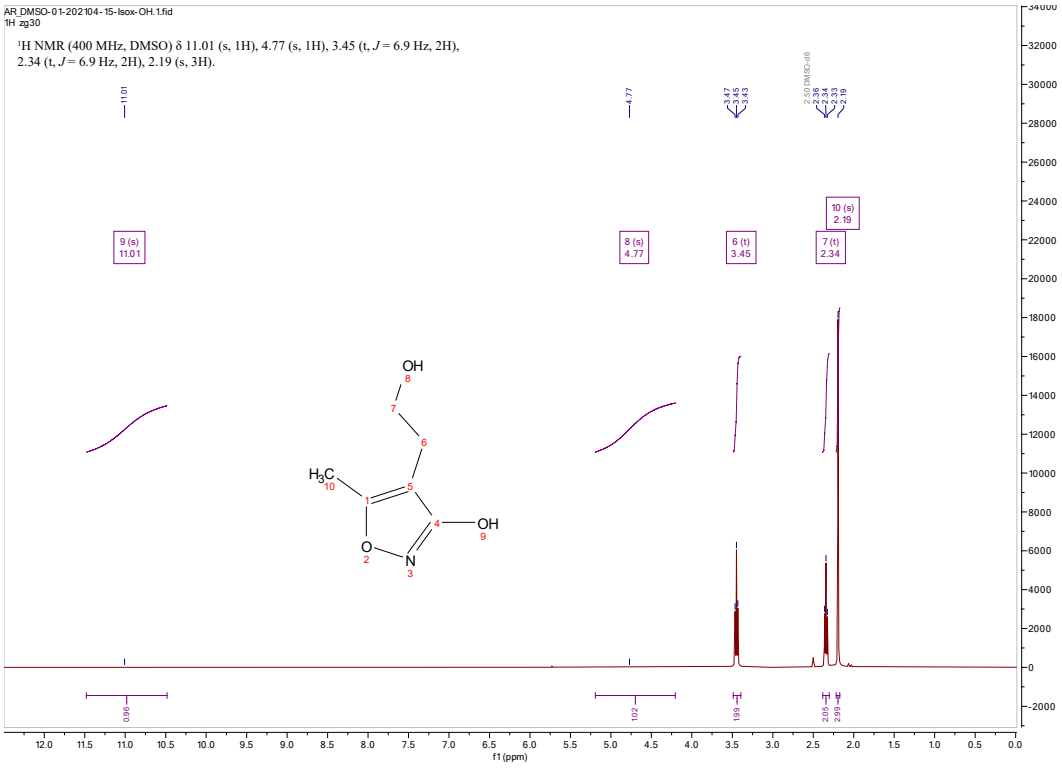



**Figure S9**. $^{1}$H NMR Spectrum of Compound **6**

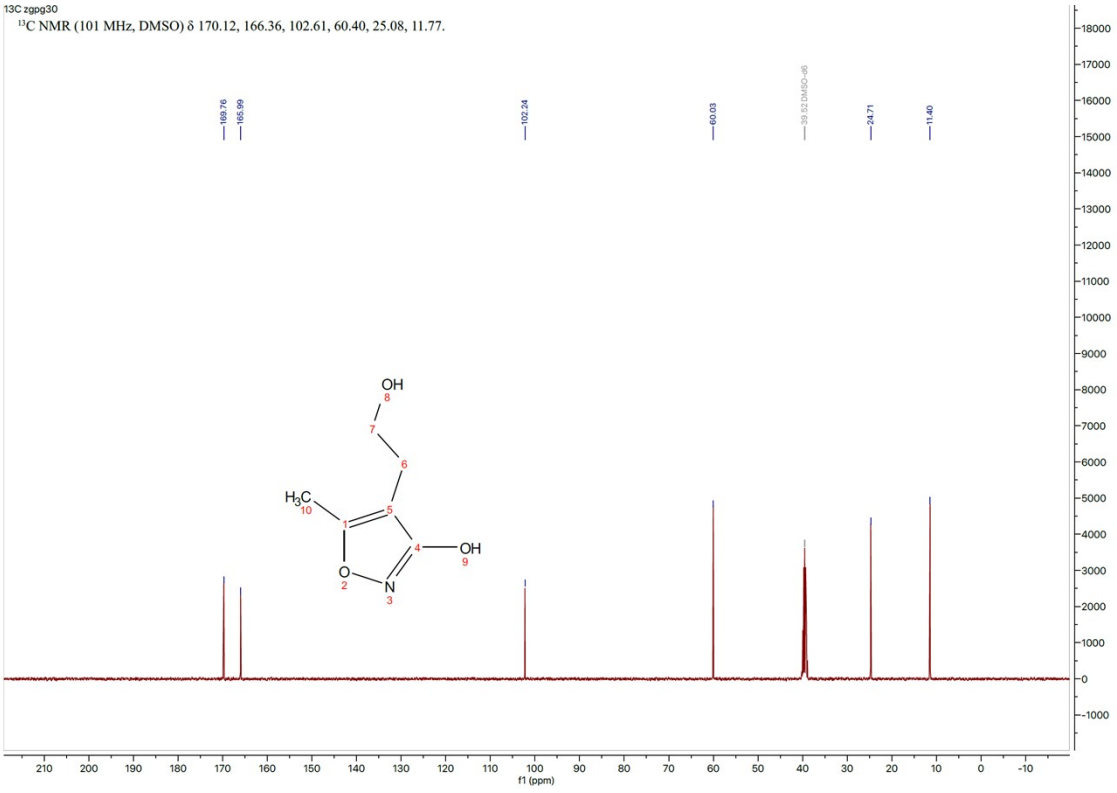



**Figure S10**. $^{13}C$ NMR Spectrum of Compound **6**

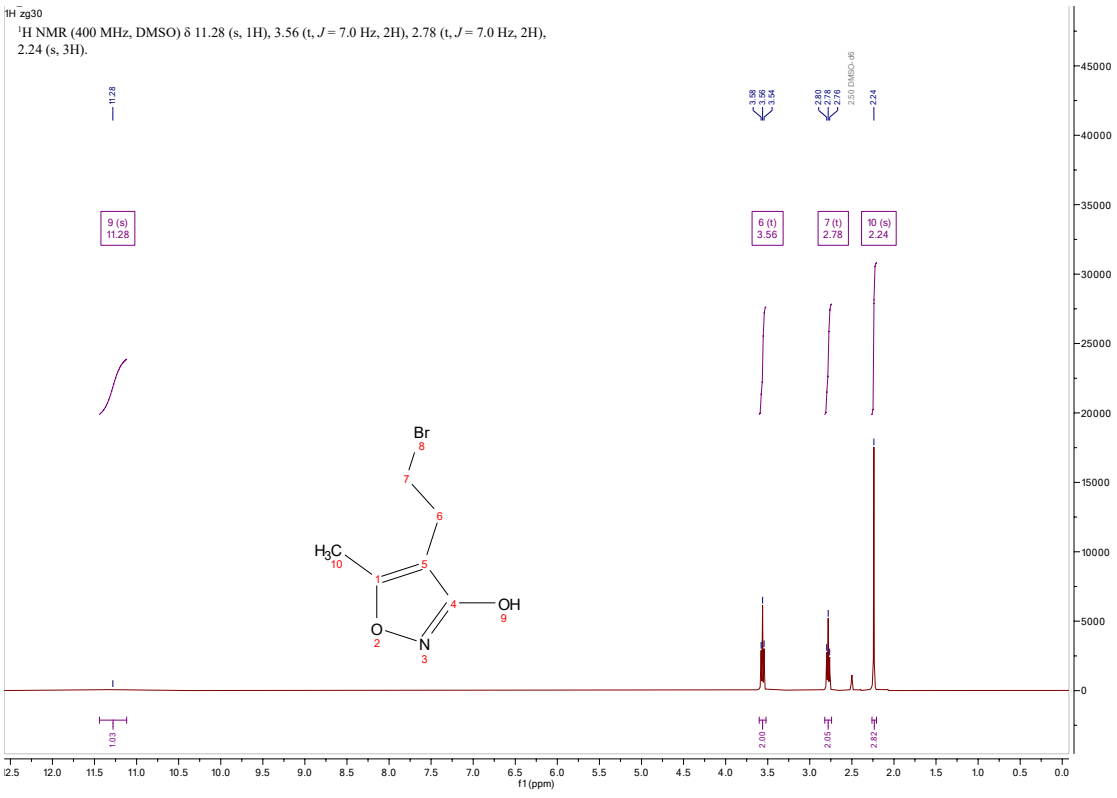



**Figure S11**. $^{1}H$ NMR Spectrum of Compound **7**

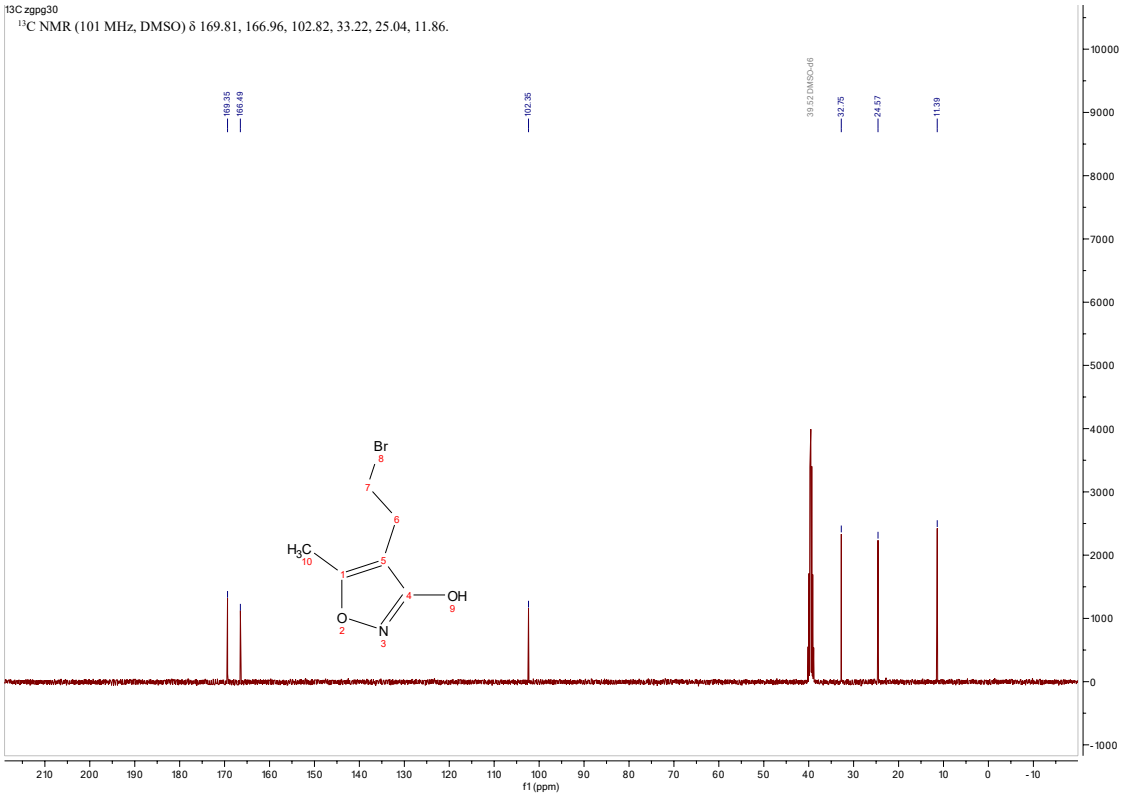



**Figure S12**. $^{13}C$ NMR Spectrum of Compound **7**


**Figure S13**. $^{1}H$ NMR Spectrum of Compound **8**

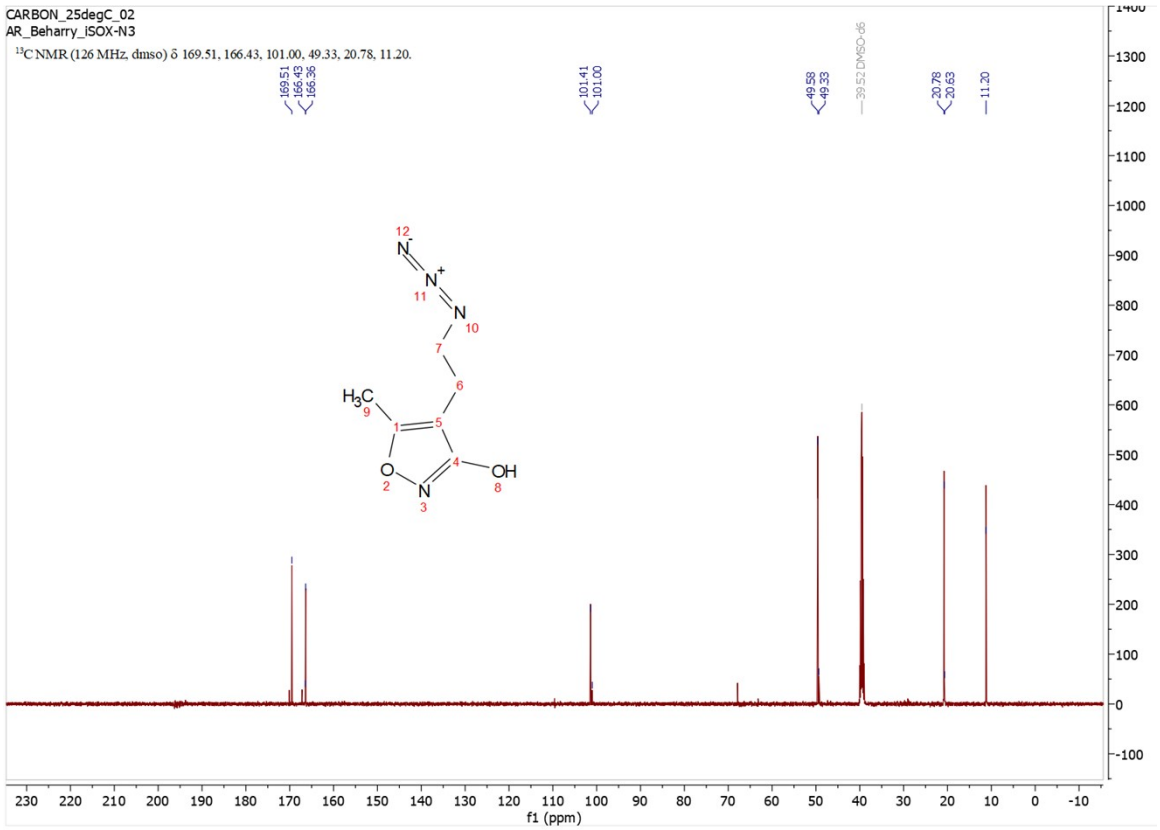



**Figure S14**. $^{13}C$ NMR Spectrum of Compound **8**

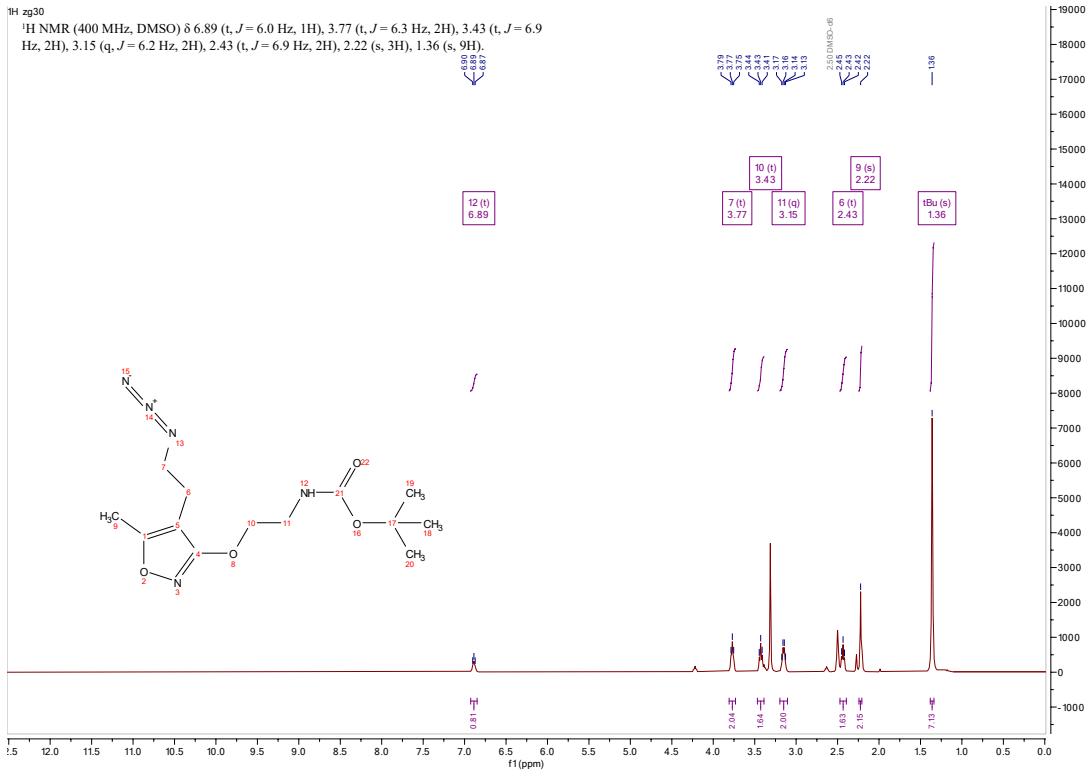



**Figure S15**. $^{1}H$ NMR Spectrum of Compound **9**

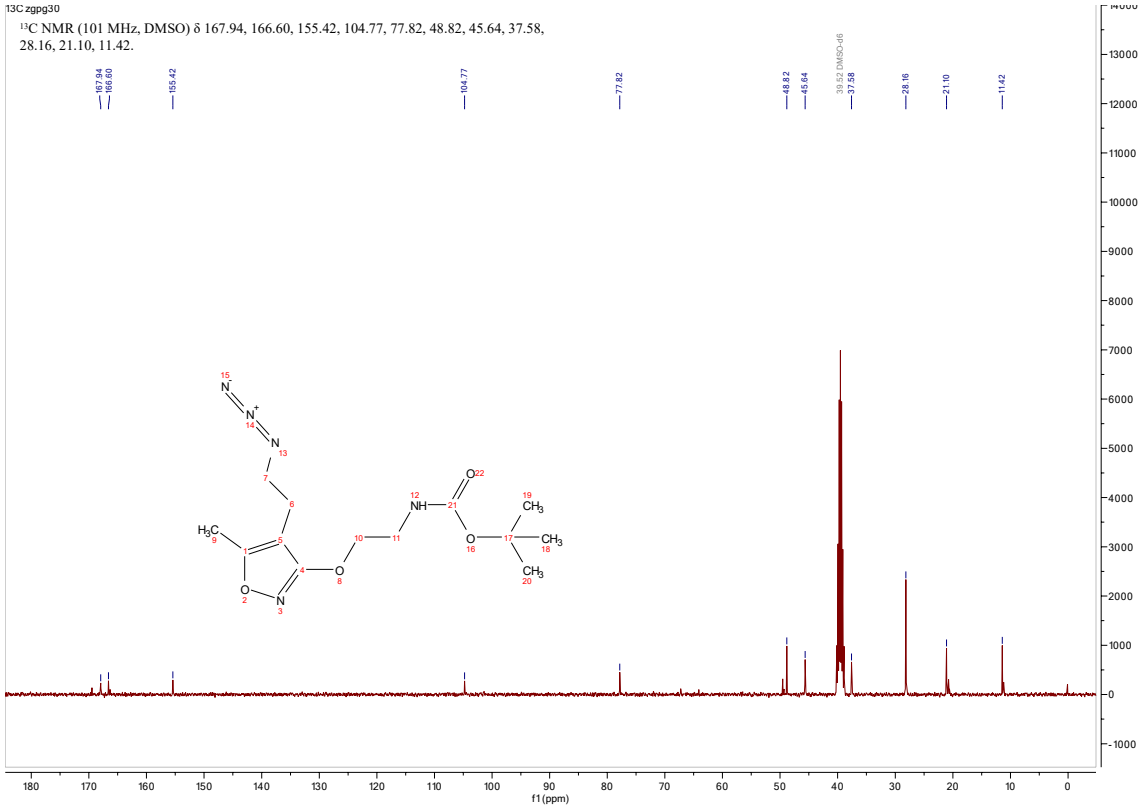



**Figure S16**. $^{13}$C NMR Spectrum of Compound **9**

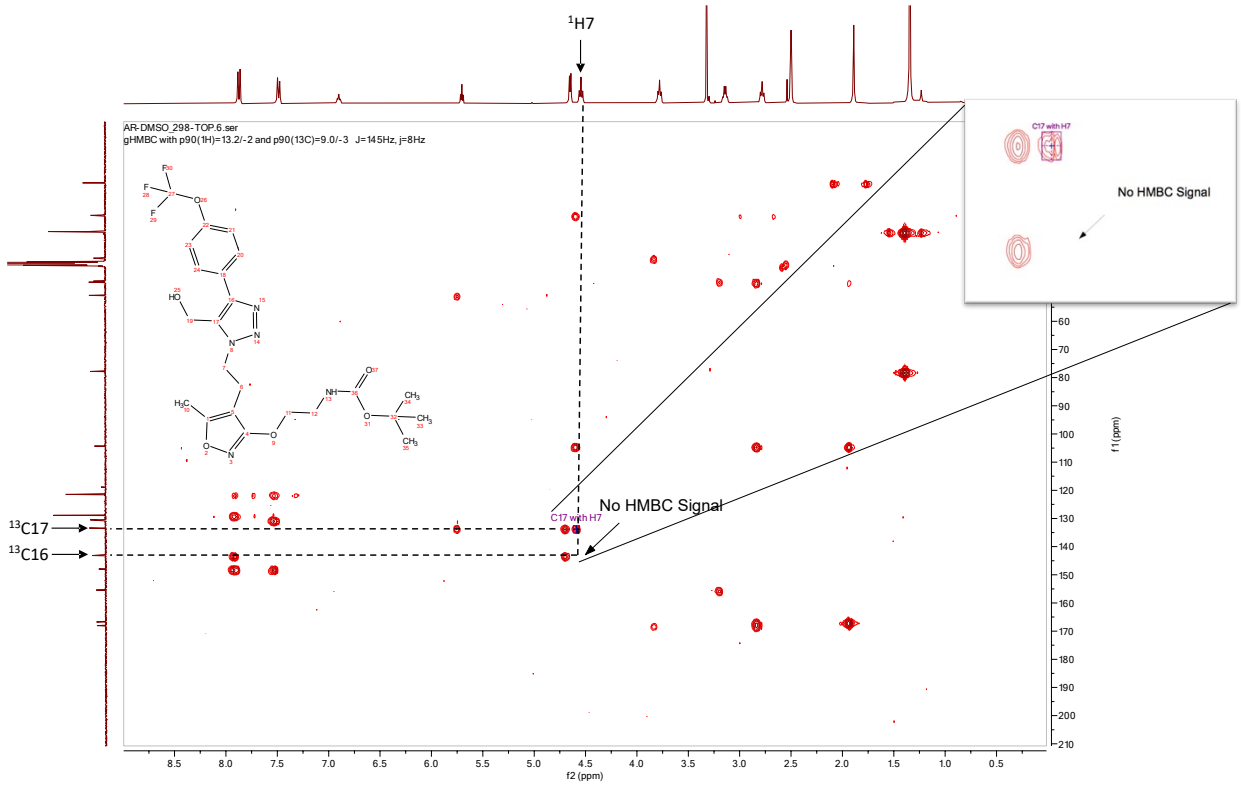



**Figure S17.** $^{1}$H-$^{13}$C HMBC NMR Spectrum of 1,4-triazole isomer (Compound **10**)

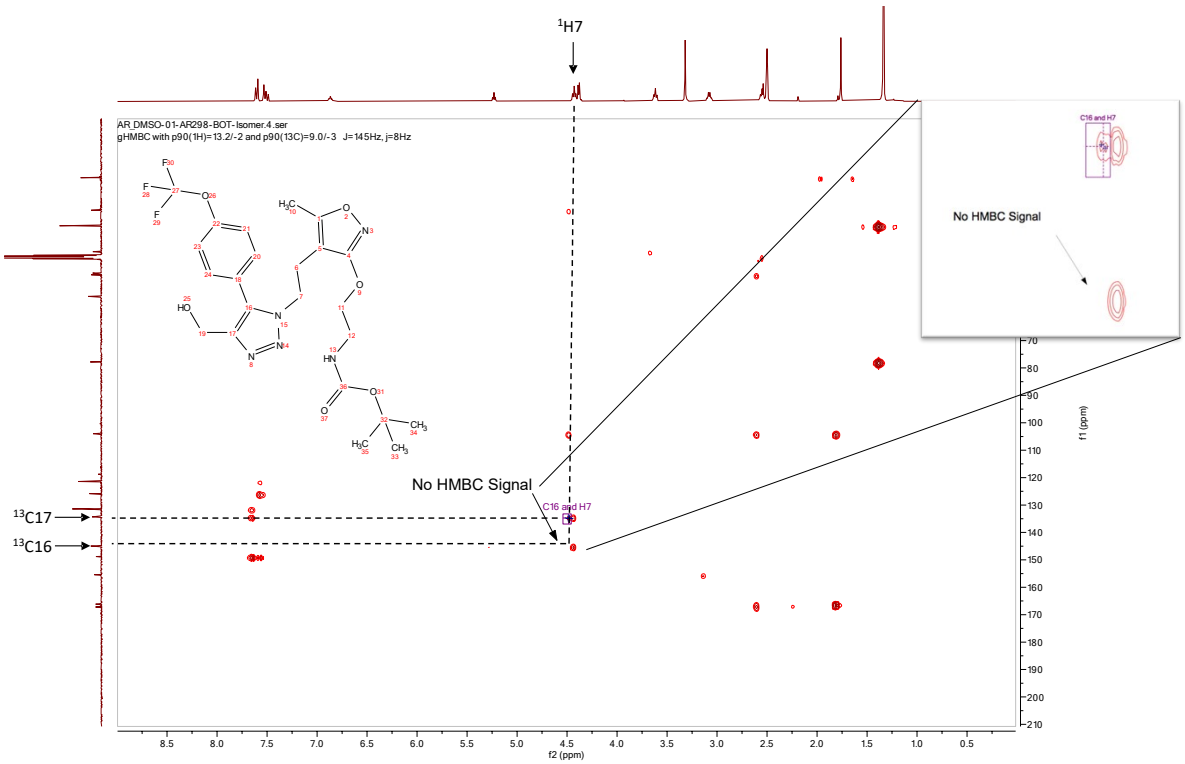



**Figure S18.** $^{1}$H-$^{13}$C HMBC NMR Spectrum of 3,4-triazole isomer

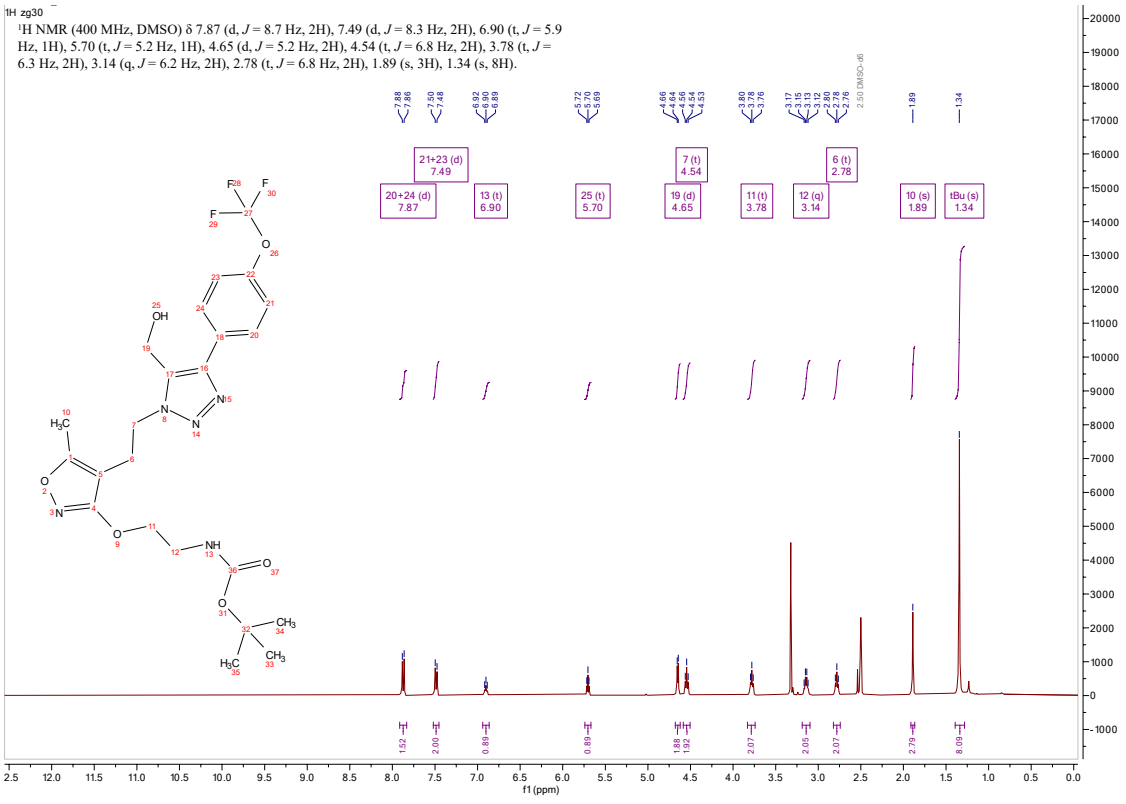



**Figure S19**. $^{1}$H NMR Spectrum of Compound **10**

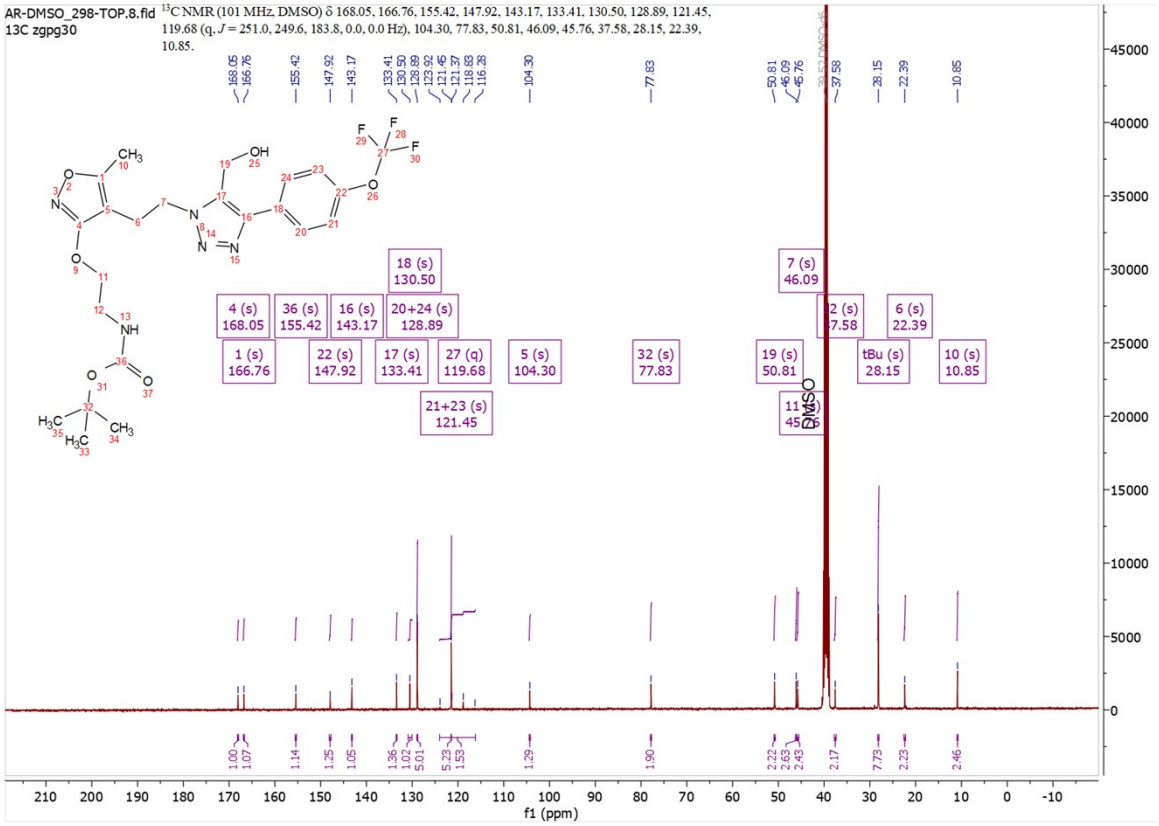



**Figure S20**. $^{13}$C NMR Spectrum of Compound **10**

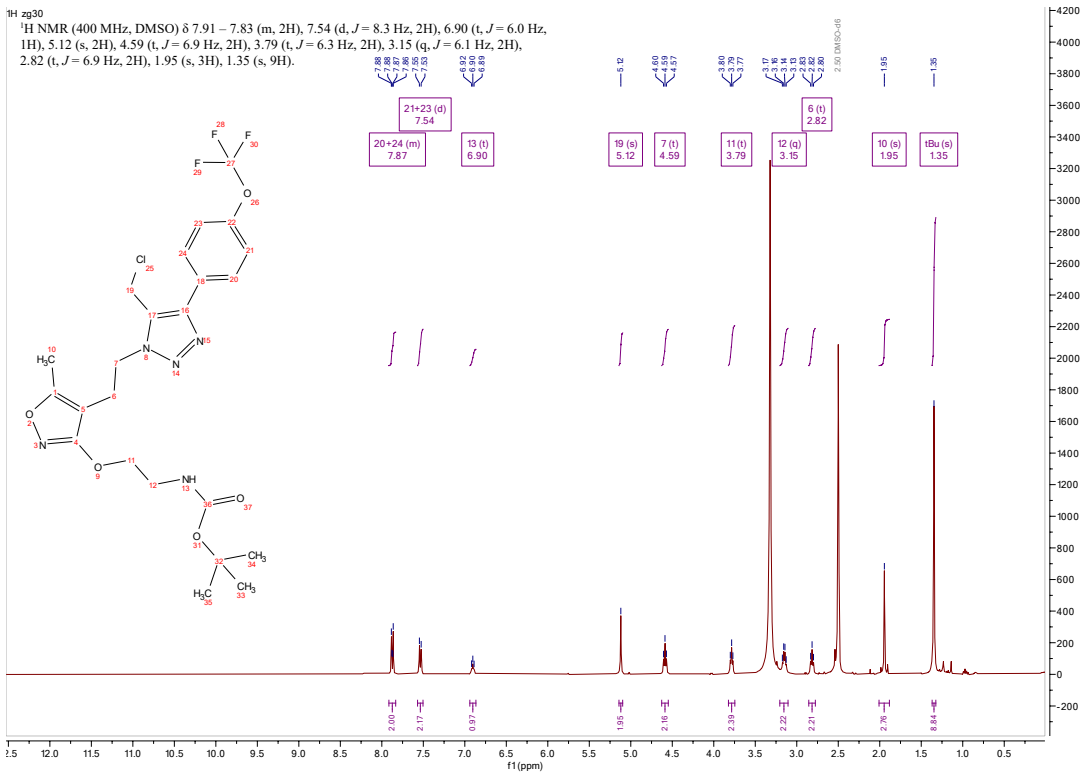



**Figure S21**. $^{1}$H NMR Spectrum of Compound **11**

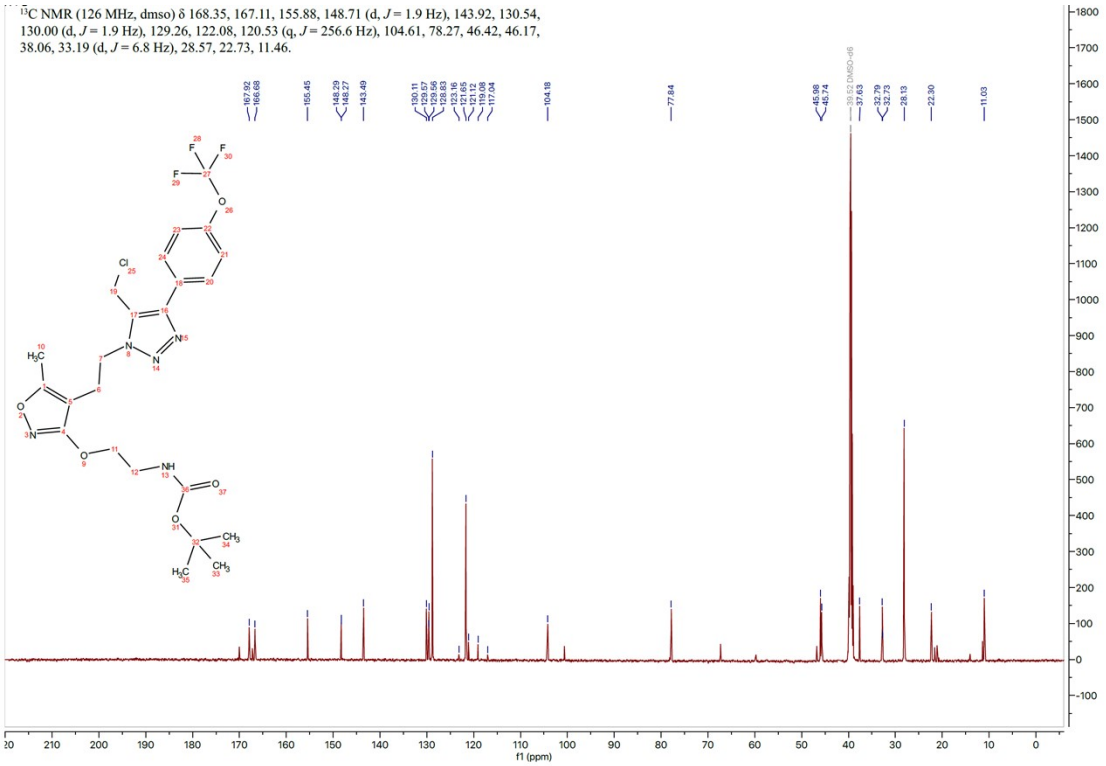



**Figure S22**. $^{13}$C NMR Spectrum of Compound **11**

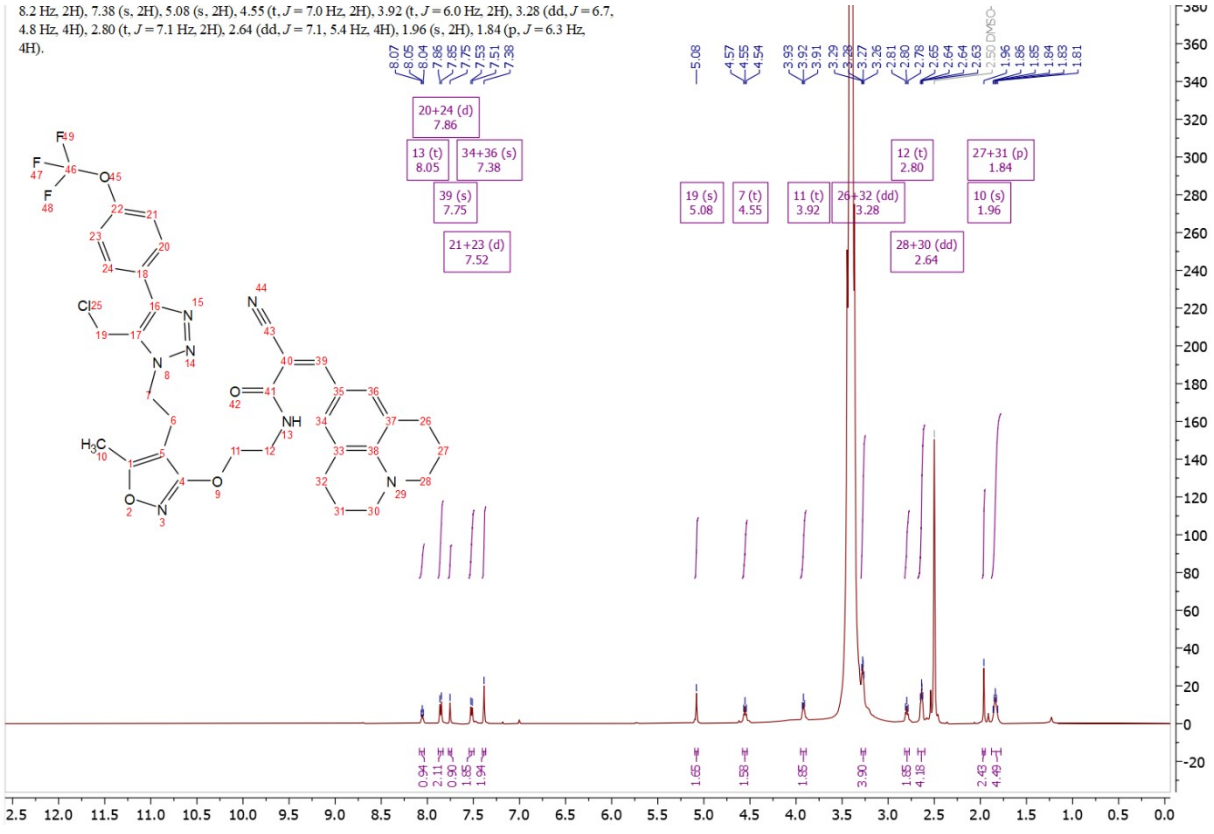



**Figure S23**. $^{1}$H NMR Spectrum of Compound **13**

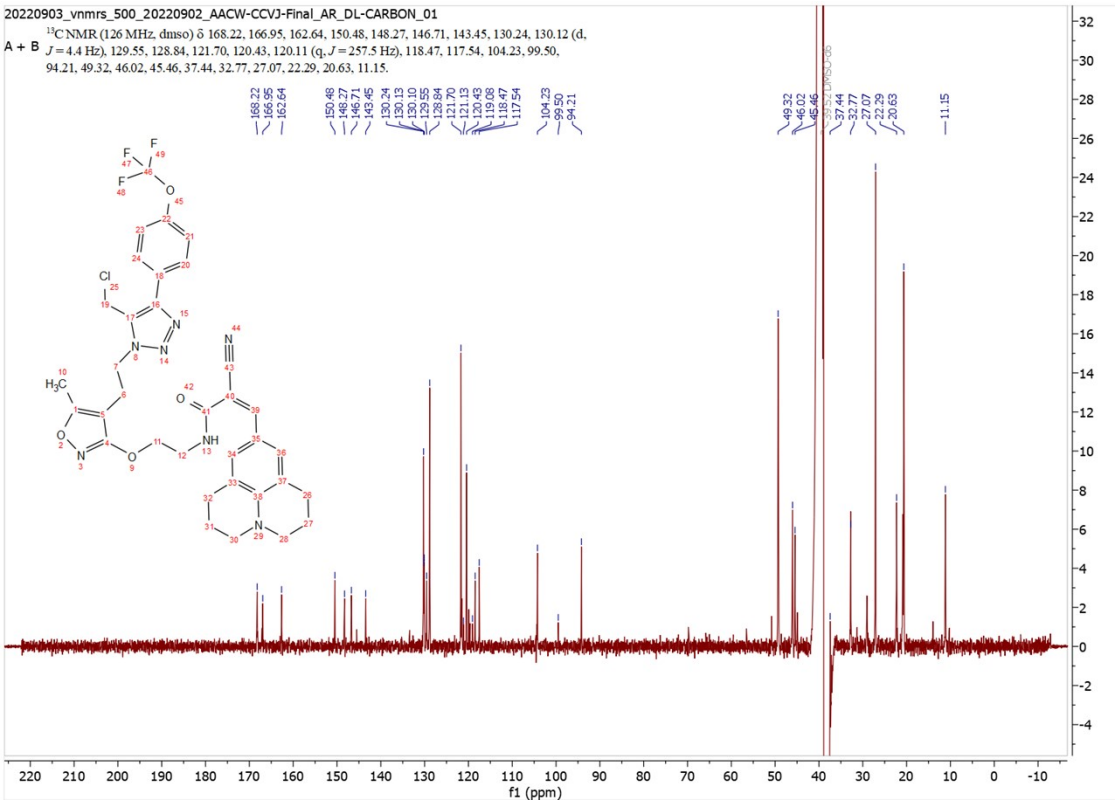



**Figure S24**. $^{13}$C NMR Spectrum of Compound **13**

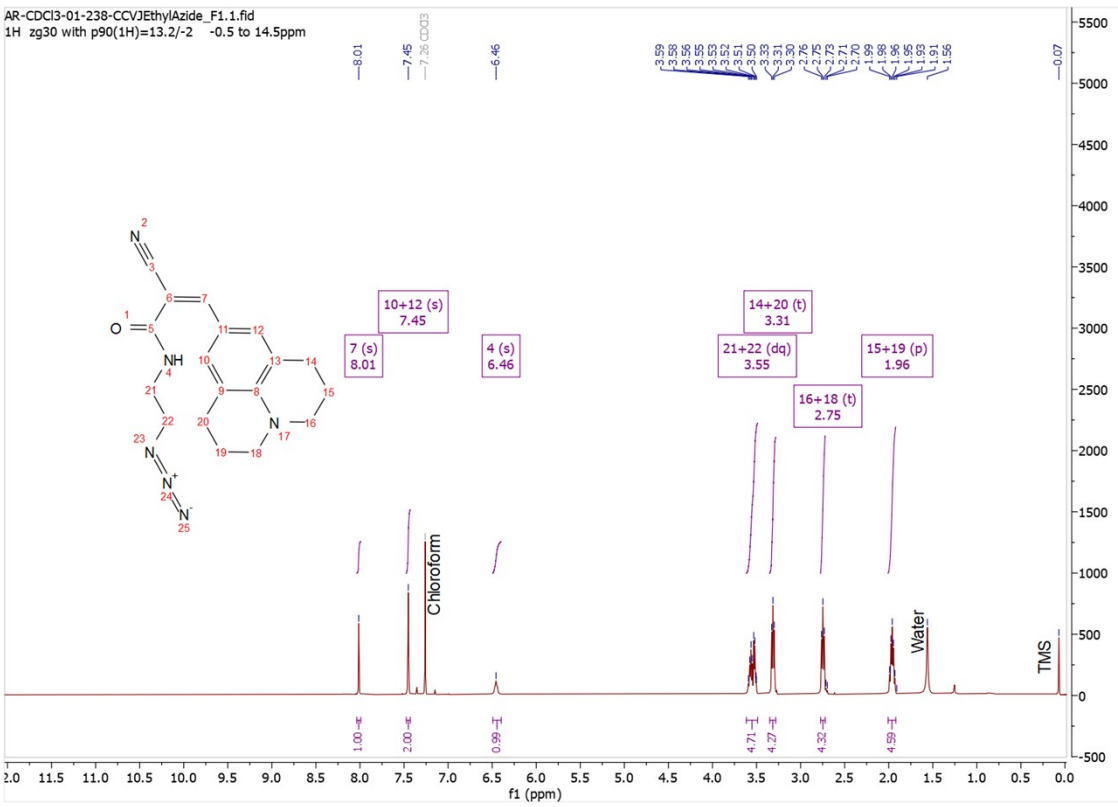



**Figure S25**. $^{1}$H NMR Spectrum of Compound **14**

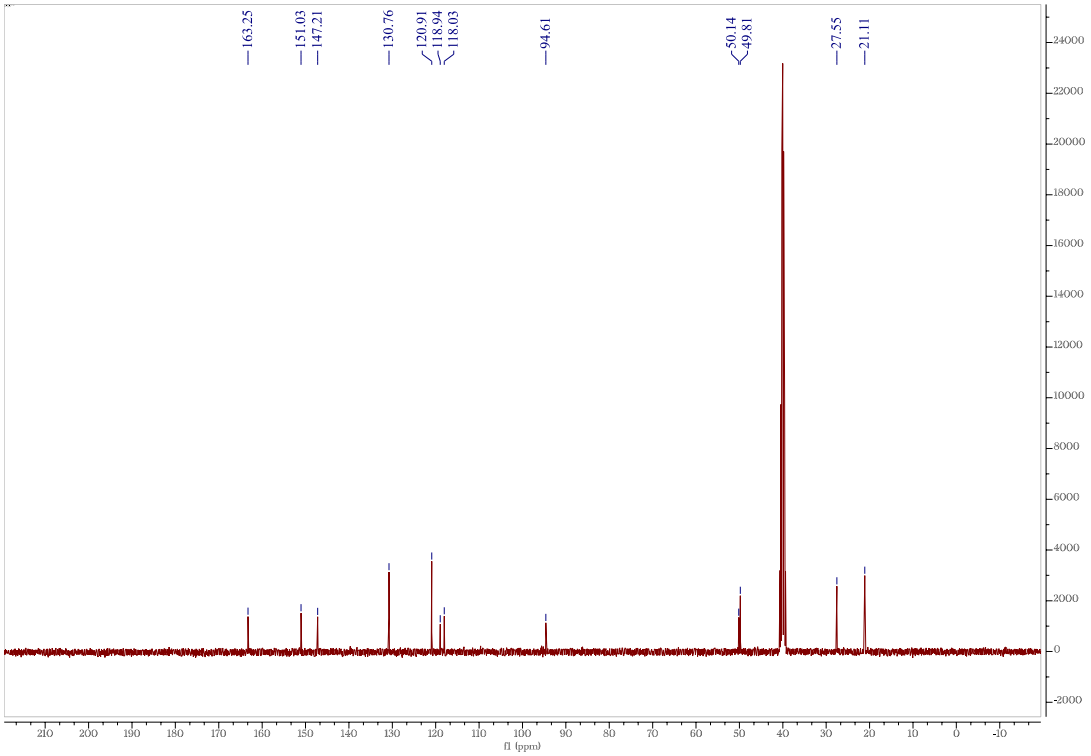



**Figure S26**. $^{13}$C NMR Spectrum of Compound **14**

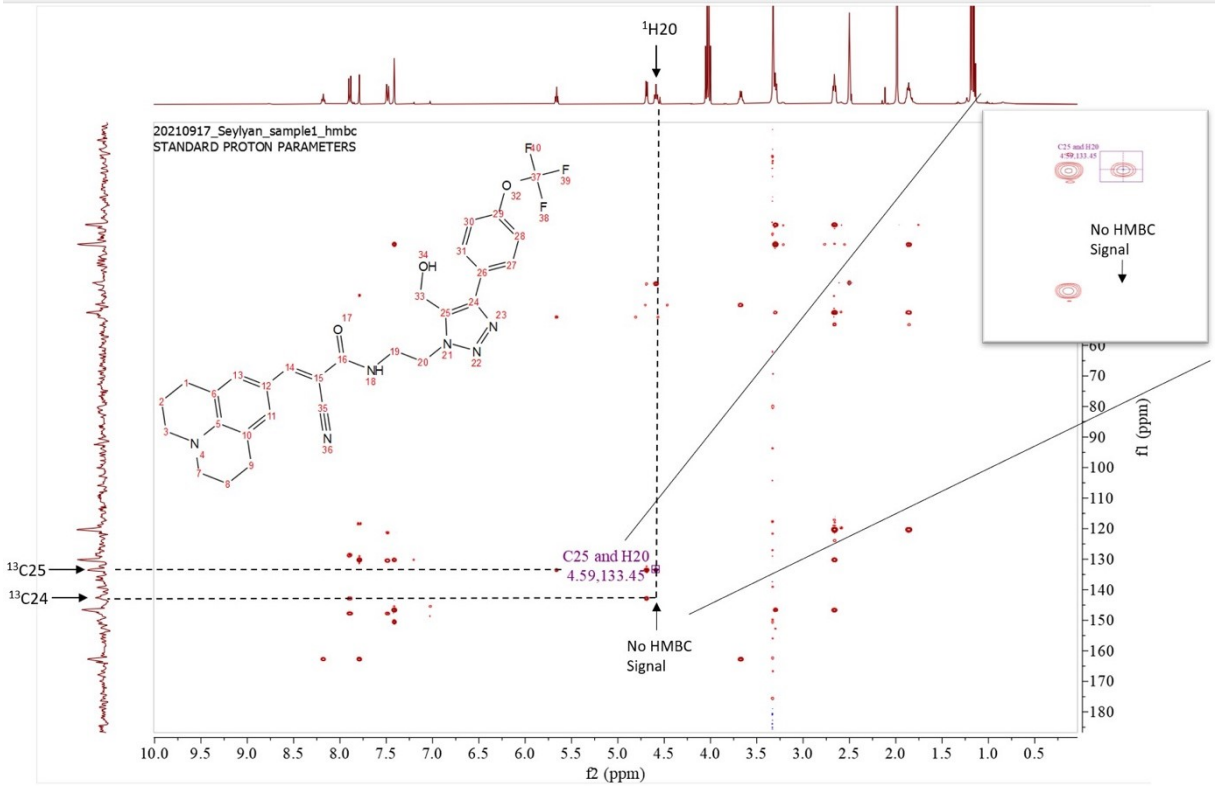



**Figure S27**. $^{1}$H-$^{13}$C HMBC NMR Spectrum of 1,4-triazole isomer (Compound **15)**

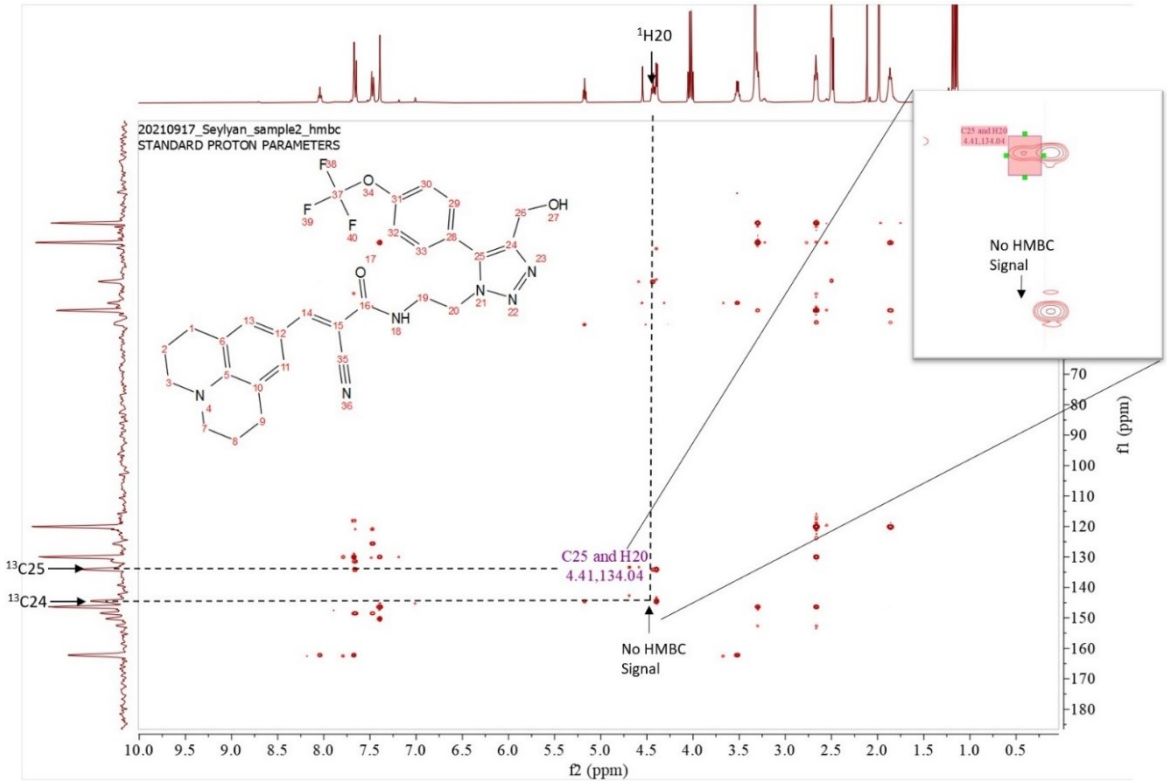



**Figure S28**. $^{1}H$-$^{13}C$ HMBC NMR Spectrum of 3,4-triazole isomer

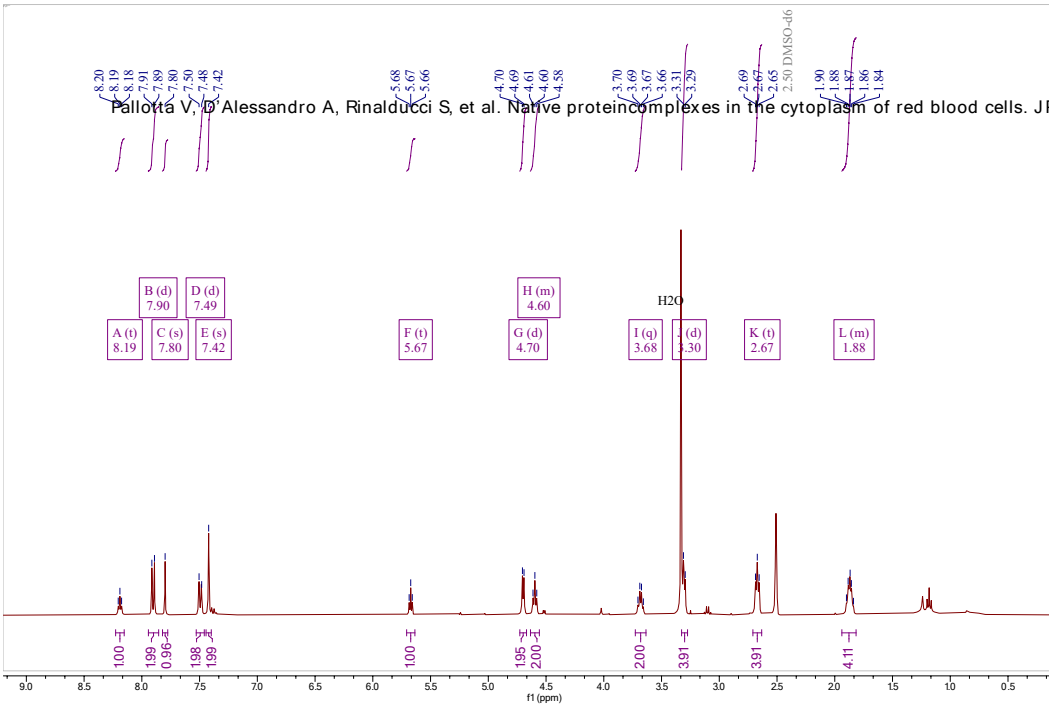



**Figure S29**. $^{1}H$ NMR Spectrum of Compound **15**

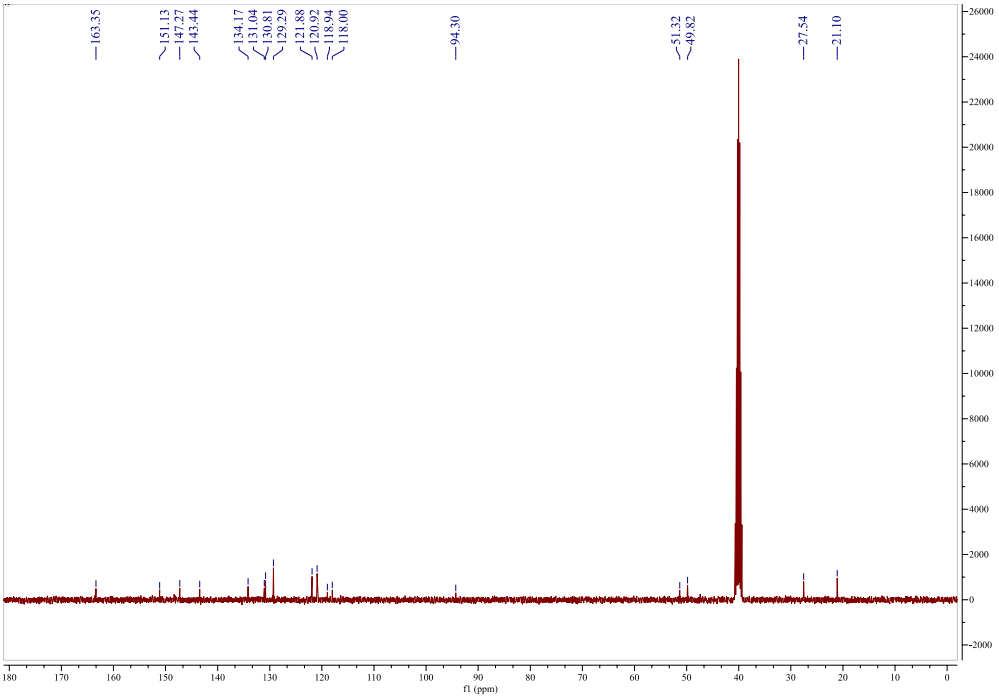



**Figure S30**. $^{13}$C NMR Spectrum of Compound **15**

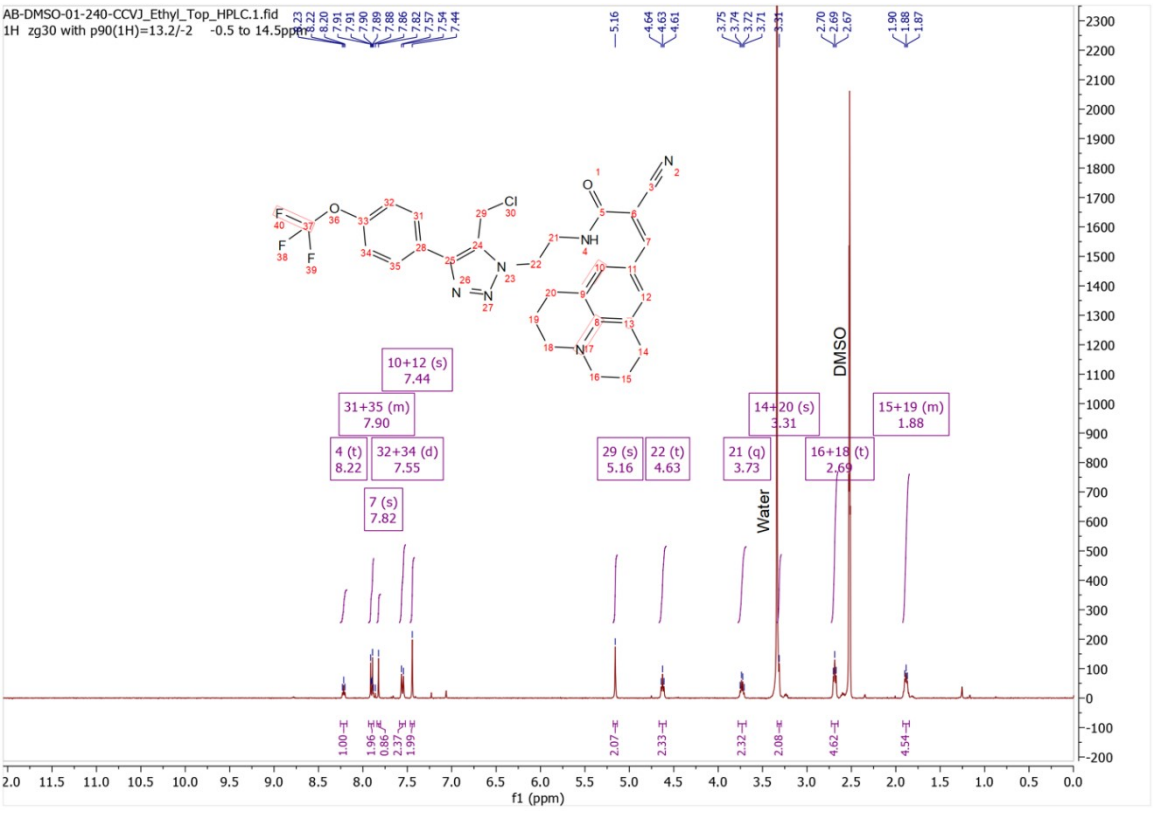



**Figure S31**. $^{1}$H NMR Spectrum of Compound **16**

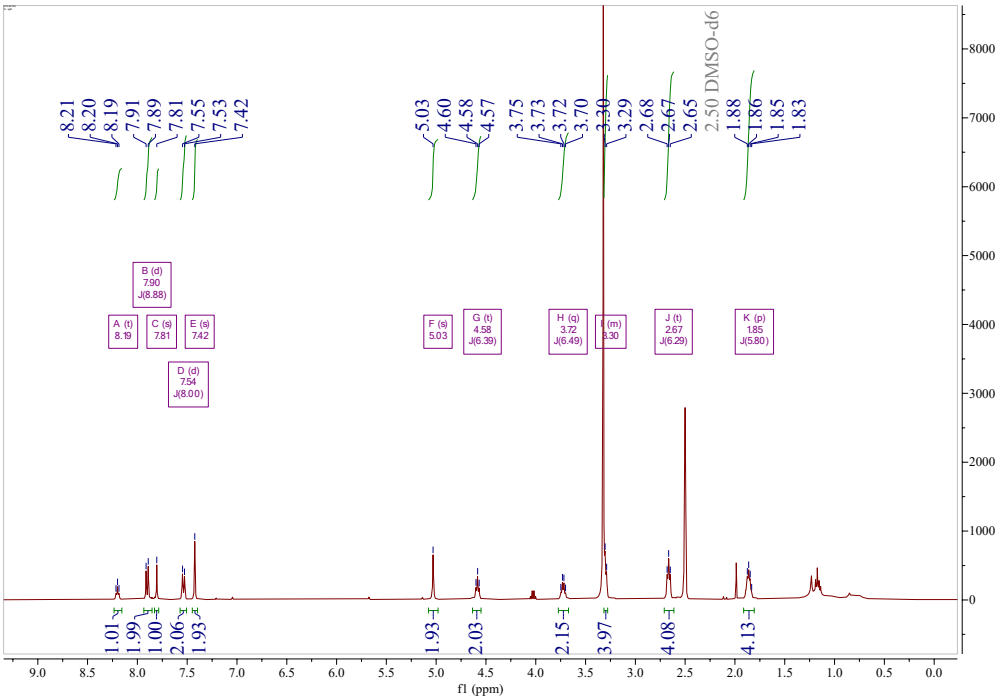



**Figure S32**. $^{1}$H NMR Spectrum of Compound **17**

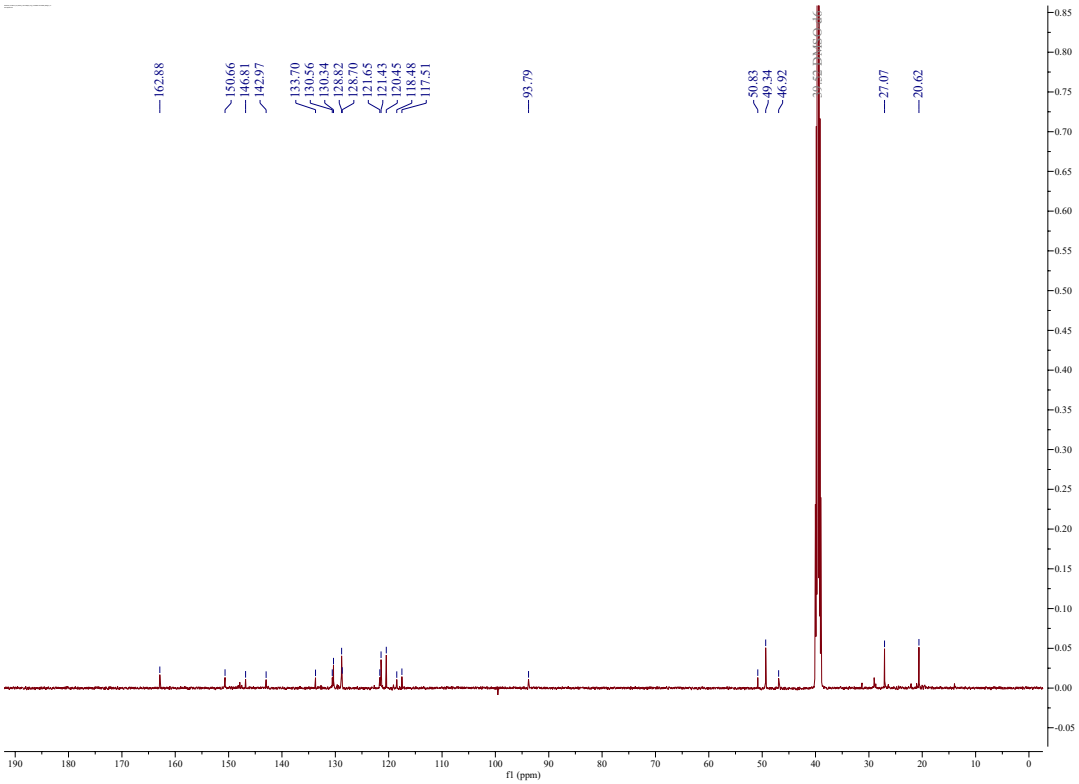



**Figure S33**. $^{13}$C NMR Spectrum of Compound **17**

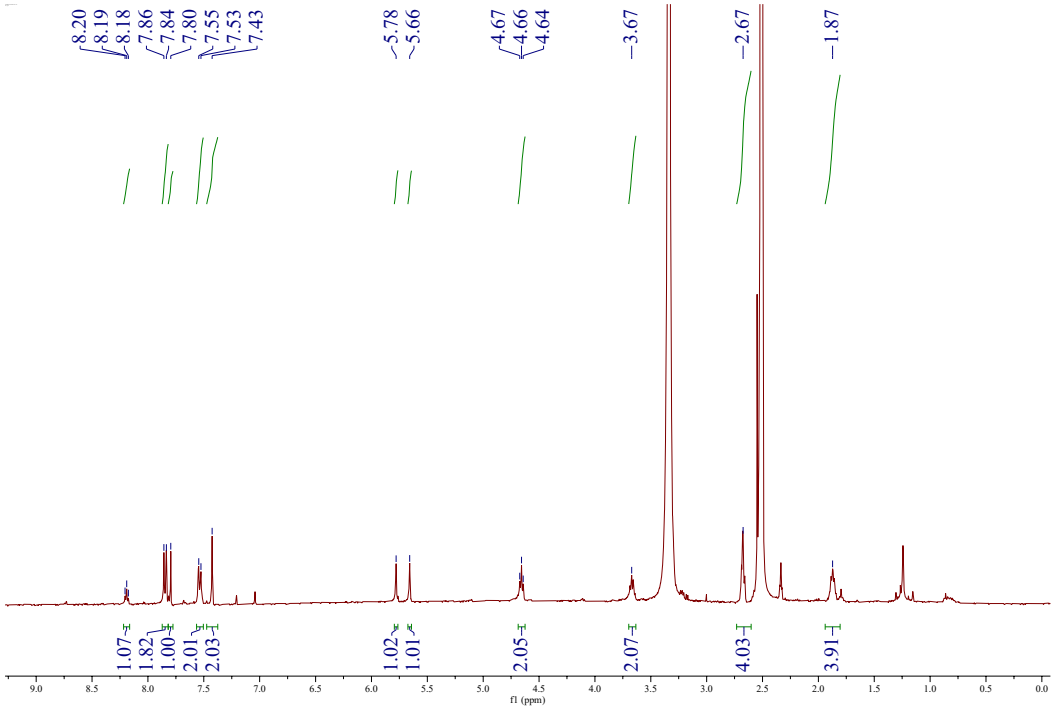



**Figure S34**. $^{1}$H NMR Spectrum of Compound **18**

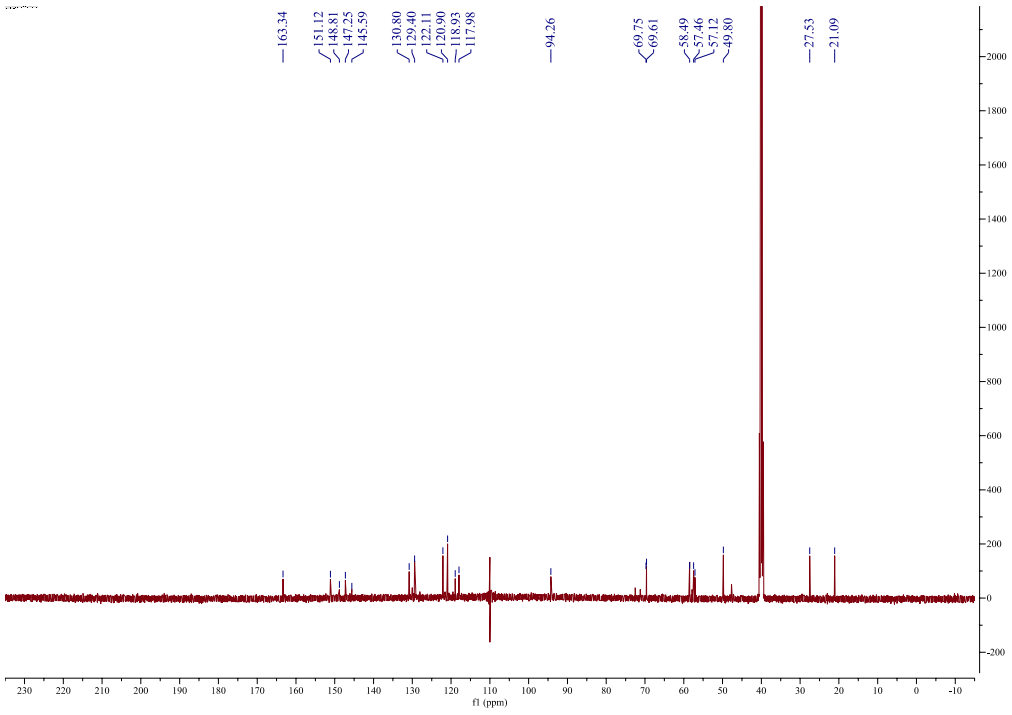



**Figure S35**. $^{13}$C NMR Spectrum of Compound **18**

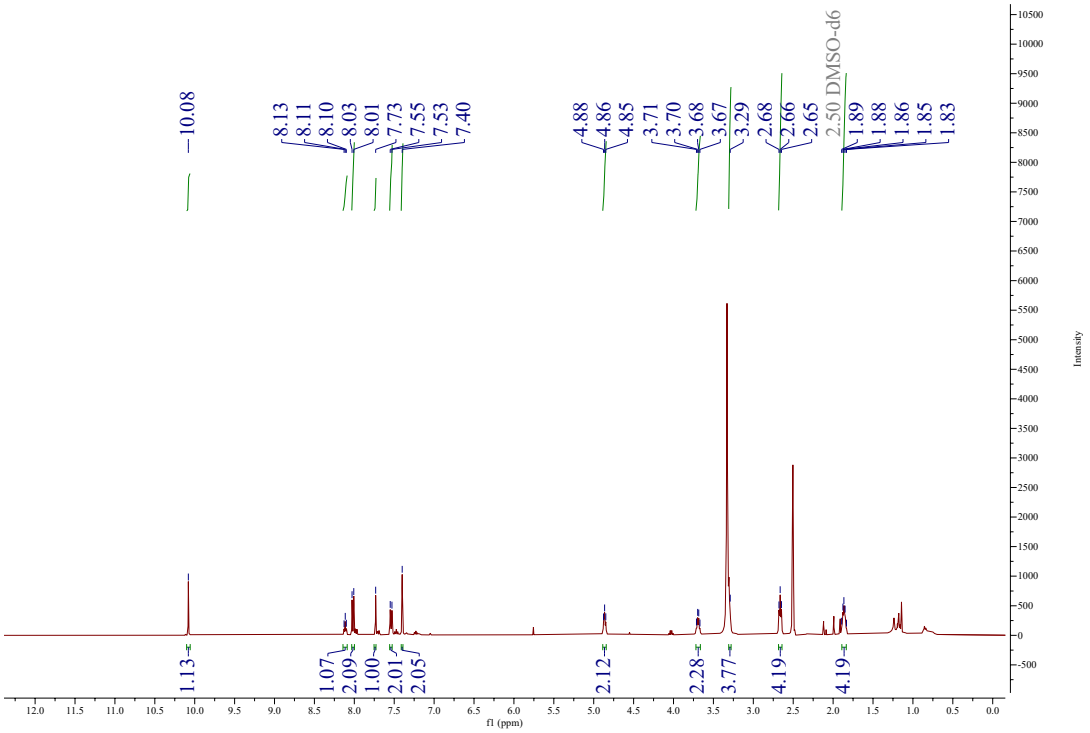



**Figure S36**. $^{1}$H NMR Spectrum of Compound **19**

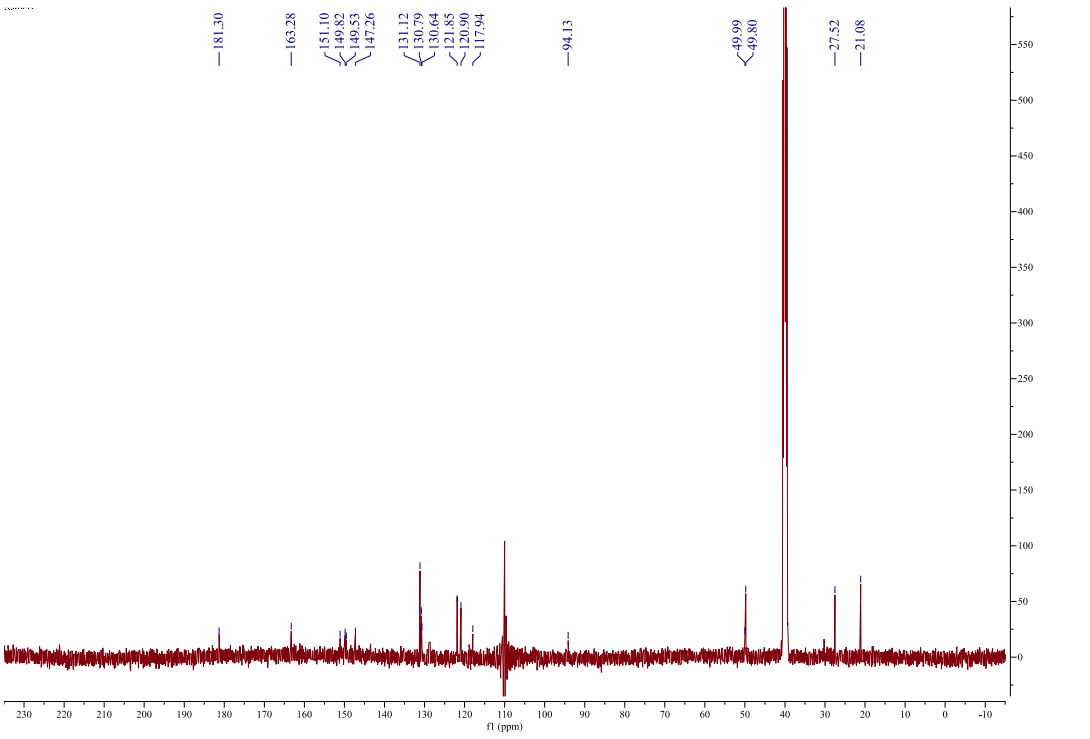



**Figure S37**. $^{13}$C NMR Spectrum of Compound **19**

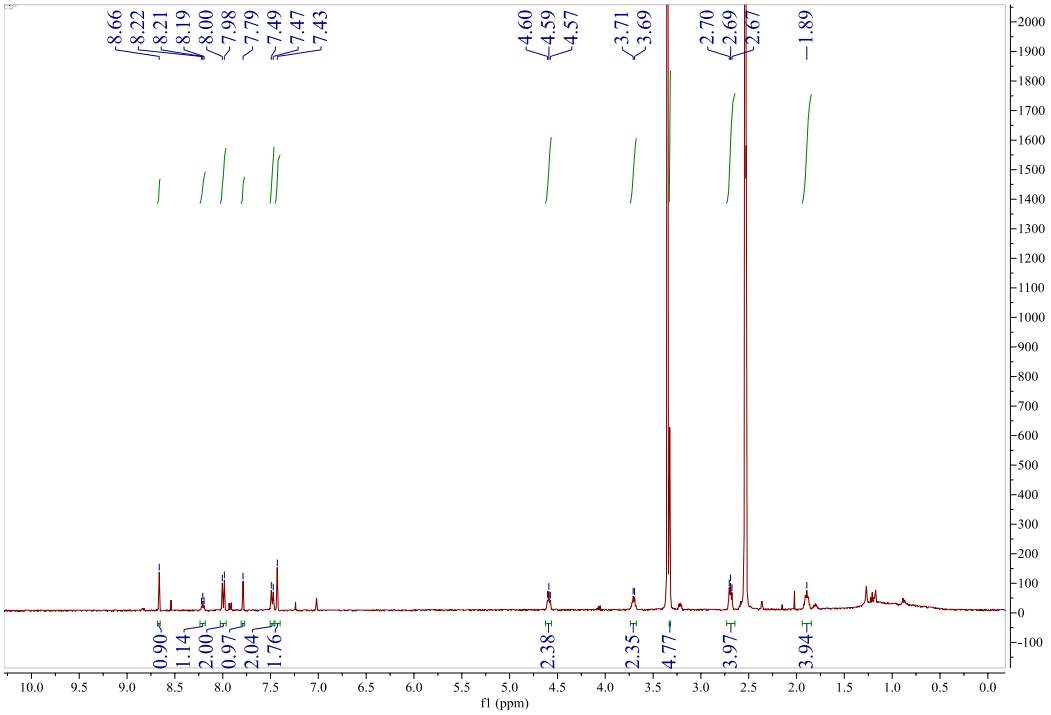



**Figure S38**. $^{1}$H NMR Spectrum of Compound **20**

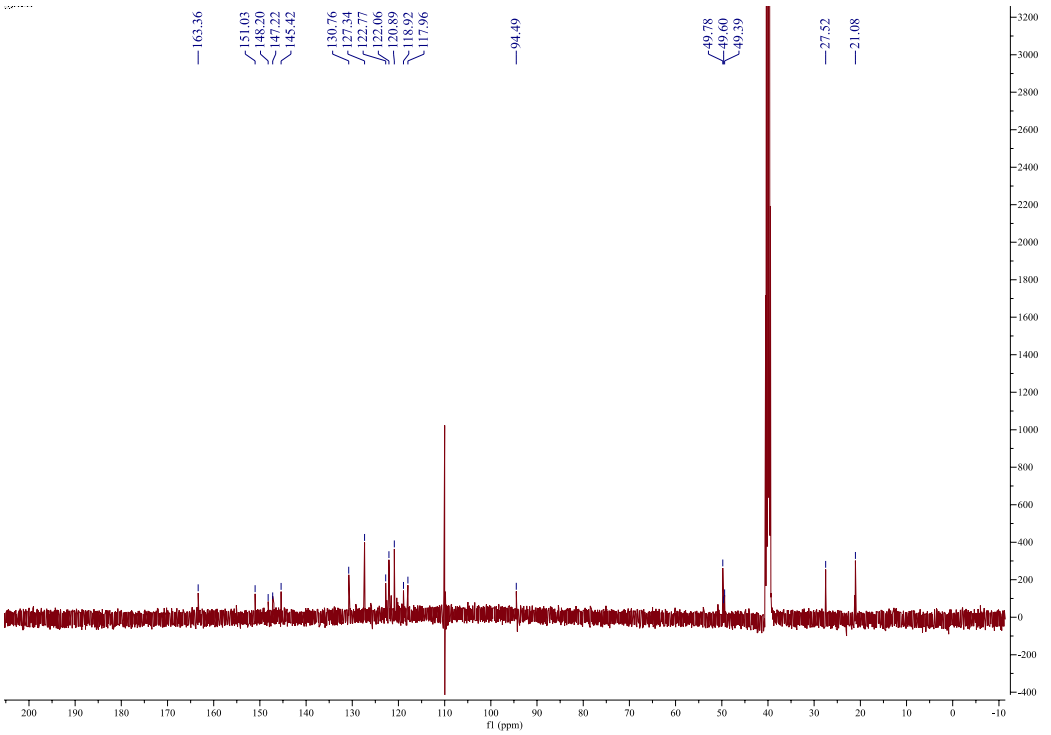



**Figure S39**. $^{13}$C NMR Spectrum of Compound **20**

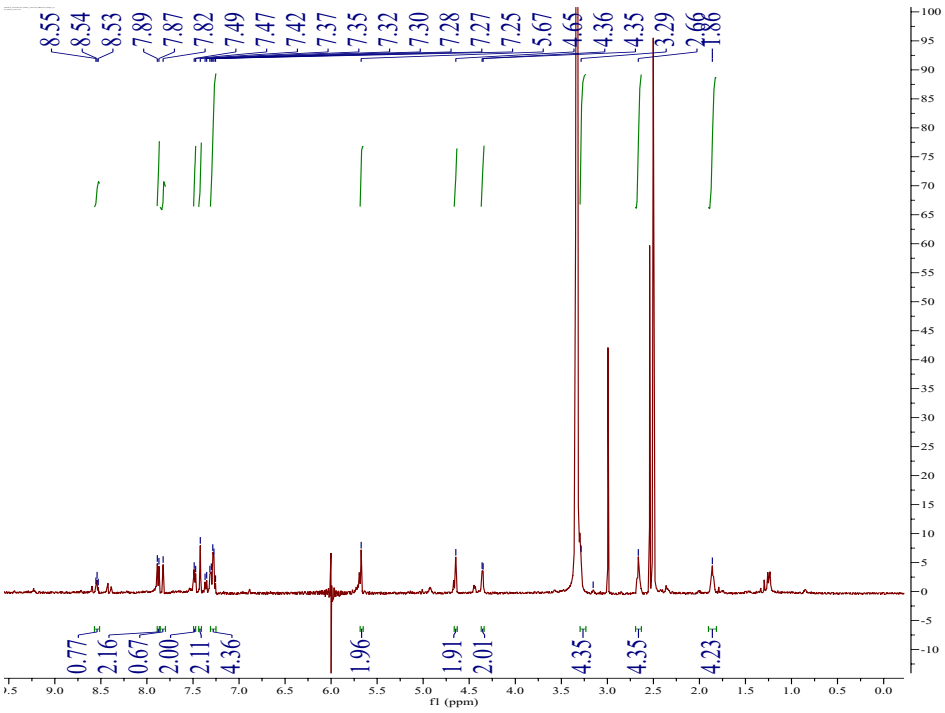



**Figure S40**. $^{1}$H NMR Spectrum of Compound **21**

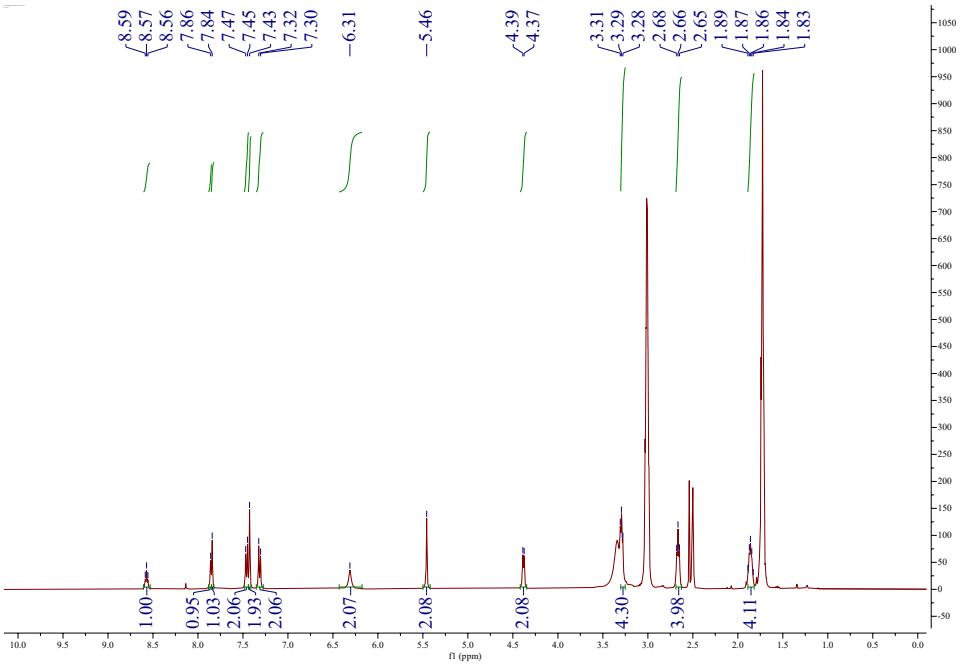



**Figure S41**. $^{1}$H NMR Spectrum of **BG-CCVJ**

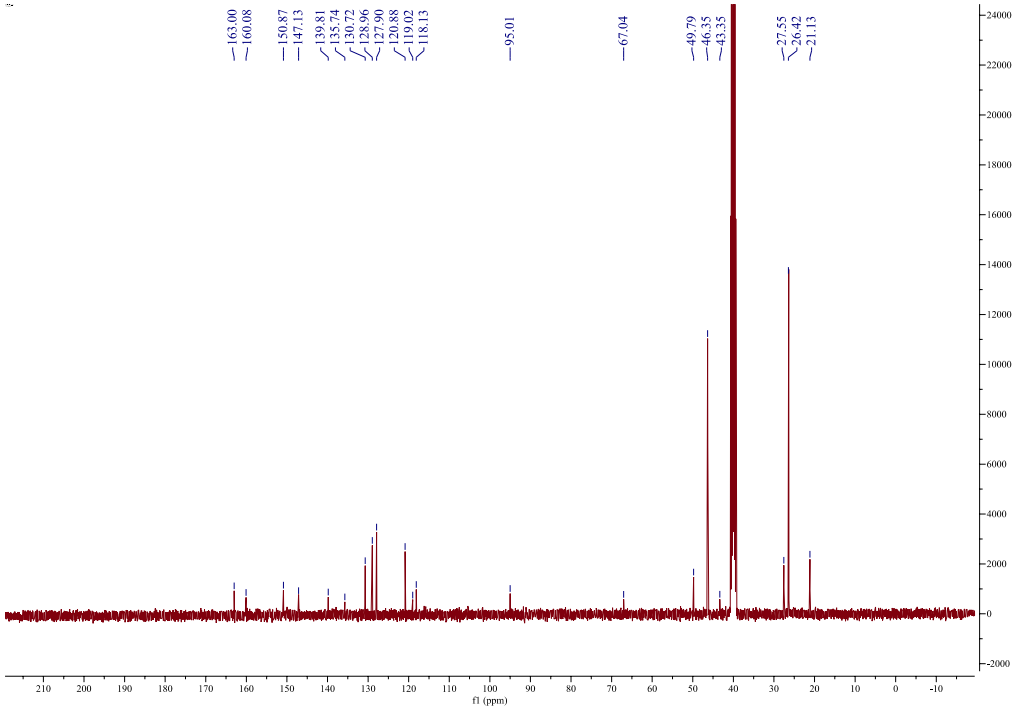



**Figure S42**. $^{13}$C NMR Spectrum of **BG-CCVJ**

## 2.3.2. HRMS Spectra

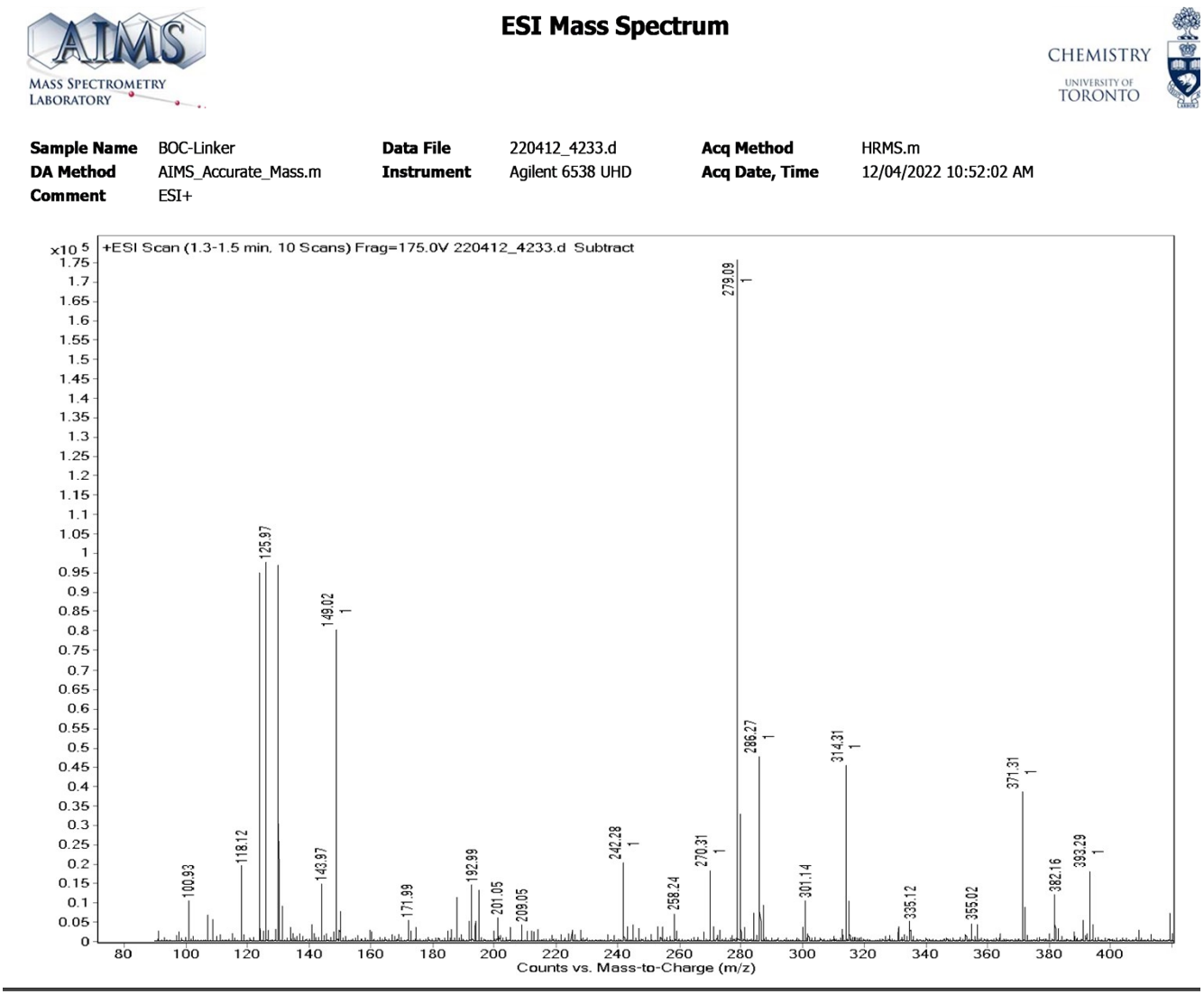



**Figure S43.** HRMS Spectrum of Compound **1**

**Figure S44.** HRMS Spectrum of Compound **5**

**Figure S45.** HRMS Spectrum of Compound **6**

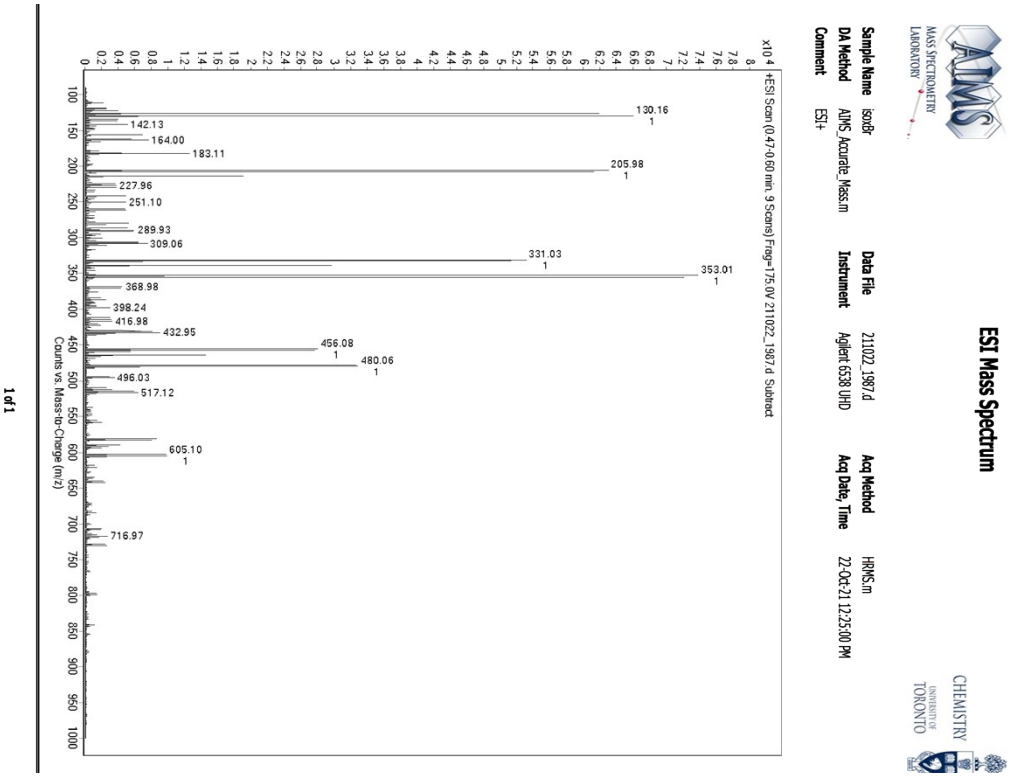



**Figure S46.** HRMS Spectrum of Compound **7**

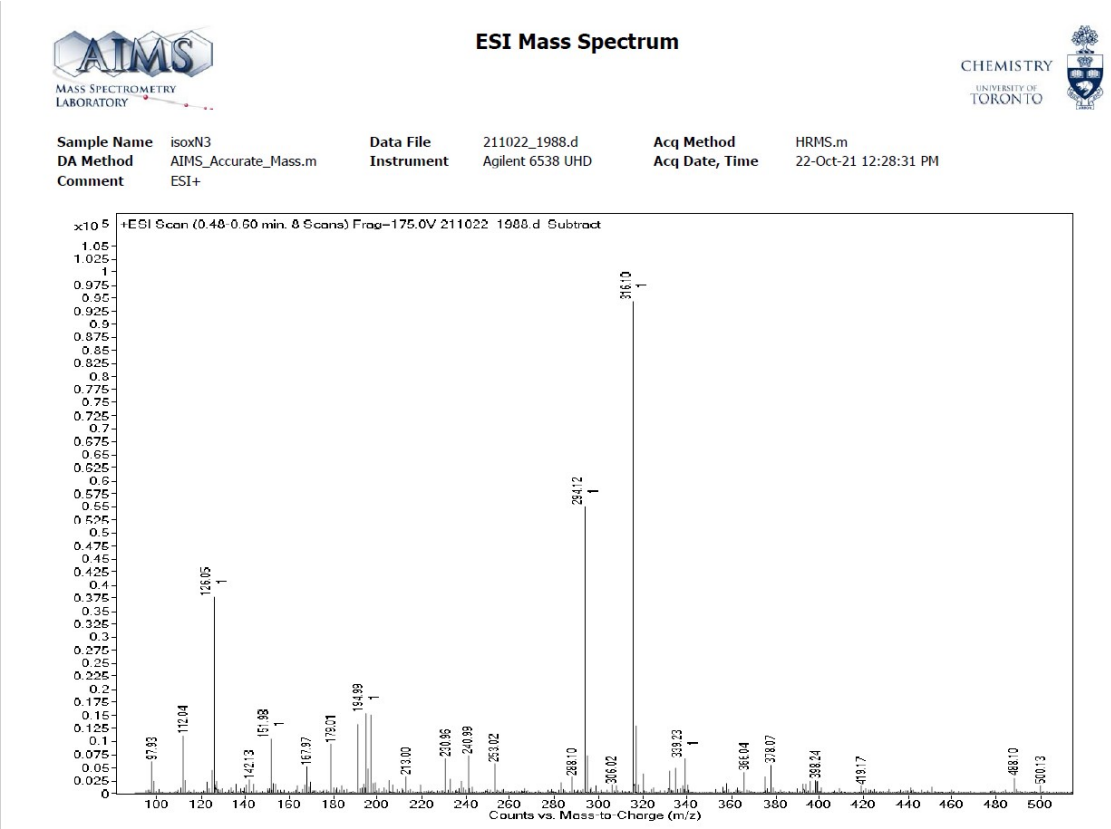



**Figure S47.** HRMS Spectrum of Compound **8**

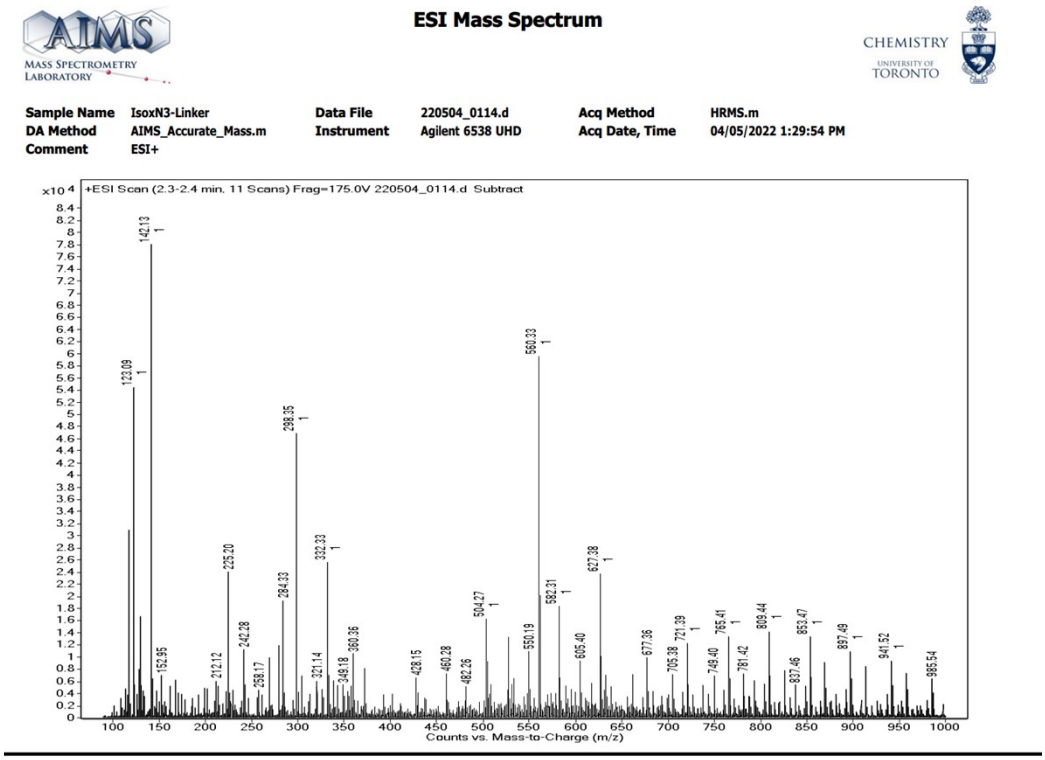



**Figure S48.** HRMS Spectrum of Compound **9**

**Figure S49.** HRMS Spectrum of Compound **10**

**Figure S50.** HRMS Spectrum of Compound **11**

**Figure S51.** HRMS Spectrum of Compound **13**

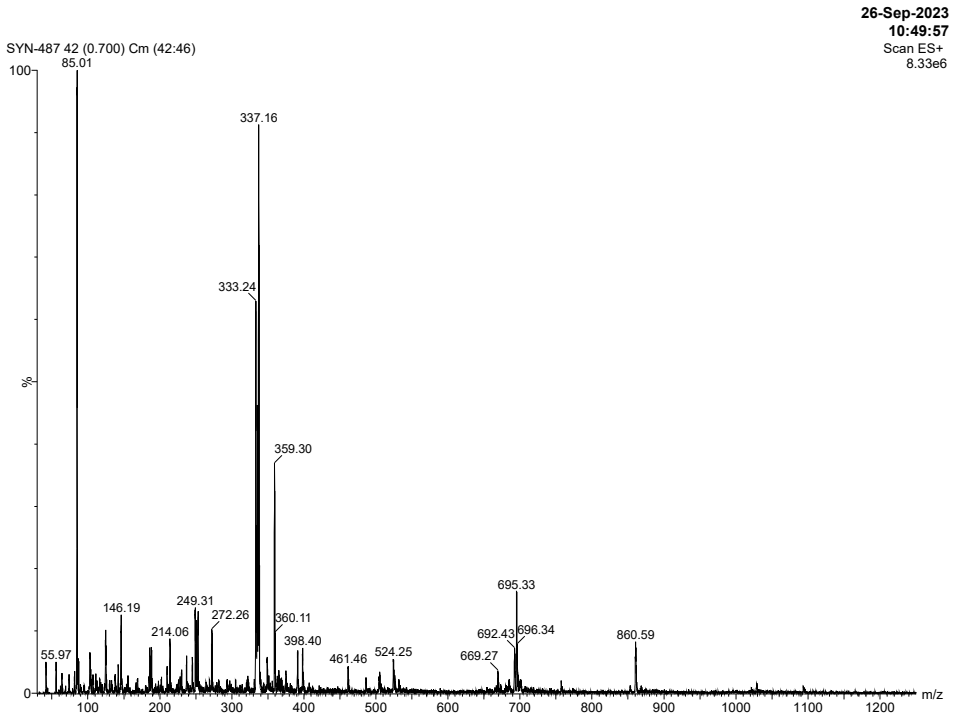



**Figure S52.** HRMS Spectrum of Compound **14**

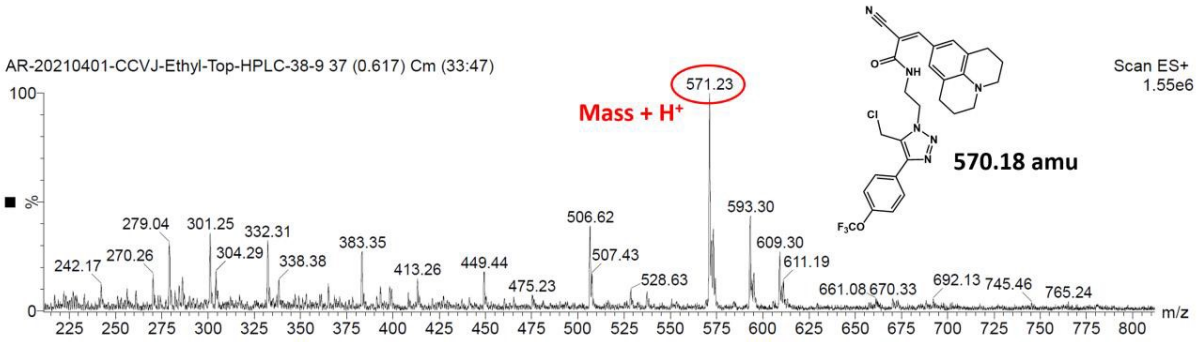



**Figure S53.** HRMS Spectrum of Compound **16**

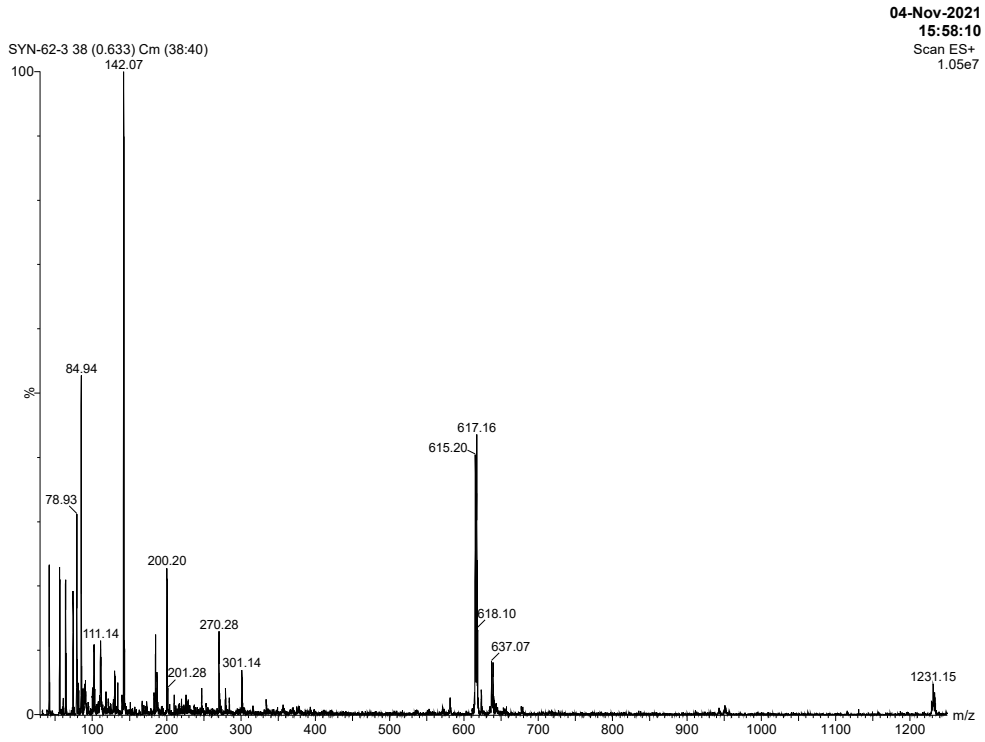



**Figure S54.** HRMS Spectrum of Compound **17**

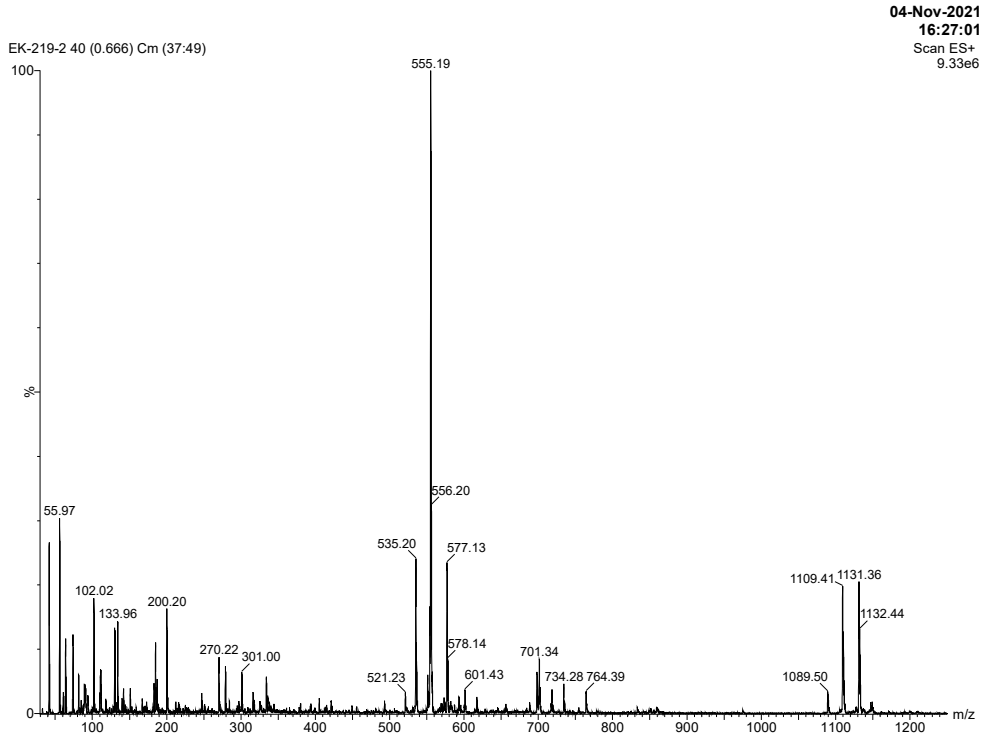



**Figure S55.** HRMS Spectrum of Compound **18**

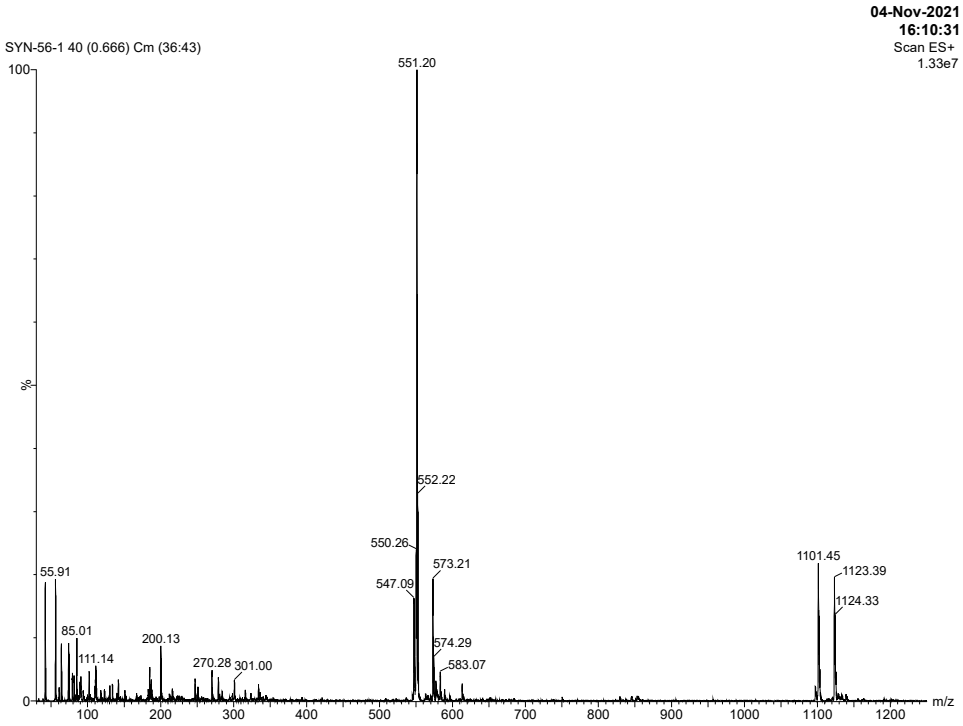



**Figure S56.** HRMS Spectrum of Compound **19**

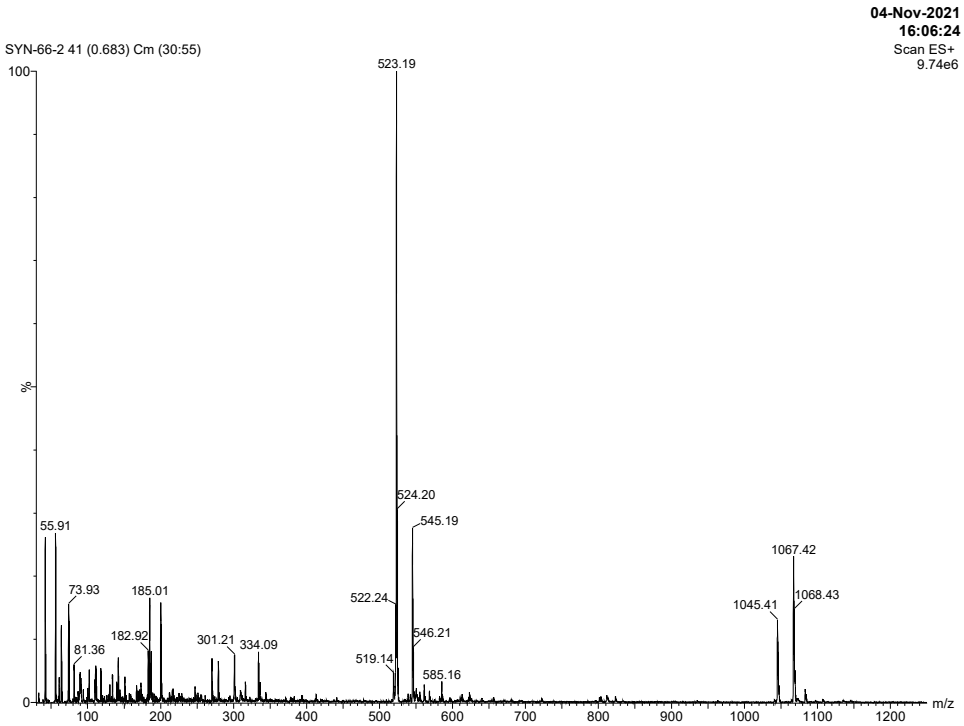



**Figure S57.** HRMS Spectrum of Compound **20**

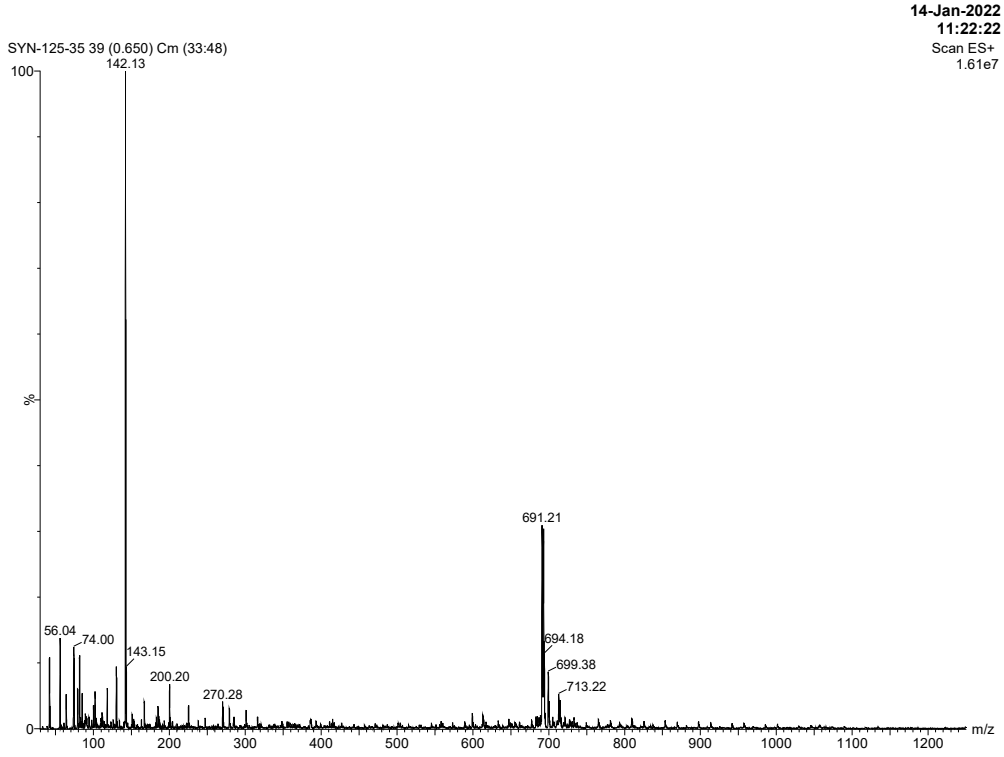



**Figure S58.** HRMS Spectrum of Compound **21**

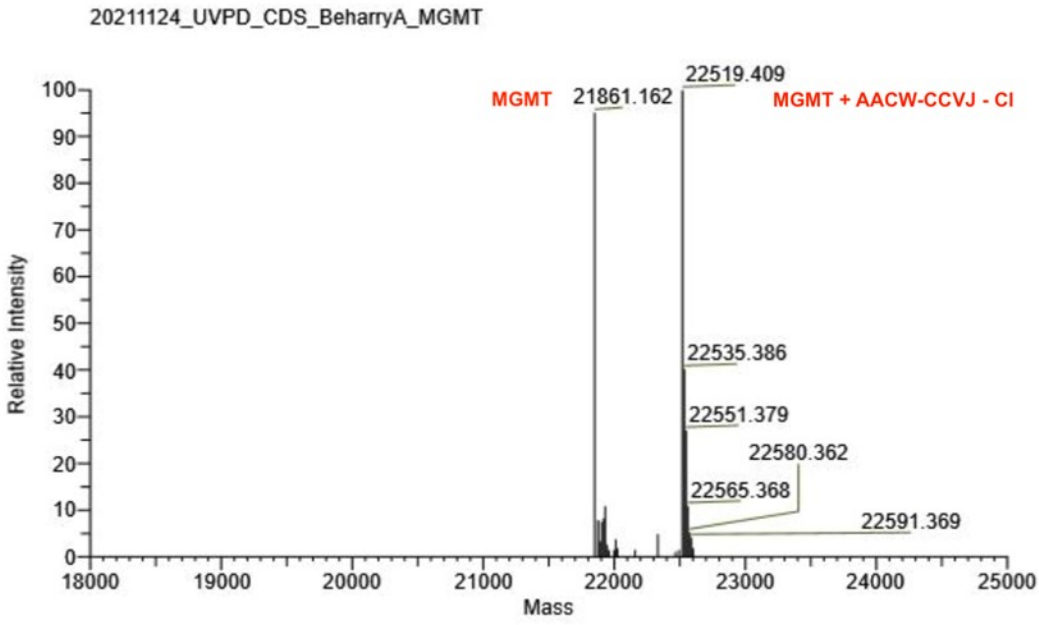



**Figure S59.** HRMS Spectrum of MGMT and MGMT+AACW-CCVJ

### 3. MGMT Purification Method

Both the MGMT protein and the C145S variant were overexpressed and purified following methods previously described in the literature.[9] Cells containing the plasmid coding for the wild-type MGMT or C145S variant were grown in a 1 L culture of LB broth + 100 μg/mL ampicillin until an OD600=0.6 was reached. The cells were then induced with 0.3 mM IPTG, incubated for 4 hours at 37℃ while shaking at 225 rpm, then harvested by centrifugation at 6000 x g at 4 °C for 30 minutes. The pellets were weighed and then resuspended in 5 mL of buffer (20 mM Tris HCl (pH 8.0), 250 mM NaCl, 20 mM β-mercaptoethanol supplemented with Complete™, Mini, EDTA-free Protease Inhibitor Cocktail Tablets) per gram of pellet. Then, the cells were homogenized by 20 strokes in a Dounce homogenizer, lysed using two rounds of French press and centrifuged at 17000 x g for 45 minutes at 4°C. The lysate was then applied to a pre-equilibrated Ni-NTA Superflow column (100 mL resuspension buffer) containing 3.5 mL of resin. The lysate was run twice through the column at a flow rate of 1 mL/minute to ensure complete binding. The column was washed with 200 mL of resuspension buffer and 20 mM of imidazole at 1 mL/minute, until the eluent had a constant OD280 reading. The protein eluted with 50 mL resuspension buffer supplemented with 200 mM imidazole at 1 mL/minute. Fractions (1 mL) were collected those which displayed protein content were pooled followed by dialysis against 4 L of dialysis buffer (50 mM Tris HCl (pH 7.6), 250 mM NaCl, 20 mM β-mercaptoethanol and 0.1 mM EDTA) using 8000 Da cut-off dialysis tubing. A yield of 8-10 mg of purified protein were typically obtained per liter of culture inoculated. The molecular weight of the MGMT protein and C145S variant were determined by ESI-MS that was run in positive ion mode on a Micromass Q-Tof Ultima API and found to be in accordance with the expected masses of 21 875 and 21 860 Da, respectively.[9]

## 4. UV-Vis and Fluorescence Assays with Purified MGMT

Absorbance spectra and fluorescence emission spectra were obtained with a Shimadzu UV-1800 Spectrophotometer and Shimadzu RF-6000 Spectro Fluorophotometer respectively. Concentrated stock solutions of all compounds were made in DMSO and diluted 100x in the cuvette containing phosphate-buffered saline (PBS) for spectroscopic readings. Fluorescence spectra was obtained with a 3 nm excitation bandwidth, 3 nm emission bandwidth, and scan speed of 600 nm/minute. Enzyme assays were performed with a 60 µL, 1 cm path length quartz cuvette, in PBS at 37 ℃. Upon probe addition an initial spectrum was acquired. The enzyme was then added, fluorescence time course was acquired until signal intensity plateaued, and then a final spectrum was acquired.

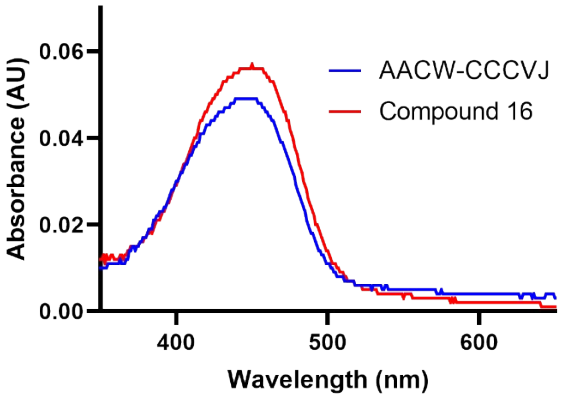



**Figure S60.** Absorbance spectra of AACW-CCVJ and Compound **16** in PBS.

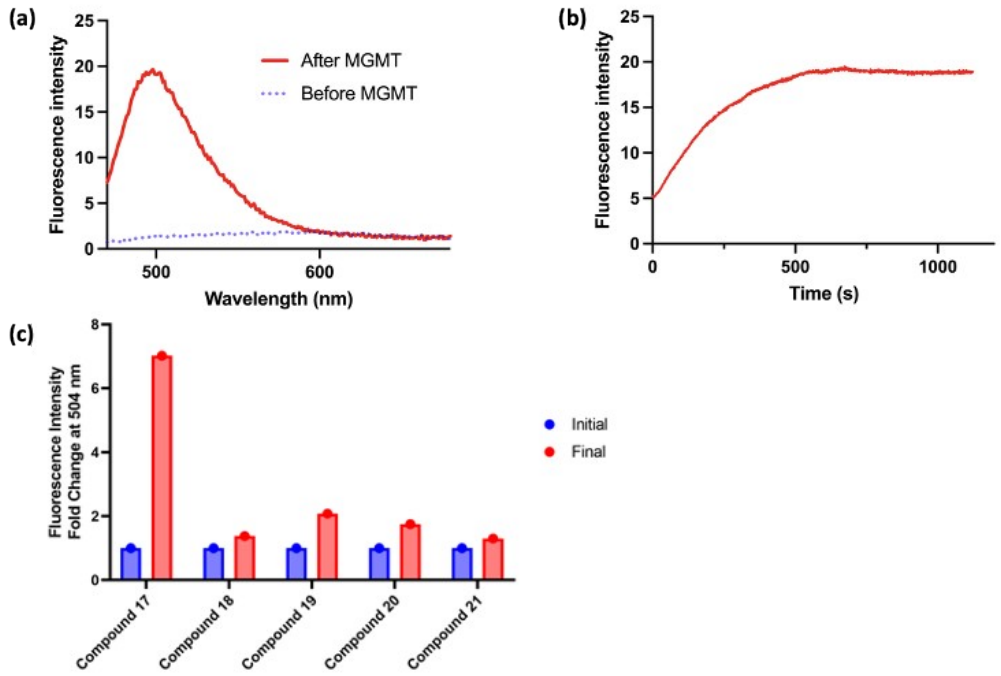



**Figure S61. (a)** Initial and final fluorescence emissions changes due to MGMT of compound **16**. **(b)** Time courses of compound **16**. (1 μM probe was incubated with 5 μM MGMT in PBS at 37 ℃. (Excitation and emission bandwidths were both set to 3 nm, and the excitation/emission wavelengths used were 450 nm and 500 nm, respectively for both (a) and (b)). **(c)** The fluorescence fold-changes due to MGMT are summarized for compounds **17**-**21** (1 μM probe was incubated with 1 μM MGMT in PBS at 37 ℃. Excitation and emission bandwidths were both set to 3 nm. The excitation and emission wavelengths were 440 nm and 504 nm, respectively.

## 5. Fluorescence Quantum Yield

The fluorescence quantum yields in PBS were determined using Perylene ($\Phi_F$ = 0.92 in EtOH) as the reference standard.[10] Separate solutions of the standard in EtOH and compounds in PBS were prepared such that the absorbances at the wavelength of maximum absorbance ($\lambda_{max}$) were between 0.04 and 0.05 AU. Emission spectra of each sample with excitation at 435 nm (1.5 nm bandwidth) were taken with emission measured (1.5 nm emission bandwidth) at 60 nm $min^{-1}$ scan speed. Recordings were performed in a quartz cell square (1 cm path length, 60 µL volume). Reference compound, AACW-CCVJ and **16** were excited at 435 nm. The fluorescence quantum yields were calculated by the following equation:

$$\Phi_s = \Phi_r \left(\frac{I_s}{I_r}\right)\left(\frac{A_r}{A_s}\right)\left(\frac{n_s^2}{n_r^2}\right)$$

where $\Phi$ represents the fluorescence quantum yield, $I$ represents the integrated fluorescence intensity, $A$ represents the absorbance at the specified wavelength, and $n$ is the refractive index of the solvent ($n$ = 1.36 for EtOH, $n$ = 1.33 for PBS). Subscripts $s$ and $r$ represent sample and reference standard, respectively. Calculated fluorescence quantum yields were shown in Figure **4**.

## 6. Kinetic Parameters

Michaelis-Menten curves were generated by addition of 100 nM MGMT to 75 – 750 nM probe in PBS at 37 ℃ and measuring the initial velocity of fluorescence increase (excitation and emission bandwidths were both set to 3 nm, and the excitation/emission wavelengths used were 450 nm and 500 nm, respectively). Each probe concentration

was performed in triplicate. The Michaelis-Menten curves were fit using GraphPad Prism 8, using the Michaelis-Menten non-linear regression function.

To calculate the second order rate constants for the reaction of AACW-CCVJ and BG-CCVJ with MGMT, the fluorescence increases of 2 – 6.5 μM probe reacting with 0.2 μM MGMT were measured. The natural log of the fluorescence values was then plotted against time (s) and the slopes of the resulting lines were the pseudo-first-order rate constants ($k_{obs}$). Plotting the $k_{obs}$ values against probe concentration yielded the second order rate constant. (excitation and emission bandwidths were both set to 3 nm, and the excitation/emission wavelengths used were 450 nm and 500 nm, respectively).

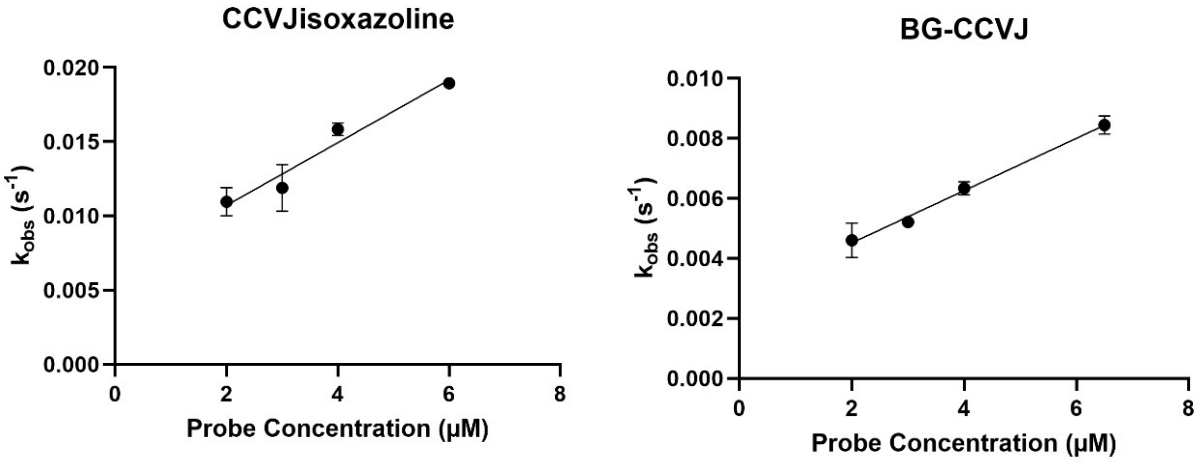



**Figure S62.**The second order rate constants for the reaction of BG-CCVJ (left) and AACW-CCVJ (right) were calculated from the fluorescence increase of 2 – 6.5 μM probe reacting with 0.2 μM MGMT and were found to be 870 ± 60 M-1s-1 for BG-CCVJ ($R^2$ = 0.98) and 2630 ± 260 M-1s-1 ($R^2$ = 0.92) for AACW-CCVJ.

**6. Detection Limit Calculation**

In order to determine the LOD, fluorescence titration experiments were conducted with increasing MGMT concentrations. Slope of the graph of emission intensity versus MGMT concentration was calculated. In addition, emission spectrum of (AACW-CCVJ) without MGMT was taken seven times and the standard deviation of the emission intensity was determined. Finally, detection limit was calculated according to given formula below; where s is the standard deviation of 7 blank measurements and m is the slope of the emission intensity versus MGMT concentration graph.

$$\text{Detection limit} = 3s/m$$

According to the formula, detection limit was found to be 0.117 μM.

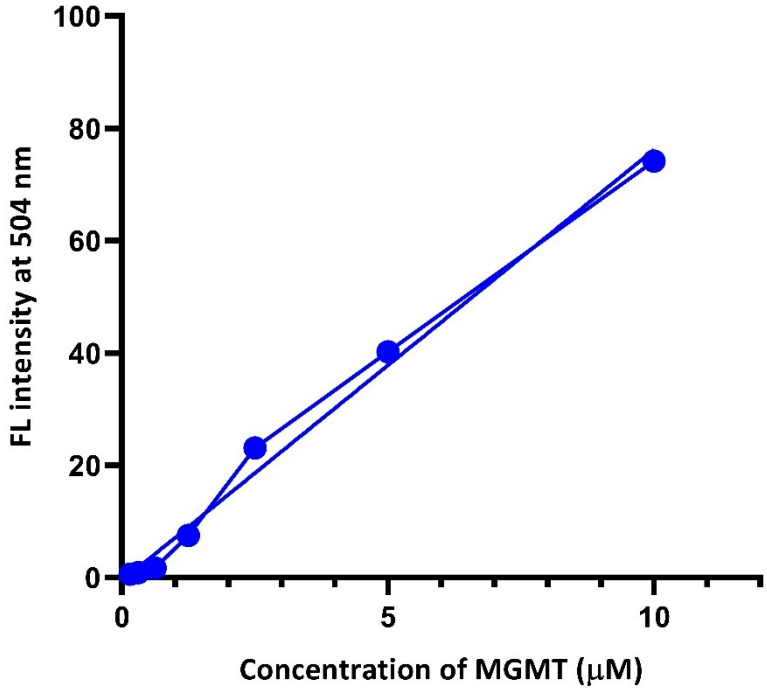



**Figure S63.** Limit of detection for the reaction of MGMT and AACW-CCVJ was calculated from the fluorescence increase of 0.15 – 10 μM MGMT reacting with 1 μM AACW-CCVJ for 1 hour at 37 ºC and was found to be 0.117 μM.

## 7. Gel electrophoresis

### 7.1 Mini SDS-PAGE Gel

The probe was incubated with MGMT in PBS with a final volume of 15 µL. After incubation at 37 ℃ for a given amount of time, the solution was 2x diluted using 2x gel loading buffer (BioRad) spiked with 50 mM DTT. The samples were submerged in a 95 ℃ water bath for 3 minutes, then loaded onto the gel (BioRad Mini-PROTEAN 4 – 20%, 10-well, 30 µL) and ran at 120 V. Fluorescence images of the gel were then taken (iBright™ 1500 or BioRad Gel Doc XR+). The gel was subsequently fixed, stained with Coomassie or silver, then imaged again.

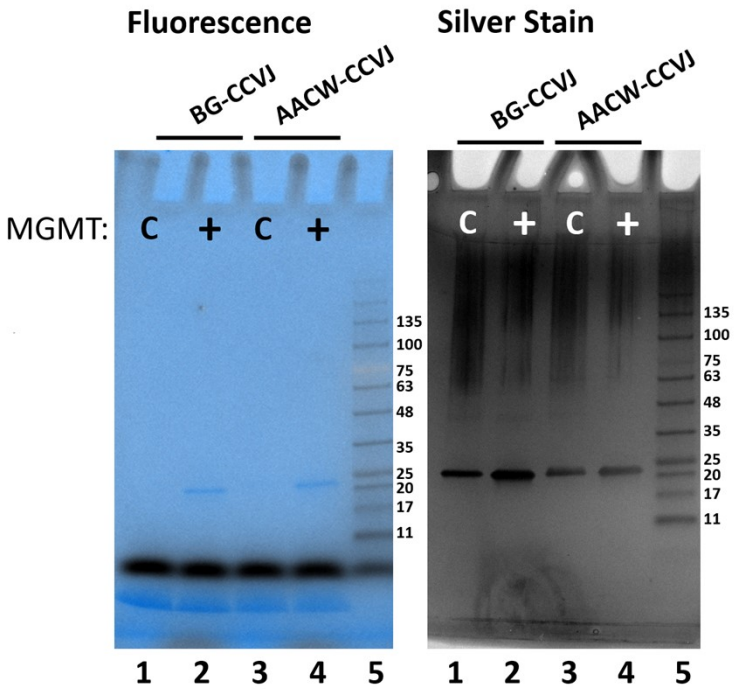



**Figure S64.** Fluorescence (left) and silver stain (right) of SDS-PAGE gel whereby 0.5 µM native MGMT (indicated by **+**) or C145S MGMT (indicated by a **C**) was incubated with 2.0 equivalents of either BG-CCVJ, AACW-CCVJ for 45 minutes at 37 ºC.

### 7.2 Large Format SDS-PAGE Gel

Casting 4-15% SDS PAGE 20 cm gel:

Plates were prepared and clamped according to the BioRad instruction manual (Instruction Manual, PROTEAN® II xi Cell IPG Conversion Kit, Rev B (Number:4106107)). 15% resolving gel was casted first leaving ¼ of the plate empty for the 4% stacking gel. MilliQ water (11.2 mL), 30% Acrylamide/Bis solution, 37.5:1 (BioRad, cat. #: 1610158, 24 mL), Tris-HCl (1.5M, pH 8.8, 12 mL), 10% SDS (480 µL in water), 10% APS (BioRad, cat. #: 1610700, 240 µL in water), and TEMED (BioRad, cat. #: 1610800, 48 µL) were added and carefully mixed in a 50 mL falcon tube. The solution was carefully added to the clamped plates. Using a syringe and needle, water was carefully added on top of the gel layer to level it. After 1hour, the water was removed from the plates. MilliQ water (12.2 mL), 30% Acrylamide/Bis solution, 37.5:1 (2.6 mL), Tris-HCl (0.5M, pH 6.8, 5 mL), 10% SDS (200 µL in water), 10% APS (100 µL in water), and TEMED (20 µL) were added to a 50 mL falcon tube and mixed gently. This solution was added on top of the resolving polymerized gel. A 15 well comb was added immediately thereafter. The gel was allowed to polymerize for another hour. Finally, the comb was removed, and wells were washed with MilliQ water and 1x SDS Tris glycine buffer. The gel was ready for use.

### 7.3 Cell Culture

U251 (provided by Dr. Sunit Das, SickKids) was cultured with Dulbecco's Modified Eagle's medium (DMEM) (Sigma-Aldrich, D6429), T98G (ATCC) and HeLa-S3 (CEDARLANE) cells were cultured with Minimum Essential Medium Eagle (EMEM) (Sigma-Aldrich, M4655). For all media, 10% FBS and 1% antibiotic-anti-mitotic (A.A.) solution was used, except for U251, where 1% Pen-strep was used instead of A.A.

Cells were grown in T75 flasks and were maintained in a 5% $CO_2$ atmosphere at 37 ºC.

### 7.4 Nucleus/Cytoplasm Fractionation

Nuclear and cytoplasmic cell lysates were prepared by using the standard osmotic shock protocol.[11] T98G and U251 cell lysates concentrations were determined by using BCA assays (PierceTM BCA protein Assay Kit, catalog #: 23225).

The **AACW-CCVJ** and compound **16** were incubated with MGMT, nuclear cell lysates of T98G and U251 in hypotonic buffer (10 mM HEPES, pH7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1x PI, and 0.5mM DTT) with a final volume of 35 µL. After incubation at 37 ℃ for 1 hour, the solution was 2x diluted using 2x gel loading buffer (BioRad) spiked with 50 mM BME. The samples were submerged in a 95 ℃ water bath for 3 minutes, then loaded onto the gel and ran at 100 V for 2 hours, then at 120 V at 48 hours. Fluorescence images of the gel were then taken (iBright ™ 1500). The gel was subsequently fixed, stained with silver, then imaged again.

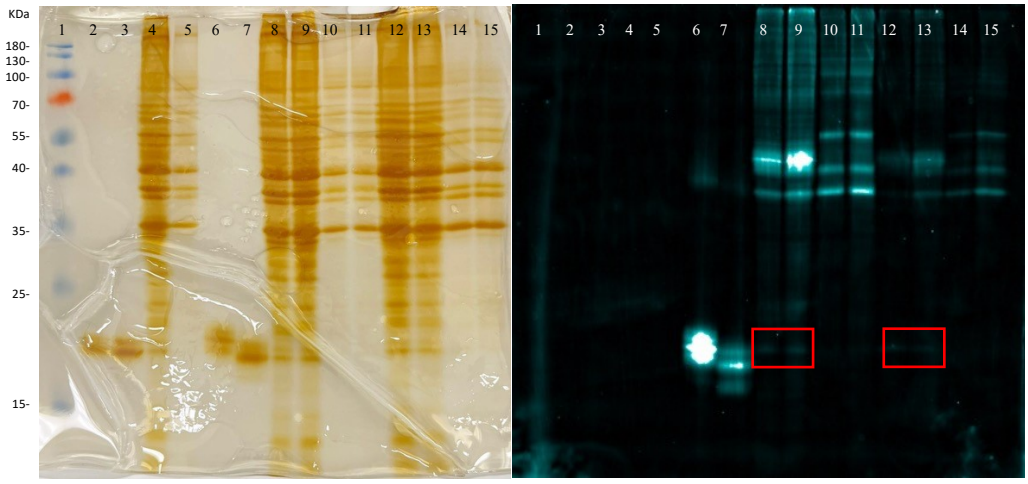



**Figure S65.** SDS-PAGE gels investigating the labelling of pure MGMT as well as T98G (MGMT positive, 100 µg) and U251 (MGMT negative, 100 µg) cell lysates by **AACW-CCVJ** and Compound **16**. Pure MGMT and lysates were incubated with either 5 µM or 10 µM **AACW-CCVJ** and **16** for 1 hour at 37 ºC, ran on an SDS-PAGE gel, then fluorescence imaging was performed (ex: 455-485 nm, em: 508-557 nm) (right), followed by Silver staining (left). Lane 1: Ladder, Lane 2: 2µg MGMT, Lane 3: 2µg MGMT, Lane 4: T98G lysate (100 µg), Lane 5: U251 lysate (100 µg), Lane 6: 4 µg MGMT + 10 µM AACW-CCVJ, Lane 7: 4 µg MGMT + 10 µM Compound 16, Lane 8: T98G lysate (100 µg) +5 µM AACW-CCVJ, Lane 9: T98G lysate (100 µg) + 10 µM AACW-CCVJ, Lane 10: U251 lysate (100 µg) + 5 µM AACW-CCVJ, Lane 11: U251 lysate (100 µg) + 10 µM AACW-CCVJ, Lane 12: T98G lysate (100 µg) + 5 µM Compound 16, Lane 13: T98G lysate (100 µg) + 10 µM Compound 16, Lane 14: U251 lysate (100 µg) + 5 µM Compound 16. The red boxes demonstrate the MGMT bands.

**8. Western Blot**

Western blot analysis of T98G, HeLa S3, and U251 lysates was performed to confirm MGMT positivity of these lysates, and cell lines (Fig. S64). Cell pellets ($10^7$ cells) were lysed using 0.5 mL RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 8.0), with 1x protease inhibitor (Roche complete mini EDTA-free tablet, 11836153001). After incubating on ice for 1 hour, lysates were centrifuged (10,000 rpm, for 10 min at 4 ℃) and the supernatant (containing the lysate) was transferred to separate tubes. BCA assay (Thermo Scientific micro-BCA protein assay kit, cat. #: 23235) was used to measure protein concentration. Lysates were then separated by SDS-PAGE (100 µg or 40 µg lysate per well) and subjected to western blotting. MGMT monoclonal antibody (cat. #: MA5-13506, Invitrogen) with dilution of 1:400, goat anti-mouse HRP-conjugated secondary antibody (cat. #: 170-5047, Bio-Rad) with dilution of 1:16,000, and beta-actin (cat. #: ab20272, Abcam) with dilution of 1:5,000 were used. The membrane was visualized by chemiluminescence (ECL) Western Blotting Substrate (cat. #. 170-50-60, Bio-Rad,) on iBright™ 1500.

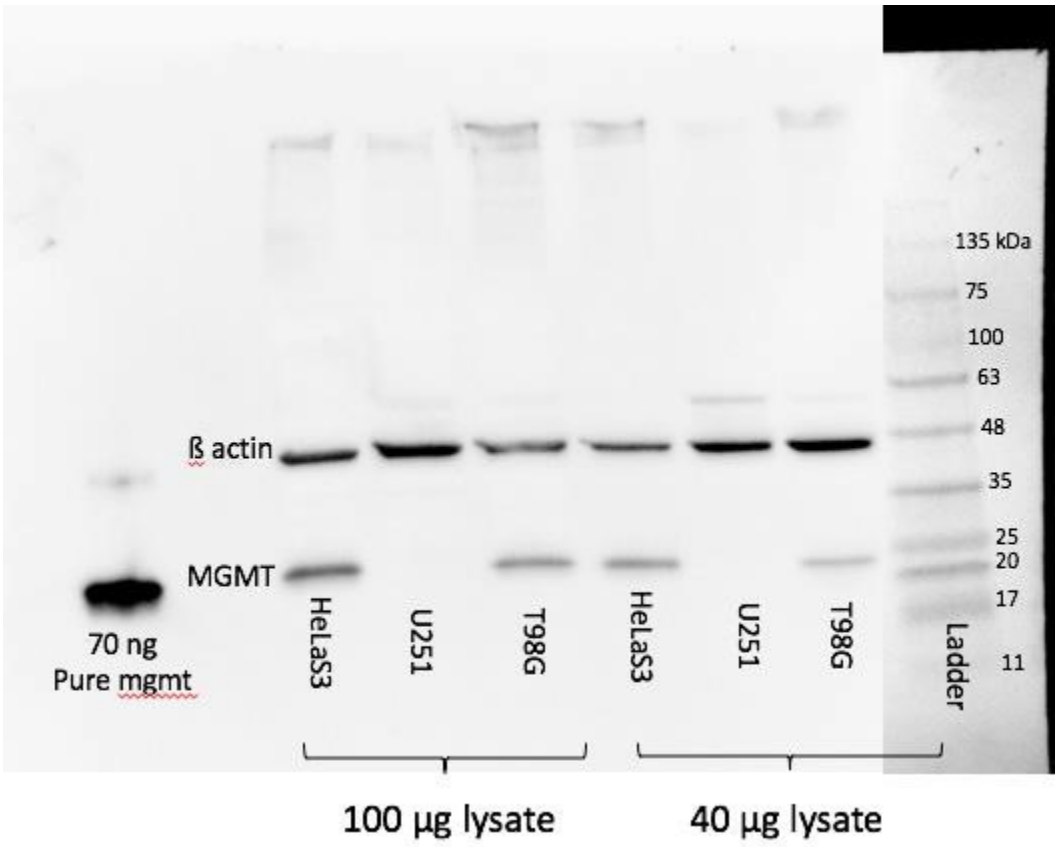



**Figure S66.** Western blot analysis for MGMT of T98G, U251 and HeLa S3 lysates

## 9. Cell Viability Assay

In order to determine the effects of AACW-CCVJ on cells, we performed MTT experiments, as previously reported in the literature.[12] Briefly, T98G cells were seeded at a density of 10,000 cells per well in 96-well plates (Thermo Scientific NunclonTM Delta Surface) and incubated with 100 µL EMEM overnight at 37 °C in 5% $CO_2$. On the following day, AACW-CCVJ was added into wells in triplicate in varying concentrations (10–0.3 µM). After the cells were incubated for 24 h, 20 µL of 5 mg/mL solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphneyltetrazolium bromide (MTT) (Alfa Aesar) in PBS was added to each well. Following 3 h of incubation, the solution was removed and replaced with 150 µL of DMSO to solubilize the formazan. Absorbance values at 565 nm of each well were determined by a Tecan Infinite M1000 plate reader.

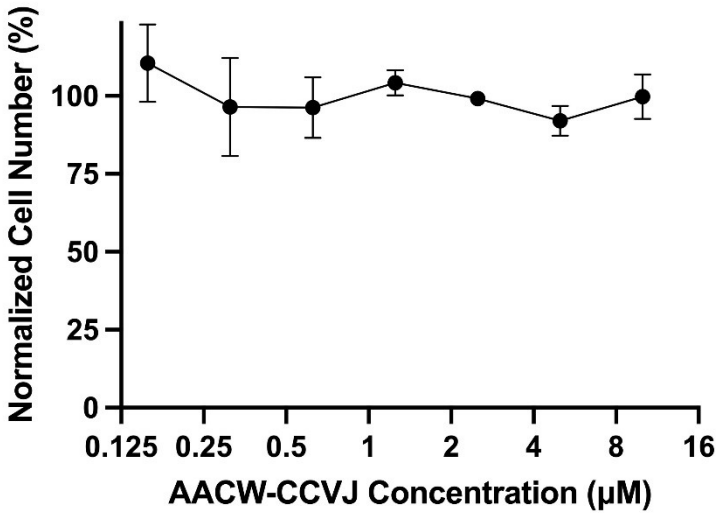



**Figure S67.** Cell viabilities at varying concentrations of AACW-CCVJ. T98G cells were treated with increasing concentrations (10–0.3 µM) of AACW-CCVJ. Data are presented as mean ± SD (n = 3).

## 10. References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