Electronic Supplementary Information (ESI)

Photoactivatable AMPA for the Study of Glutamatergic Neuronal Transmission Using Two-Photon Excitation

Davide Deodato,[a] Naeem Asad,[a] and Timothy M. Dore*[a,b]

[a]New York University Abu Dhabi, PO Box 129188, Abu Dhabi, United Arab Emirates.
[b]Department of Chemistry, University of Georgia, Athens, GA 30602, USA.

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General

Reagents and solvents were obtained from commercial sources and used as purchased without further purification. Compounds **1** and **4c** were prepared as previously described.[1, 2] For reactions carried out above room temperature, an oil bath was used as the source of heating. Anhydrous methanol was obtained with a PureSolv solvent purification system (Innovative Technology). Purification was carried out using flash chromatography on an Isolera Spektra 4 with Biotage SNAP cartridges packed with KPSIL silica. HPLC analysis and preparative HPLC purifications were carried on an Agilent Infinity series system fitted with an autosampler and diode array detector using Zorbax Eclipse C-18 reverse phase columns, having a mobile phase composed of water with 0.1% TFA and acetonitrile. HRMS was performed on an Agilent 6540 HD Accurate Mass QTOF/LC/MS with electrospray ionization (ESI). UV spectra were recorded on a Cary 5000 UV−vis−near-infrared (NIR) spectrophotometer (Agilent). Emission spectra were obtained with a Perkin Elmer LS 55 fluorescence spectrometer. ^{1}H NMR and ^{13}C NMR spectra were recorded using a Bruker Avance III HD 500 MHz NMR spectrometer. KMOPS buffer consisted of 100 mM KCl and 10 mM MOPS (3-(N-morpholino)propanesulfonic acid) titrated to pH 7.2 with NaOH 0.1 N.

Synthesis

4-Methoxy-7-(methoxymethoxy)-2-methylquinoline-8-carbonitrile (**2a**). Sodium (28 mg, 1.22 mmol) was dissolved in dry MeOH (20 mL) under argon atmosphere with stirring. After complete dissolution, a solution of **1**[1] (200 mg, 0.76 mmol) in dry MeOH (20 mL) was added dropwise. The mixture was stirred at refluxing conditions for 4 h. After cooling the reaction was quenched with 5% citric acid (30 mL) and extracted with EtOAc (3 × 30 mL), washed with H_2O (3 × 20 mL) and brine (3 × 20 mL), dried over $MgSO_4$, and concentrated to dryness. The resulting residue was purified by column chromatography (0 to 70% EtOAc in n-hexane, gradient elution) to yield compound **2a** as a light yellow powder (180 mg, 0.70 mmol, 92% yield). ^{1}H NMR (500 MHz, chloroform-*d*): δ 8.11 (d, *J* = 9.3 Hz, 1H), 7.28 (d, *J* = 9.3 Hz, 1H), 6.56 (s, 1H), 5.38 (s, 2H), 3.99 (s, 3H), 3.54 (s, 3H), 2.65 (s, 3H); ^{13}C NMR (126 MHz, chloroform-*d*): δ 164.0, 162.3, 162.1, 149.6, 128.0, 115.2, 115.0, 113.0, 100.7, 98.5, 95.0, 56.8, 55.9, 26.1; HRMS (ESI-QTOF) *m/z*: $[M + H]^+$ calcd for $C_{14}H_{14}N_2O_3$, 259.1077; found, 259.1067.

7-(Methoxymethoxy)-2-methyl-4-(3,4,5-trimethoxyphenyl)quinoline-8-carbonitrile (**2b**). Compound **1** (700 mg, 0.67 mmol), 3,4,5-trimethoxyphenylboronic acid (284 mg, 1.34 mmol), sodium iodide (201 mg, 1.34 mmol) and cesium carbonate (655 mg, 2.01 mmol) were dissolved in anhydrous dioxane (50 mL) under a N_2 atmosphere. A solution of RuPhos (31 mg, 0.067 mmol) and palladium acetate (15 mg, 0.067 mmol) in anhydrous dioxane (2 mL) was added dropwise. The mixture was heated to 80 °C and stirred under a N_2 atmosphere for 5 h. After cooling the mixture was diluted with EtOAc (200 mL) and filtered through celite. The filtrate was washed with H_2O (3 × 50 mL), saturated $NaHCO_3$ solution (2 × 50 mL), and brine (2 × 50 mL). The organic layer was dried over $MgSO_4$, concentrated to dryness, and purified by column chromatography (0 to 80% EtOAc in n-hexane, gradient elution) furnishing compound **2b** as a yellow powder (80 mg, 72% yield). The ^{1}H NMR and HRMS spectra of **2b** matched the published data.[1]

2-(Hydroxymethyl)-4-methoxy-7-(methoxymethoxy)quinoline-8-carbonitrile (**3a**). To a suspension of selenium dioxide (456 mg, 4.07 mmol) in dioxane (30 mL), an aqueous 70% solution of tert-butyl hydroperoxide (0.2 mL, 1.36 mmol) was added and the mixture was stirred at 50 °C for 15 min. A solution compound **2a** (350 mg, 1.36 mmol) in dioxane (20 mL) was added dropwise. The mixture was heated to 70 °C and stirred for 4 h. After cooling, the mixture was diluted with EtOAc (200 mL) and filtered through a pad of celite. The filtrate was washed with H_2O (3 × 50 mL), saturated $NaHCO_3$ solution (2 × 50 mL), and brine (3 × 50 mL). The organic layer was dried over $MgSO_4$, concentrated under reduced pressure and the residue was dissolved in ethanol (50 mL). Sodium borohydride (205 mg, 5.40 mmol) was added in small portions, and the mixture was stirred at room temperature for 6

h. The solvent was evaporated under reduced pressure and the residue was dissolved in EtOAc (200 mL), washed with H_2O (3 × 50 mL) and brine (3 × 50 mL), dried over $MgSO_4$, and concentrated to dryness. The resulting residue was purified by column chromatography (0 to 80% EtOAc in n-hexane, gradient elution) to yield the benzylic alcohol **3a** as a sticky oil (335 mg, 1.22 mmol, 94% yield). ^{1}H NMR (500 MHz, chloroform-*d*): δ 8.21 (d, *J* = 9.2 Hz, 1H), 7.38 (d, *J* = 9.3 Hz, 1H), 6.63 (s, 1H), 5.44 (s, 2H), 4.86 (s, 2H), 4.06 (s, 3H), 3.59 (s, 3H); ^{13}C NMR (126 MHz, chloroform-*d*): δ 164.0, 162.9, 162.3, 148.4, 128.3, 115.9, 114.6, 113.7, 98.7, 96.9, 95.0, 64.4, 56.9, 56.2; HRMS (ESI-QTOF) *m*/*z*: $[M + H]^+$ calcd for $C_{14}H_{14}N_2O_4$, 275.1026; found, 275.1025.

2-(Hydroxymethyl)-7-(methoxymethoxy)-4-(3,4,5-trimethoxyphenyl)quinoline-8-carbonitrile (**3b**)**.** Prepared from **2b** as previously described.[1]

General procedure for the preparation of methanesulfonate esters **4a**-**c**.[2] A round-bottomed flask containing a solution of the alcohol precursor (0.68 mmol) in CH_2Cl_2 (20 mL) was placed into an ice bath and cooled to 0 °C. Triethylamine (0.38 mL, 2.72 mmol) was added followed by slow dropwise addition of methanesulfonyl chloride (0.16 mL, 2.05 mmol). After stirring at 0 °C for 15 min the ice bath was removed and the mixture was stirred at room temperature for 12 h. The reaction was diluted with CH_2Cl_2 (150 mL), washed with H_2O (3 × 100 mL) and brine (3 × 100 mL), dried over $MgSO_4$, and concentrated to dryness. The resulting residue was purified by column chromatography (0 to 70% EtOAc in n-hexane, gradient elution) to yield the corresponding methanesulfonate ester.

(8-Cyano-4-methoxy-7-(methoxymethoxy)quinolin-2-yl)methyl methanesulfonate (**4a**). (167 mg, 0.47 mmol, 75% yield). ^{1}H NMR (500 MHz, chloroform-*d*): δ 8.33 (d, *J* = 9.6 Hz, 1H), 7.48 (d, *J* = 9.5 Hz, 1H), 6.90 (s, 1H), 5.49 (s, 2H), 5.46 (s, 2H), 4.12 (d, *J* = 7.8 Hz, 3H), 3.60 (s, 3H), 3.30 (s, 3H); ^{13}C NMR (126 MHz, chloroform-*d*): δ 163.6, 162.7, 158.6, 149.5, 128.4, 115.9, 114.8, 114.6, 99.1, 98.4, 95.0, 72.1, 56.9, 56.3, 38.4; HRMS (ESI-QTOF) *m*/*z*: $[M + H]^+$ calcd for $C_{15}H_{16}N_2O_6S$, 353.0802; found, 353.0816.

(8-Cyano-7-(methoxymethoxy)-4-(3,4,5-trimethoxyphenyl)quinolin-2-yl)methyl methanesulfonate (**4b**). (222 mg, 0.46 mmol, 68% yield). ^{1}H NMR (500 MHz, chloroform-*d*): δ 8.15 (d, *J* = 9.5 Hz, 1H), 7.53 (d, *J* = 9.5 Hz, 1H), 7.49 (s, 1H), 6.65 (s, 2H), 5.58 (s, 2H), 5.46 (s, 2H), 3.95 (s, 3H), 3.90 (s, 6H), 3.59 (s, 3H), 3.31 (s, 3H); ^{13}C NMR (126 MHz, chloroform-*d*): δ 162.3, 156.5, 153.5, 150.5, 149.0, 138.8, 132.2, 132.0, 121.6, 119.0, 116.0, 114.7, 106.7, 99.8, 95.0, 71.6, 61.0, 57.0, 56.4, 38.4; HRMS (ESI-QTOF) *m*/*z*: $[M + H]^+$ calcd for $C_{23}H_{24}N_2O_8$, 489.1326; found, 489.1316.

(8-Cyano-7-(methoxymethoxy)quinolin-2-yl)methyl methanesulfonate (**4c**). (190 mg, 0.59 mmol, 87% yield); HRMS (ESI-QTOF) *m*/*z*: $[M + H]^+$ calcd for $C_{14}H_{14}N_2O_5$, 323.0696; found, 323.0702. The ^{1}H NMR spectrum of **4c** matched the published data.[2]

General procedure for the preparation of caged hymexazol derivatives. To a stirred solution of 3-hydroxy-5-methylisoxazole (hymexazol) (20 mg, 0.06 mmol) in acetone (3 mL), potassium carbonate (25 mg, 0.18 mmol) was added. The mixture was stirred at room temperature for 10 min and then a solution of the MOM-protected methanesulfonate ester **4a**-**c** (0.8 mmol) in acetone (2 mL) was added dropwise. The resulting mixture was stirred at refluxing conditions until the reaction was completed (1-3 h), monitoring by TLC or HPLC. The solvent was evaporated under reduced pressure and the residue was dissolved in CH_2Cl_2 (20 mL), washed with H_2O (3 × 10 mL) and brine (3 × 10 mL), dried over $MgSO_4$ and concentrated to dryness. The resulting residue was purified by column chromatography (0 to 80% EtOAc in n-hexane, gradient elution) to yield the corresponding MOM-protected hymexazol derivative, which was dissolved in CH_2Cl_2 (2 mL). TFA (0.2 mL) was added dropwise, and the reaction was stirred at room temperature in the dark until HPLC showed complete consumption of the starting material (2-5 h). After evaporation of the solvent, the resulting residue was purified by trituration with diethyl ether, affording the caged hymexazol derivatives **5a**-**c**.

7-Hydroxy-4-methoxy-2-(((5-methylisoxazol-3-yl)oxy)methyl)quinoline-8-carbonitrile (**5a**). (13 mg, 0.04 mmol, 67% yield). ^{1}H NMR (500 MHz, methanol-d_4): δ 8.30 (d, *J* = 9.2 Hz, 1H), 7.23 (d, *J* = 9.2 Hz, 1H), 7.15 (s, 1H), 5.99 (s, 1H), 5.48 (s, 2H), 4.14 (s, 3H), 2.37 (s, 3H); ^{13}C NMR (126 MHz, methanol-d_4): δ 171.8, 171.5, 165.0, 164.6, 160.2, 148.6, 128.2, 116.7, 114.3, 114.0, 97.9, 93.3, 92.4, 71.1, 55.8, 11.3; HRMS (ESI-QTOF) *m/z*: [M + H]$^+$ calcd for $C_{16}H_{13}N_3O_4$, 312.0979; found, 312.0732.

7-Hydroxy-2-(((5-methylisoxazol-3-yl)oxy)methyl)-4-(3,4,5-trimethoxyphenyl)quinoline-8-carbonitrile (**5b**). (22 mg, 0.05 mmol, 83% yield). ^{1}H NMR (500 MHz, methanol-d_4): δ 8.07 (dd, *J* = 9.4, 1.6 Hz, 1H), 7.50 (d, *J* = 1.7 Hz, 1H), 7.26 (dd, *J* = 9.4, 1.7 Hz, 1H), 6.81 (d, *J* = 1.6 Hz, 2H), 5.96 (s, 1H), 5.51 (d, *J* = 1.7 Hz, 2H), 3.89 (dd, *J* = 11.5, 1.6 Hz, 9H), 2.36 (s, 3H); ^{13}C NMR (126 MHz, methanol-d_4): δ 171.8, 171.4, 164.1, 158.2, 158.2, 153.4, 150.2, 149.1, 138.4, 132.9, 131.9, 112.0, 117.9, 114.8, 106.8, 94.7, 94.8, 71.6, 59.8, 55.4, 11.3; HRMS (ESI-QTOF) *m/z*: [M + H]$^+$ calcd for $C_{24}H_{21}N_3O_6$, 448.1503; found, 448.1398.

7-Hydroxy-2-(((5-methylisoxazol-3-yl)oxy)methyl)quinoline-8-carbonitrile (**5c**). (12 mg, 0.04 mmol, 66% yield). ^{1}H NMR (500 MHz, methanol-d_4): δ 8.31 (d, *J* = 8.4 Hz, 1H), 8.04 (d, *J* = 9.1 Hz, 1H), 7.59 (d, *J* = 8.4 Hz, 1H), 7.29 (d, *J* = 9.1 Hz, 1H), 5.97 (d, *J* = 1.1 Hz, 1H), 5.51 (s, 2H), 2.37 (d, *J* = 0.9 Hz, 3H);^{13}C NMR (126 MHz, methanol-d_4): δ 171.9, 171.4, 164.3, 158.8, 148.5, 137.3, 133.9, 121.7, 117.9, 117.4, 114.7, 94.3, 92.5, 71.7, 11.3; HRMS (ESI-QTOF) *m/z*: [M + H]$^+$ calcd for $C_{15}H_{11}N_3O_3$, 282.0873; found, 282.0847.

4-(Bromomethyl)-2-(methoxymethyl)-5-methylisoxazol-3(2H)-one (**6**). 3-hydroxy-5-methylisoxazole (hymexazol) (5.0 g, 51.0 mmol) and 1,3,5-trioxane (9.2 g, 102.0 mmol) were dissolved in 50 mL of 47% aqueous hydrobromic acid and sulfuric acid (3 mL) was added dropwise. The resulting mixture was heated at 65 °C and stirred at this temperature for 12 h. After cooling, the mixture was extracted with CH_2Cl_2 (5 × 50 mL) and MeOH (125 mL) was added to the combined organic phases. The solution was stirred at room temperature for 2 h and then diluted with CH_2Cl_2 (500 mL). The solution was washed with H_2O (3 × 200 mL), dried over $MgSO_4$ and concentrated to dryness. The resulting residue was purified by column chromatography (0 to 100% EtOAc in n-hexane, gradient elution) affording compound **6** as a clear oil (1.9 g, 8.1 mmol, 16% yield). ^{1}H NMR (500 MHz, chloroform-*d*): δ 5.13 (s, 2H), 4.17 (s, 2H), 3.38 (s, 3H), 2.30 (s, 3H); ^{13}C NMR (126 MHz, chloroform-*d*) δ 169.0, 166.0, 107.4, 75.5, 57.2, 18.7, 12.2; HRMS (ESI-QTOF) *m/z*: [M + H]$^+$ calcd for $C_7H_{10}BrNO_3$, (^{79}Br) 235.9917 and (^{81}Br) 237.9897; found, (^{79}Br) 235.9853 and (^{81}Br) 237.9732.

Diethyl 2-acetamido-2-((2-(methoxymethyl)-5-methyl-3-oxo-2,3-dihydroisoxazol-4-yl)methyl)malonate (**7**). To a solution of diethyl acetamidomalonate (1.6 g, 4.68 mmol) in DMF (20 mL), a 60% suspension of sodium hydride in mineral oil (221 mg, 5.53 mmol) was added portion-wise over a period of 15 min. A solution of 6 (1.0 g, 4.25 mmol) in DMF (5 mL) was added dropwise and the mixture was stirred at room temperature for 12 h. Acetic acid (1 mL) was added and the mixture was partially evaporated under reduced pressure. The residue was diluted with CH_2Cl_2 (200 mL), washed with H_2O (2 × 50 mL), brine (2 × 50 mL) and 10% LiCl (2 × 30 mL), dried over $MgSO_4$, and concentrated to dryness. The resulting residue was purified by column chromatography (0 to 80% EtOAc in n-hexane, gradient elution) affording compound **7** as a white powder (1.2 g, 3.2 mmol, 75% yield). ^{1}H NMR (500 MHz, chloroform-*d*): δ 5.08 (s, 2H), 4.32 – 4.17 (m, 4H), 3.36 (s, 3H), 3.28 (s, 2H), 2.16 (s, 3H), 2.01 (s, 3H), 1.27 (td, *J* = 7.2, 4.6 Hz, 6H); ^{13}C NMR (126 MHz, chloroform-*d*): δ 169.6, 168.8, 167.8, 167.5, 103.8, 75.1, 65.5, 62.8, 57.1, 26.0, 22.9, 13.9, 11.7; HRMS (ESI-QTOF) *m/z*: [M + H]$^+$ calcd for $C_{16}H_{24}N_2O_8$, 373.1605; found, 373.1567.

1-Carboxy-2-(3-hydroxy-5-methylisoxazol-4-yl)ethan-1-aminium 2,2,2-trifluoroacetate (± AMPA). Compound **7** (300 mg, 0.81 mmol) was dissolved in a 1 M aqueous solution of TFA (10 mL). The

mixture was stirred at 120 °C for 12 h. After cooling, the solution was diluted with H_2O (5 mL) and concentrated under reduced pressure (this process was repeated three times). Cold acetonitrile (3 mL) was added to the viscous oil and the resulting precipitate was filtered and carefully washed with cold acetonitrile (3 mL). The white solid obtained (96 mg) was carried to the next step. Further 34 mg were obtained from the mother liquor after evaporation and purification with preparative HPLC. AMPA was isolated as a TFA salt (130 mg, 0.43 mmol, 55% yield). ^{1}H NMR (500 MHz, deuterium oxide): δ 4.24 (td, *J* = 6.0, 1.3 Hz, 1H), 3.70 (d, *J* = 0.8 Hz, 2H), 2.97 – 2.93 (m, 2H), 2.24 (s, 3H); ^{13}C NMR (126 MHz, deuterium oxide): δ 171.1, 170.4, 170.1, 162.8 (q, $^2J_{C-F}$ = 36.2 Hz), 116.3 (q, $^1J_{C-F}$ = 292.6 Hz), 100.4, 52.3, 22.2, 10.9; HRMS (ESI-QTOF) *m/z*: $[M + H]^+$ calcd for $C_7H_{10}N_2O_4$, 187.1745; found, 187.0642.

Ethyl 2-((tert-butoxycarbonyl)amino)-3-(3-hydroxy-5-methylisoxazol-4-yl)propanoate (**8**). A round-bottomed flask containing a suspension of AMPA (130 mg, 0.70 mmol) in EtOH (7 mL) was placed into an ice bath and cooled to 0 °C. After the addition of acetyl chloride (0.5 mL, 7.0 mmol) the ice bath was removed and the mixture was allowed to reach room temperature over a period of 1 h. The flask was then placed into an oil bath and the solution stirred at reflux temperature for 12 h. After cooling, the solvent was evaporated under reduced pressure furnishing the ethyl ester derivative which was dissolved in H_2O (4 mL). Triethylamine (0.4 mL, 2.90 mmol) and a solution of di-*tert*-butyl dicarbonate (183 mg, 0.84 mmol) in THF (4 mL) were added. The resulting mixture was stirred at room temperature for 12 h. The solvent was evaporated under reduced pressure and the residue was dissolved in H_2O (20 mL) and extracted with diethyl ether (3 × 30 mL). The aqueous layer was acidified to pH 2 with a 5% solution of citric acid and further extracted with EtOAc (3 × 30 mL). The combined organic phases were dried over $MgSO_4$ and concentrated to dryness. The resulting residue was purified by column chromatography (0 to 80% EtOAc in n-hexane, gradient elution) affording compound **8** as a dark white solid (130 mg, 0.41 mmol, 60% yield). ^{1}H NMR (500 MHz, chloroform-*d*): δ 8.13 (s, 1H), 4.44 (s, 1H), 4.36 – 4.00 (m, 2H), 2.84 (qd, *J* = 20.0, 17.9, 10.8 Hz, 2H), 2.27 (d, *J* = 3.6 Hz, 3H), 1.53 – 1.40 (m, 9H), 1.28 (td, *J* = 7.1, 5.1 Hz, 3H; ^{13}C NMR (126 MHz, chloroform-*d*): δ 171.5, 170.1, 167.7, 155.2, 101.0, 80.0, 67.1, 61.7, 28.3, 24.3, 14.0, 11.5; HRMS (ESI-QTOF) *m/z*: $[M - H]^-$ calcd for $C_{14}H_{22}N_2O_6$, 313.1405; found, 313.1325.

Ethyl 2-((tert-butoxycarbonyl)amino)-3-(3-((8-cyano-7-(methoxymethoxy)-4-(3,4,5-trimethoxyphenyl)quinolin-2-yl)methoxy)-5-methylisoxazol-4-yl)propanoate (**9**). To a stirred solution of **8** (109 mg, 0.22 mmol) in acetonitrile (6 mL), cesium carbonate (220 mg, 0.68 mmol) was added. The mixture was stirred at room temperature for 10 min and then a solution of **4b** (78 mg, 0.25 mmol) in acetonitrile (6 mL) was added dropwise. The resulting mixture was stirred at reflux temperature for 12 h. The solvent was evaporated under reduced pressure and the residue was dissolved in CH_2Cl_2 (50 mL), washed with a 5% solution of citric acid (3 × 20 mL) and H_2O (3 × 10 mL), dried over $MgSO_4$, and concentrated to dryness. The resulting residue was purified by column chromatography (0 to 100% EtOAc in n-hexane, gradient elution) to yield compound **9** as a light orange powder (47 mg, 0.07 mmol, 32% yield). ^{1}H NMR (500 MHz, chloroform-*d*): δ 8.15 (t, J = 9.5 Hz, 1H), 7.57 – 7.48 (m, 3H), 6.72 (s, 2H), 5.67 (s, 2H), 5.47 (s, 2H), 5.27 (d, J = 8.0 Hz, 1H), 4.47 (q, J = 6.6 Hz, 1H), 3.96 (s, 3H), 3.91 (s, 6H), 3.60 (s, 3H), 2.89 (qd, J = 14.7, 6.0 Hz, 2H), 2.30 (s, 3H), 1.36 (s, 9H), 1.17 (t, J = 7.1 Hz, 3H); ^{13}C NMR (126 MHz, chloroform-d): δ 171.4, 170.5, 168.3, 162.0, 158.9, 153.5, 149.9, 148.9, 138.6, 132.5, 132.1, 121.5, 118.7, 115.5, 114.7, 106.9, 100.2, 100.0, 95.0, 72.3, 67.1, 61.6, 61.0, 56.9, 56.4, 56.3, 53.0, 28.2, 24.5, 14.0, 11.6; HRMS (ESI-QTOF) m/z: $[M + H]^+$ calcd for $C_{36}H_{42}N_4O_{11}$, 707.2923; found, 707.2775.

1-Carboxy-2-(3-((8-cyano-7-hydroxy-4-(3,4,5-trimethoxyphenyl)quinolin-2-yl)methoxy)-5-methylisoxazol-4-yl)ethan-1-aminium 2,2,2-trifluoroacetate (**TMP-CyHQ-AMPA**). To a stirred solution of **9** (47 mg, 0.07 mmol) in THF (4 mL), a solution of lithium hydroxide (6 mg, 0.14 mmol) in H_2O (4 mL) was added dropwise. The resulting mixture was stirred at room temperature for 12 h.

The reaction was quenched with a 5% solution of citric acid (20 mL) and extracted with CH_2Cl_2 (3 × 30 mL). The combined organic phases were dried over $MgSO_4$ and concentrated to dryness. The resulting residue was purified by column chromatography (n-hexane/EtOAc gradient, then EtOAc/MeOH gradient) affording the free carboxylic acid derivative, which was dissolved in CH_2Cl_2 (2 mL). TFA (0.2 mL) was added dropwise, and the reaction was stirred at room temperature in the dark until HPLC showed complete consumption of the starting material (3 h). After evaporation of the solvent, the resulting residue was purified by trituration with diethyl ether, affording the caged AMPA derivative TMP-CyHQ-AMPA as a yellow powder (29 mg, 0.04 mmol, 63% yield). ^{1}H NMR (500 MHz, dimethylsulfoxide-d_6): δ 12.21 (s, 1H), 8.33 (s, 2H), 8.14 (d, J = 9.3 Hz, 1H), 7.96 (s, 1H), 7.59 (s, 1H), 7.39 (d, J = 9.4 Hz, 1H), 6.84 (s, 2H), 5.52 (s, 2H), 4.08 (s, 1H), 3.83 (s, 6H), 3.76 (s, 3H), 2.90 (d, J = 6.6 Hz, 2H), 2.29 (s, 3H); ^{13}C NMR (126 MHz, dimethylsulfoxide-d_6): δ 170.7, 170.5, 169.3, 164.6, 158.5, 158.3, 158.2, 153.5, 149.7, 149.3, 138.4, 132.7, 132.6, 119.7, 119.2, 118.2, 116.1, 107.5, 99.4, 94.7, 72.2, 60.6, 56.6, 52.0, 22.8, 11.9; HRMS (ESI-QTOF) m/z: $[M + H]^+$ calcd for $C_{27}H_{26}N_4O_8$, 535.1823; found, 535.1777.

UV and Emission Spectra

UV-vis spectra were obtained from a 0.1 mM solution of compound in KMOPS buffer. A blank solution of KMOPS was used to subtract baseline absorption. Fluorescence spectra were recorded from 1 µM solutions of compound in 0.01 N NaOH. A blank solution of 0.01 N NaOH was used to subtract baseline emission. Each measure was repeated in triplicate and the emission values were averaged.

Calculation of the Molar Extinction Coefficient (ε)

The values of ε at λ = 365 or 405 nm were calculated using the Beer-Lambert law: $\varepsilon = A(cl)^{-1}$, where *A* is the absorbance value measured at 365 or 405 nm, *c* the concentration of the sample, and *l* the cuvette length (1 cm).

Photolysis Reactions Initiated through 1PE

Stock solutions (10 mM) of substrates in DMSO were diluted with KMOPS buffer (KCl 100 mM, MOPS 10 mM, pH 7.2) to a final concentration of 0.1 mM. Solutions were placed in a 3-mL quartz cuvette (fitted with a stirring bar) and irradiated with a LED lamp (Cairn OptoLED Lite) at the appropriate wavelength with stirring. Aliquots (70 µL) were sampled at different time intervals and analyzed by reverse-phase uHPLC, using an external standard calibration method for quantification. All experiments were repeated in triplicate. HPLC analyses were performed on an Agilent 1290 Infinity series uHPLC using a Zorbax Eclipse Plus C18 column, monitoring the AUC at 320 nm. Separations were obtained with a gradient elution (flux rate of 0.3 mL/min) using a mobile phase composed of A = 0.1% trifuoroacetic acid in water and B = acetonitrile (starting from 5% B to 100% over 10 min and re-equilibrating to 5% B before the next run). The quantification of the percentage of the starting material remaining was obtained by comparison of the AUC measured with calibration curves generated from known concentrations of the substrate. The percentages remaining were plotted versus time and the $t_{90\%}$ values (time in seconds for 90% of reaction) were obtained by fitting a single exponential decay curve to the data using the software DeltaGraph (Red Rock Software). The quantum efficiency (Φ_u) of the photolysis reaction was calculated from the following equation:

$$\Phi_u = (I\ \sigma\ t_{90\%})^{-1}$$

where *I* represents the lamp intensity in Einstein $cm^{-2}\ s^{-1}$ (measured by ferrioxalate actinometry)[3] and σ is the decadic extinction coefficient (1000 × ε, molar extinction coefficient).[4-6] The release of hymexazol was quantified following an external standard calibration method (monitoring the AUC at 235 nm) and plotted vs. time, fitting an exponential rise to max curve to the data. The quantification of released AMPA was performed according to the published *ortho*-phthaladehyde (OPA) method,[7] since the low UV absorption of AMPA prevented the direct quantification from the HPLC traces. Briefly, photolyzed solutions (70 µL) of TMP-CyHQ-AMPA where treated with 5 µL of OPA reagent -

prepared as reported by Gardenr and Miller[7] - and analyzed by HPLC (Agilent 1290 Infinity series uHPLC using a Zorbax Eclipse Plus C18 column and fitted with a fluorescence detector). Traces were obtained with excitation wavelength 340 nm and emission wavelength 450 nm. After separation, the fluorescence output of the OPA-AMPA complex was used to quantify the concentration of AMPA comparing to an external standard calibration curve.

Photolysis Reactions Initiated through 2PE

Working solutions were prepared as described above for 1PE-mediated photolysis. Solutions (25 μL) were placed into a microcuvette (26.10F-Q-10, Starna, 10 × 1 × 1 mm illuminated dimensions) and irradiated for different time intervals (typically 5, 10, and 30 min) with 740-nm light (720 nm for **5a**) from a fs-pulsed and mode-locked Ti:sapphire laser (Mai Tai HP DeepSee, Spectra-Physics) focused on the center of the cuvette chamber. The average power used was 400-500 mW (depending on the experiment) measured after passing through the cuvette. Samples were analyzed by reverse-phase uHPLC to quantify the percentage of starting material remaining, as described for the photolysis mediated by 1PE, which was plotted versus time. The resulting data were plotted using DeltaGraph (Red Rock Software) software and fit to a single exponential decay curve. The two-photon uncaging action cross-section (δ_u) values were determined following a previously reported procedure,[4-6] using fluorescein as an external standard and the following equation:

$$\delta_u = \frac{N_\mathrm{p}\phi Q_{\mathrm{f}2}\delta_{\mathrm{a}\mathrm{F}}C_\mathrm{f}}{\langle F(t)\rangle C_\mathrm{s}}$$

where N_p is the number of product molecule formed per second determined by HPLC analysis, ϕ is the collection efficiency of the fluorescence detector positioned at a right angle to the excitation beam, Q_{f2} is the 2-photon fluorescence quantum yield of fluorescein (0.9),[8, 9] δ_{aF} is the fluorescein absorbance cross-section (30 GM at 740 nm),[10] C_f is the concentration of fluorescein, $\langle F(t)\rangle$ is the time-averaged fluorescent photon flux (photon/s) from the emission of the fluorescein standard measure by the detector, and C_s is the concentration of substrate. Quantification of hymexazol and AMPA release was performed as described above for the 1PE experiments.

Photolysis Time Courses

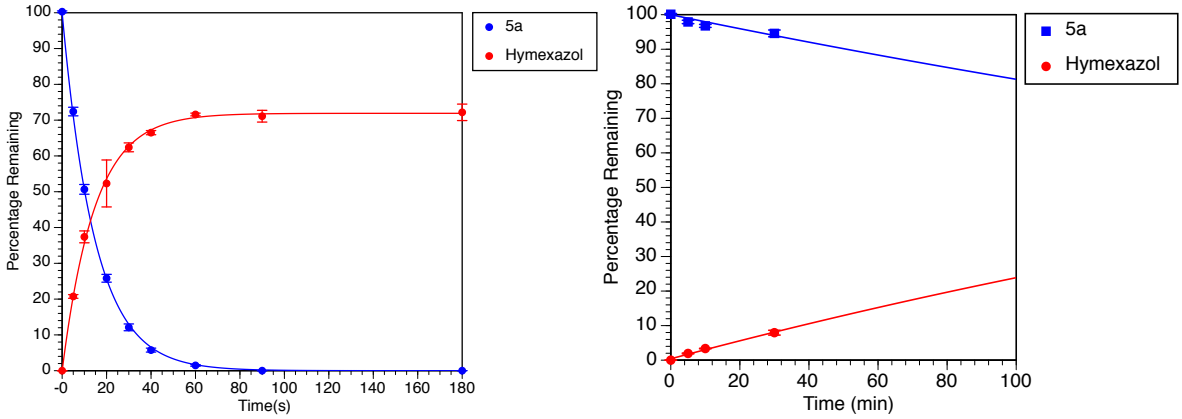


Figure S1 Time course of the photolysis of **5a** with 1PE (left) and 2PE (720 nm) (right).

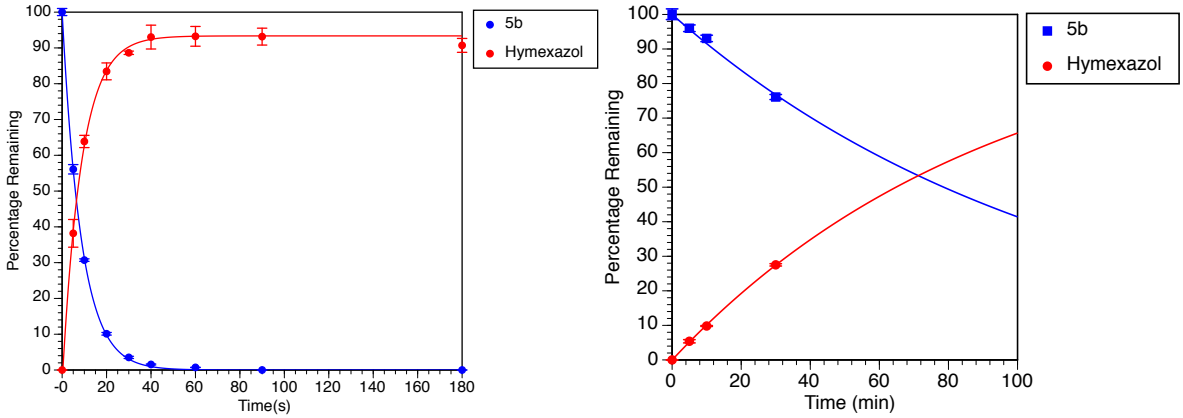


Figure S2 Time course of the photolysis of **5b** with 1PE (left) and 2PE (right).

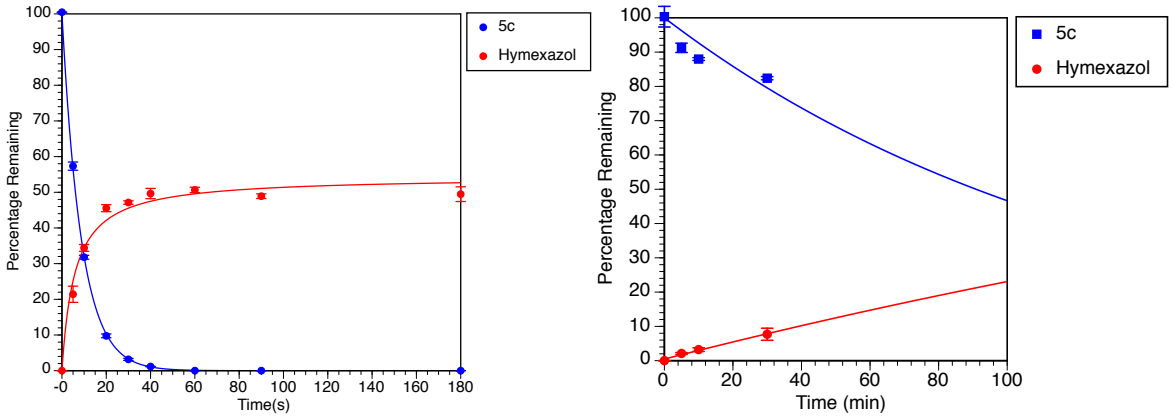
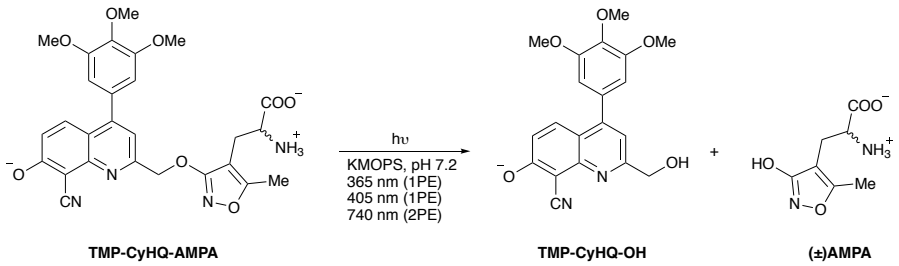


Figure S3 Time course of the photolysis of **5c** with 1PE (left) and 2PE (right).

HPLC Analysis of the Photolysis of TMP-CyHQ-AMPA



TMP-CyHQ-AMPA **TMP-CyHQ-OH** **(±)AMPA**

Figure S4 Photolysis reaction of TMP-CyHQ-AMPA.

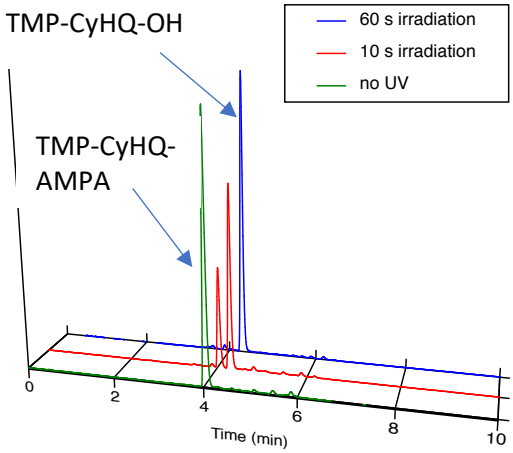


Figure S5 HPLC traces of the photolysis of TMP-CyHQ-AMPA initiated through 1PE at increasing irradiation times.
Traces were acquired at 320 nm.

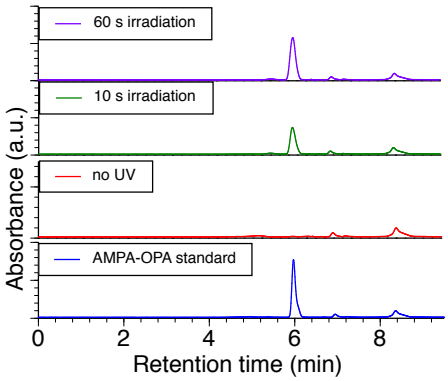


Figure S6 HPLC traces of the photolysis of TMP-CyHQ-AMPA initiated through 1PE at increasing irradiation times.
Traces were acquired with a fluorescence detector at excitation 340 nm and emission 450 nm.
Shown is the appearance of the AMPA-OPA complex compared to a standard.

Tables of Photochemical Data

Table S1 Extended photophysical and photochemical data for caged hymexazoles **5a**-**c**.[a]

Compound	λ_{abs} (nm)	λ_{em} (nm)	Stokes shift (nm)	ε_{365} (M^{-1} cm^{-1})	I (Ein cm^{-2} s^{-1})	$t_{90\%}$ (s)	yield (%)[b]	Φ_u	sensitivity (ε Φ_u)	δ_u (GM)[c]	solubility (μM)
5a	354	428	74	4000	1.6×10^{-8}	34	72	0.46	1820	0.85 [d]	>100
5b	372	481	109	3900	1.6×10^{-8}	20	93	0.80	3104	1.69	>100
5c	365	453	88	5170	1.6×10^{-8}	20	51	0.59	3038	1.00	>100

[a]0.1 mM solution in KMOPS buffer, pH 7.2. [b]Chemical yield of released hymexazol under 1PE. [c]GM = 10^{-50} cm^4 s/photon. [d]Irradiated at 720 nm.

Table S2 Extended photophysical and photochemical data for TMP-CyHQ-AMPA.[a]

λ_{abs} (nm)	λ_{em} (nm)	Stokes shift (nm)	ε_{365} (M^{-1} cm^{-1})	ε_{405} (M^{-1} cm^{-1})	$t_{90\%}$ $_{365}$ (s)	$t_{90\%}$ $_{405}$ (s)	yield $_{365}$ (%)[b]	yield $_{405}$ (%)[b]	Φ_{u365}	Φ_{u405}	sensitivity (ε_{365} Φ_u)	sensitivity (ε_{405} Φ_u)	δ_u (GM)[c]	t_d[d]	solubility (μM)
371	474	103	6200	1230	22	24	99	98	0.65	0.71	4054	880	1.71	n.h.[e]	>100

[a]0.1 mM solution in KMOPS buffer, pH 7.2. [b]Chemical yield of released AMPA. [c]GM = 10^{-50} cm^4 s/photon. [d]Time constant of spontaneous hydrolysis in buffer under dark conditions. [e]No hydrolysis (<2% detected after 6 days)

Spontaneous Hydrolysis in the Dark

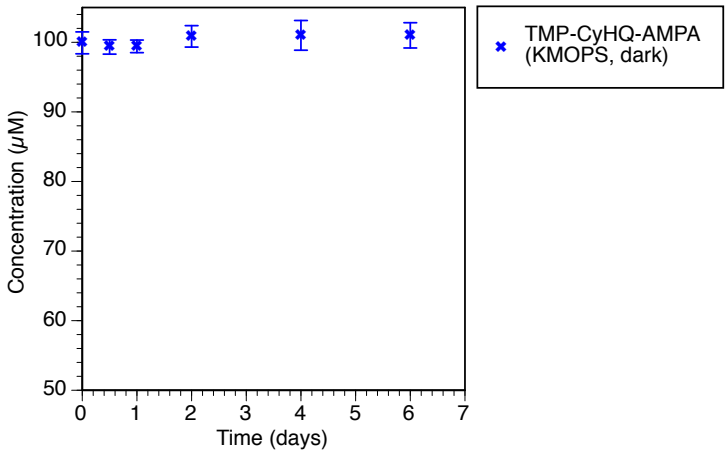


Figure S7 Time course of spontaneous hydrolysis in the dark.
Solutions of TMP-CyHQ-AMPA (0.1 mM) in KMOPS buffer were incubated in the dark and the remaining concentration plotted against the time (days). No hydrolysis was observed up to 6 days. The experiment was performed in triplicate.

Power Variation Experiment

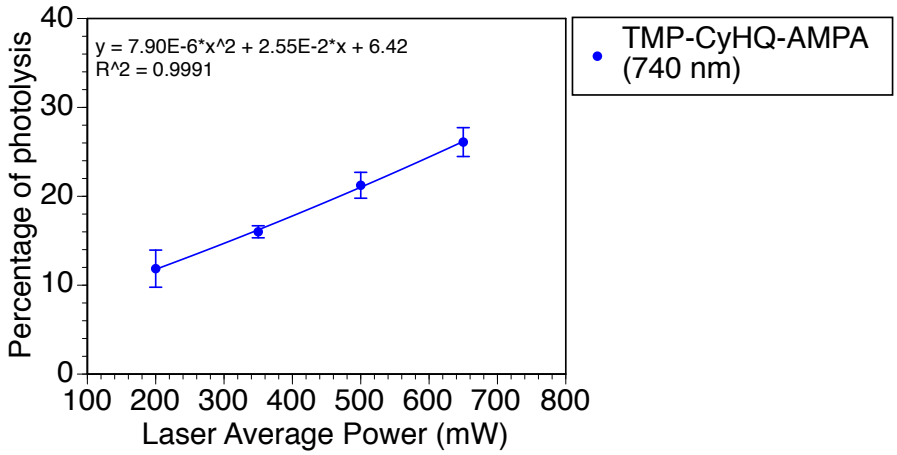


Figure S8 Correlation between the photolysis rate and the average laser power.
TMP-CyHQ-AMPA was photolyzed at different laser powers for 15 min. The remaining concentrations were determined by HPLC analysis and are the average of three runs. Lines are least-squares fits of a quadratic equation ($y = ax^2 + bx + c$). $a = 7.90 \times 10^{-6}$, $b = 2.50 \times 10^{-2}$, $c = 6.42$. $R^2 = 0.9991$. Error bars represent the standard deviations of the mean.

Computational Docking Experiments

The co-crystal structure of AMPA in the GluR2 receptor was downloaded from the Protein Data Bank (PDB 1FTM).[11] The protein was prepared and minimized using the protein preparation tool in Maestro (Schrödinger). A receptor grid was generated using the cognate molecule as centroid. The volume of the grid was increased from the standard setting to allow docking of bigger ligands. (grid volume: x = 15 Å; y = 15 Å; z =15 Å). 3-*O*-substituted AMPA analogs (Figure S9) were generated and prepared using the Ligprep module. Docking experiments were performed using the standard precision function of Glide.

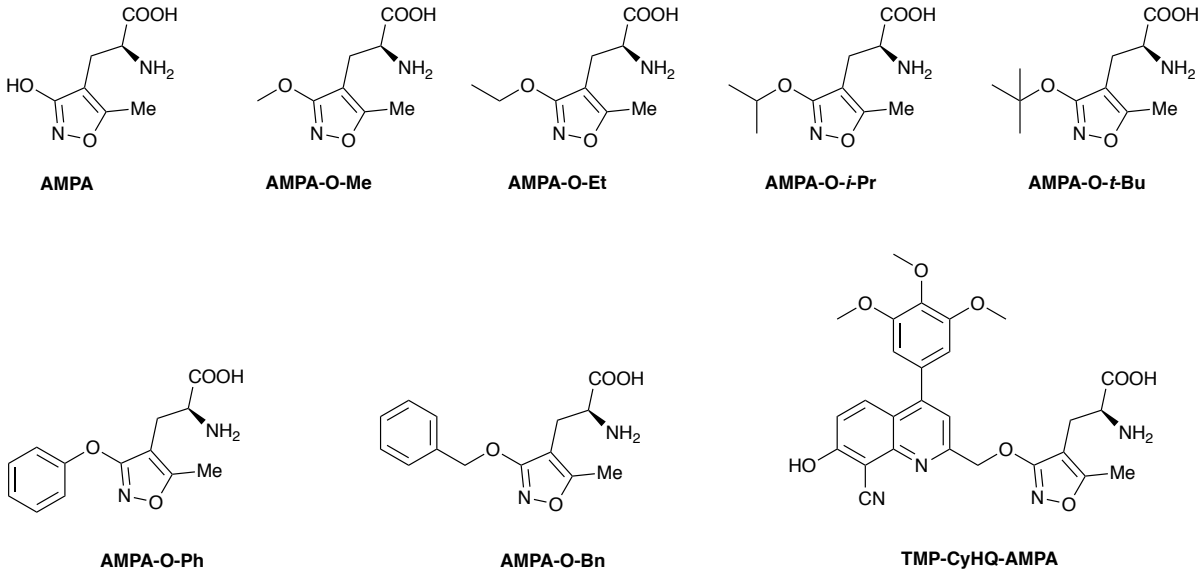


Figure S9 Structures of 3-*O*-substituted AMPA used for the docking experiments.

Table S3 Predicted docking scores of 3-*O*-substituted AMPA analogs.

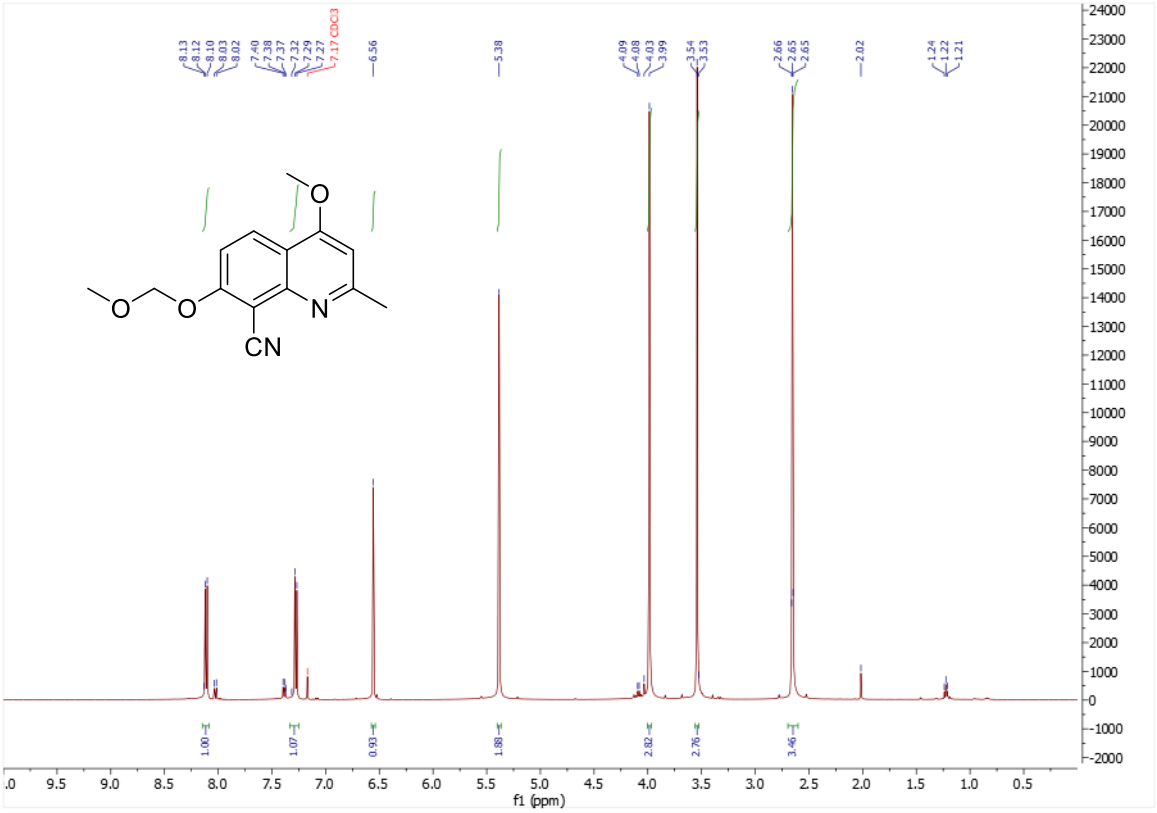
Ligand	Docking Score
AMPA	-10.277
AMPA-O-Me	-9.421
AMPA-O-Et	-9.593
AMPA-O-*i*-Pr	-9.720
AMPA-O-*t*-Bu	-8.768
AMPA-O-Ph	-8.155
AMPA-O-Bn	-6.419
TMP-CyHQ-AMPA	No poses[a]

[a] No binding pose was found in the active core of GluR2.

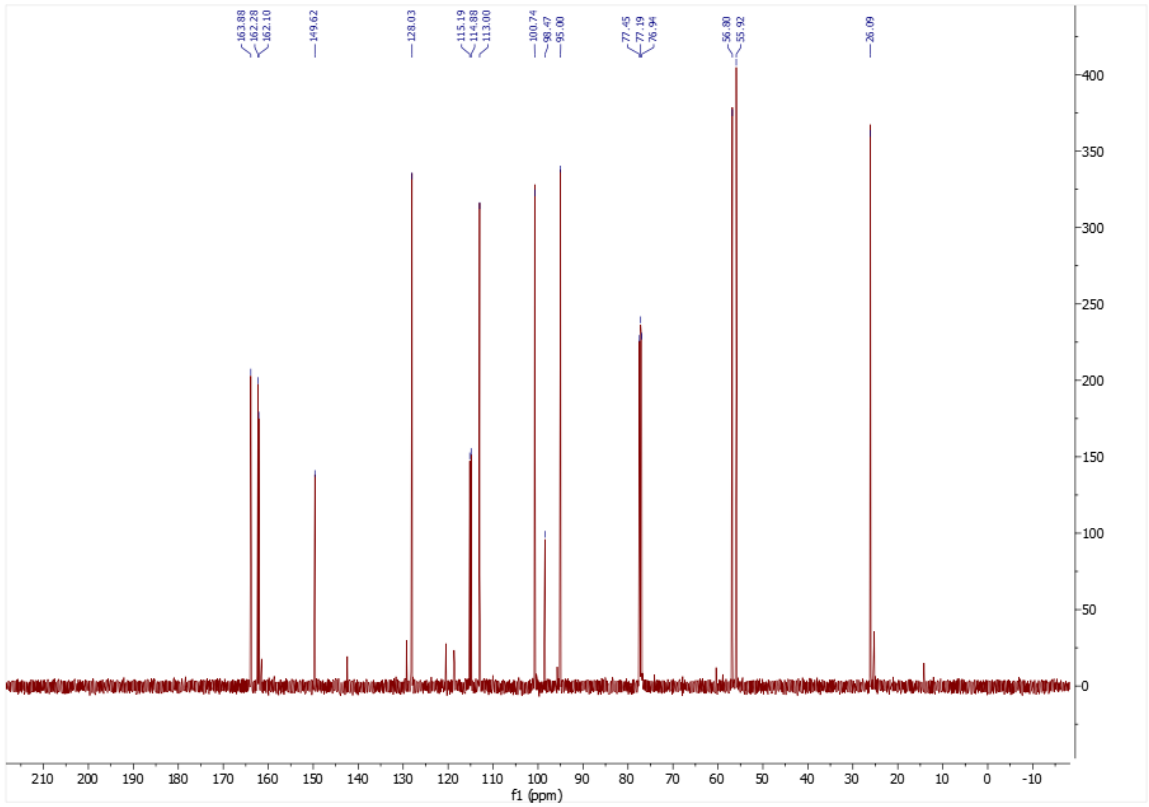
NMR Spectra

4-Methoxy-7-(methoxymethoxy)-2-methylquinoline-8-carbonitrile (**2a**)

^{1}H NMR (500 MHz, chloroform-*d*)

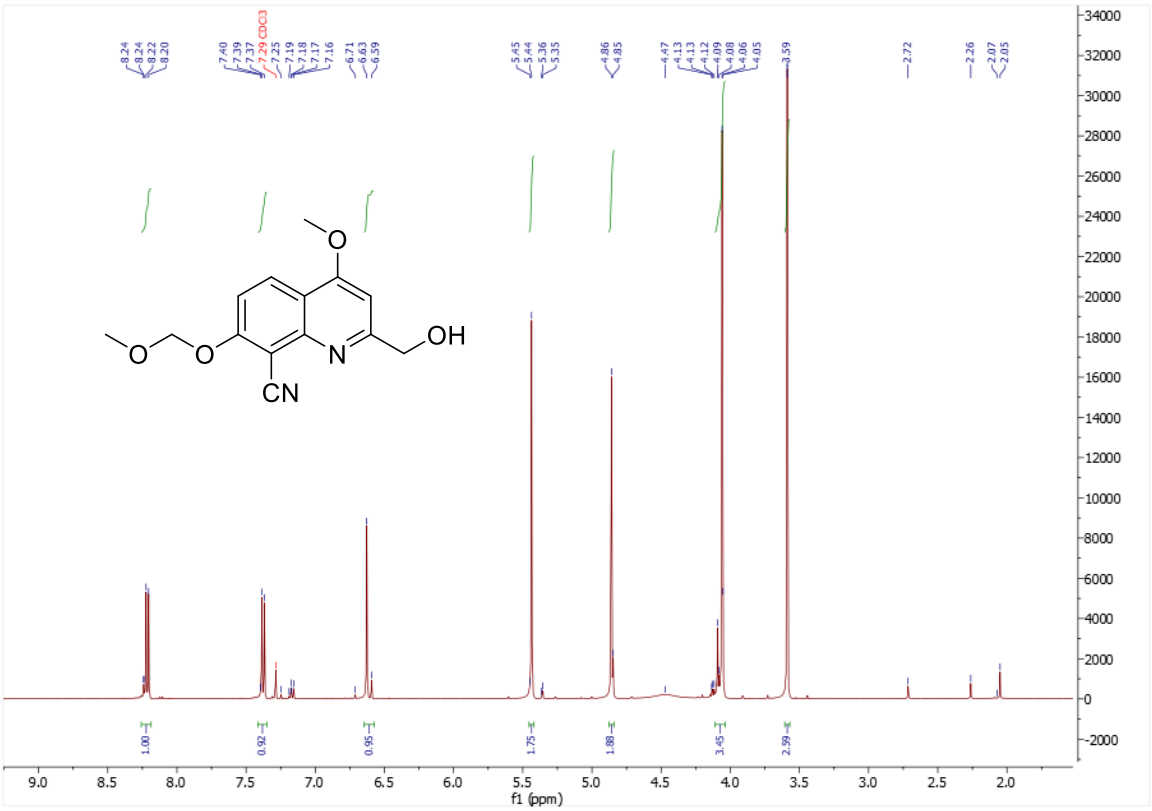


^{13}C NMR (126 MHz, chloroform-*d*)

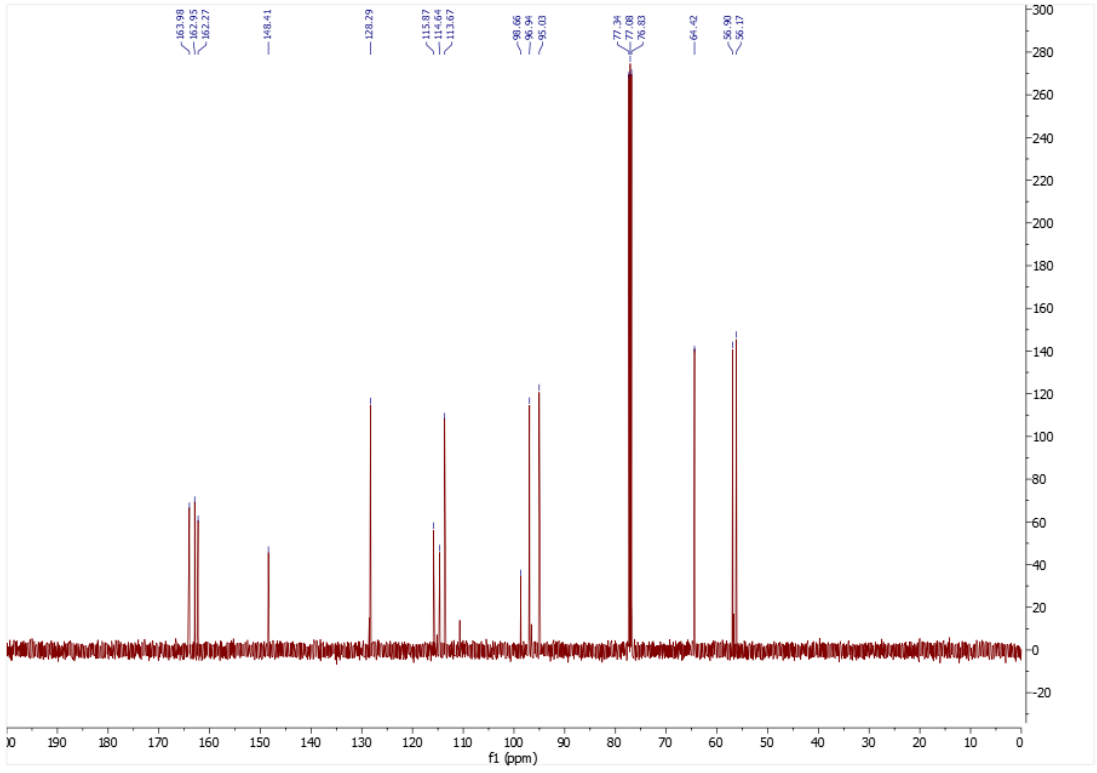


2-(Hydroxymethyl)-4-methoxy-7-(methoxymethoxy)quinoline-8-carbonitrile (**3a**)

^{1}H NMR (500 MHz, chloroform-*d*)

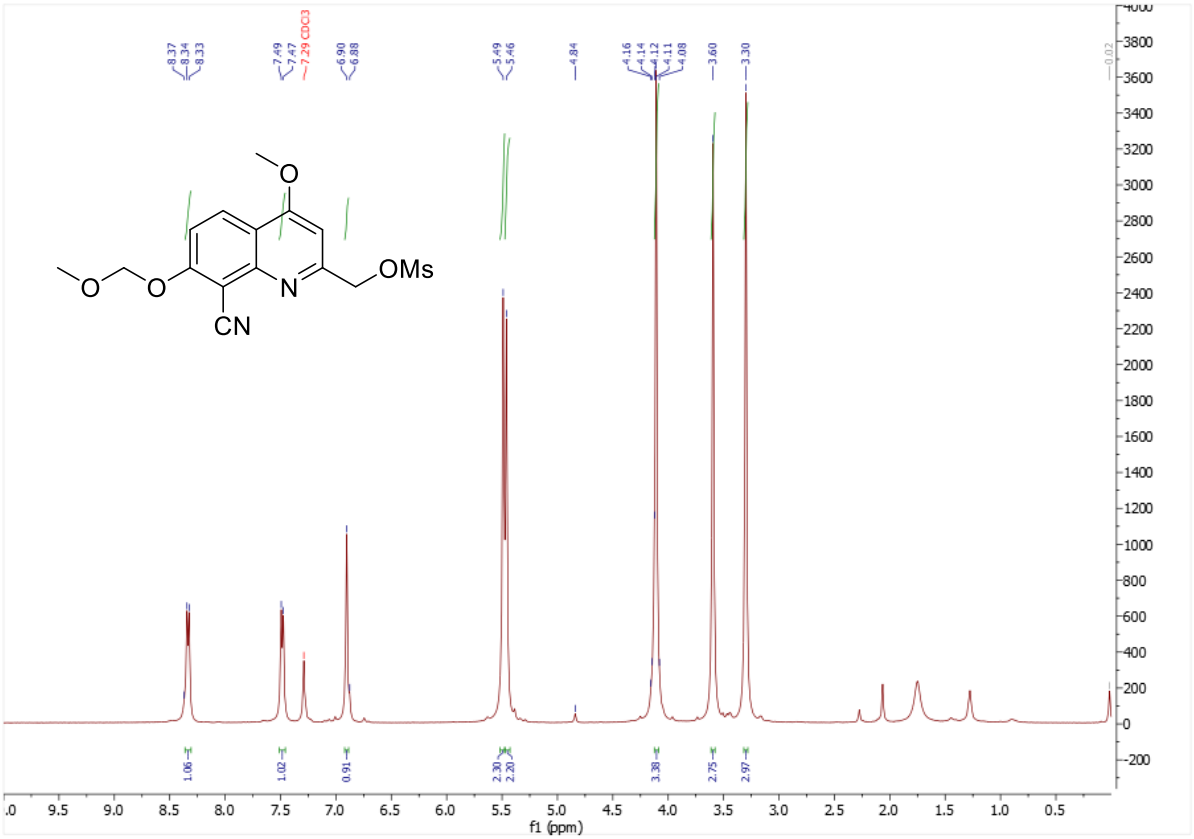


^{13}C NMR (126 MHz, chloroform-*d*)

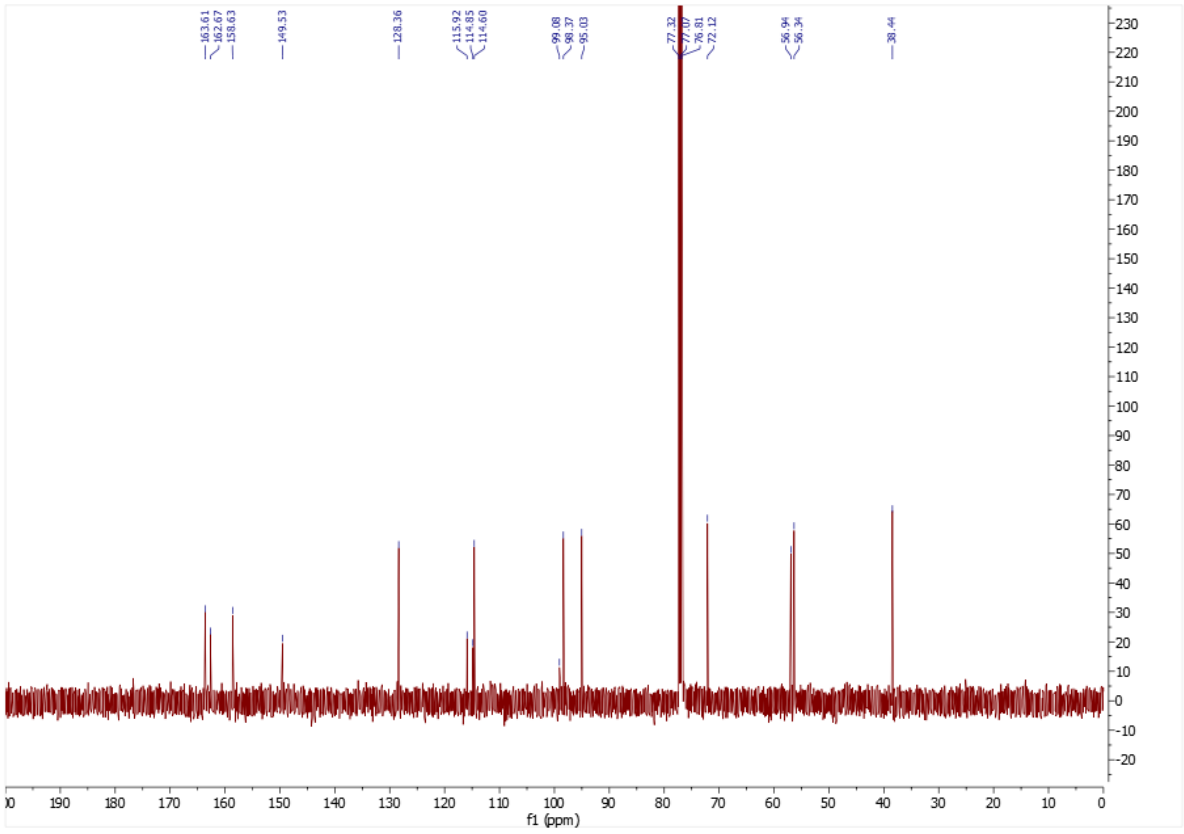


(8-Cyano-4-methoxy-7-(methoxymethoxy)quinolin-2-yl)methyl methanesulfonate (**4a**)

^{1}H NMR (500 MHz, chloroform-*d*)

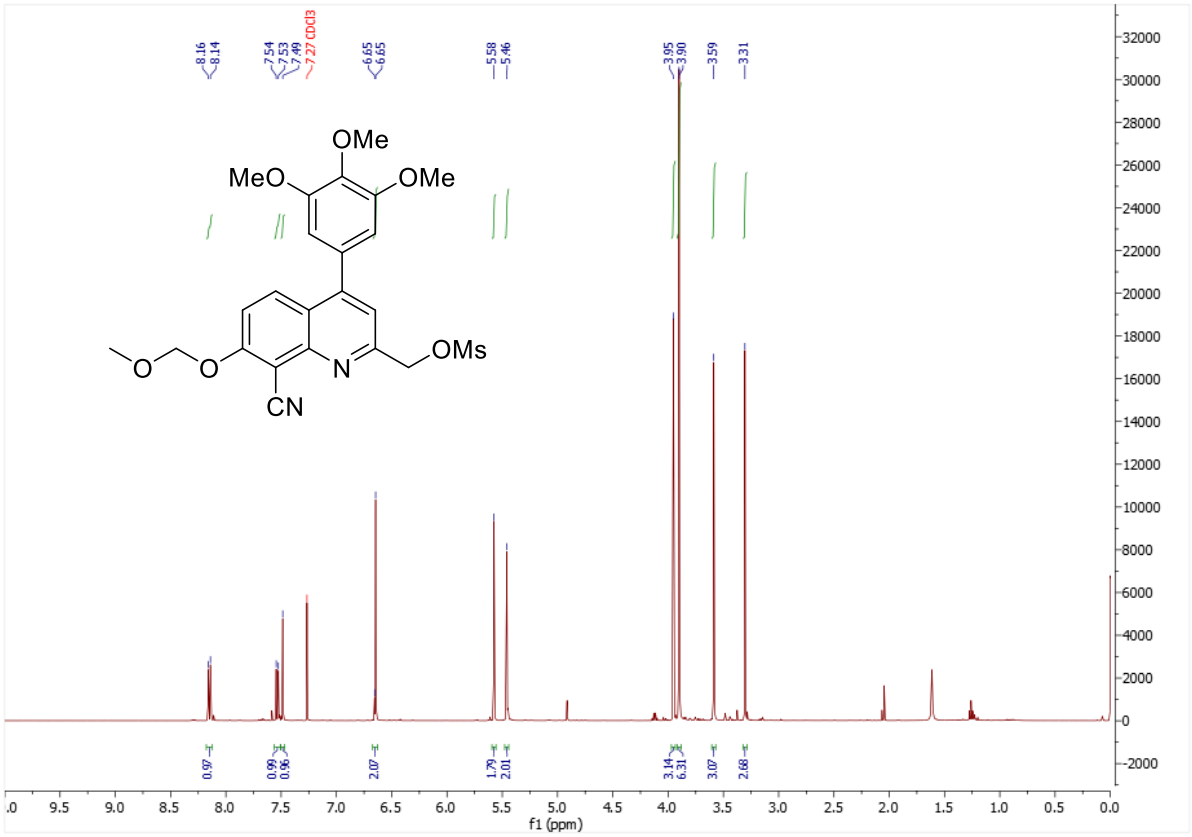


^{13}C NMR (126 MHz, chloroform-*d*)

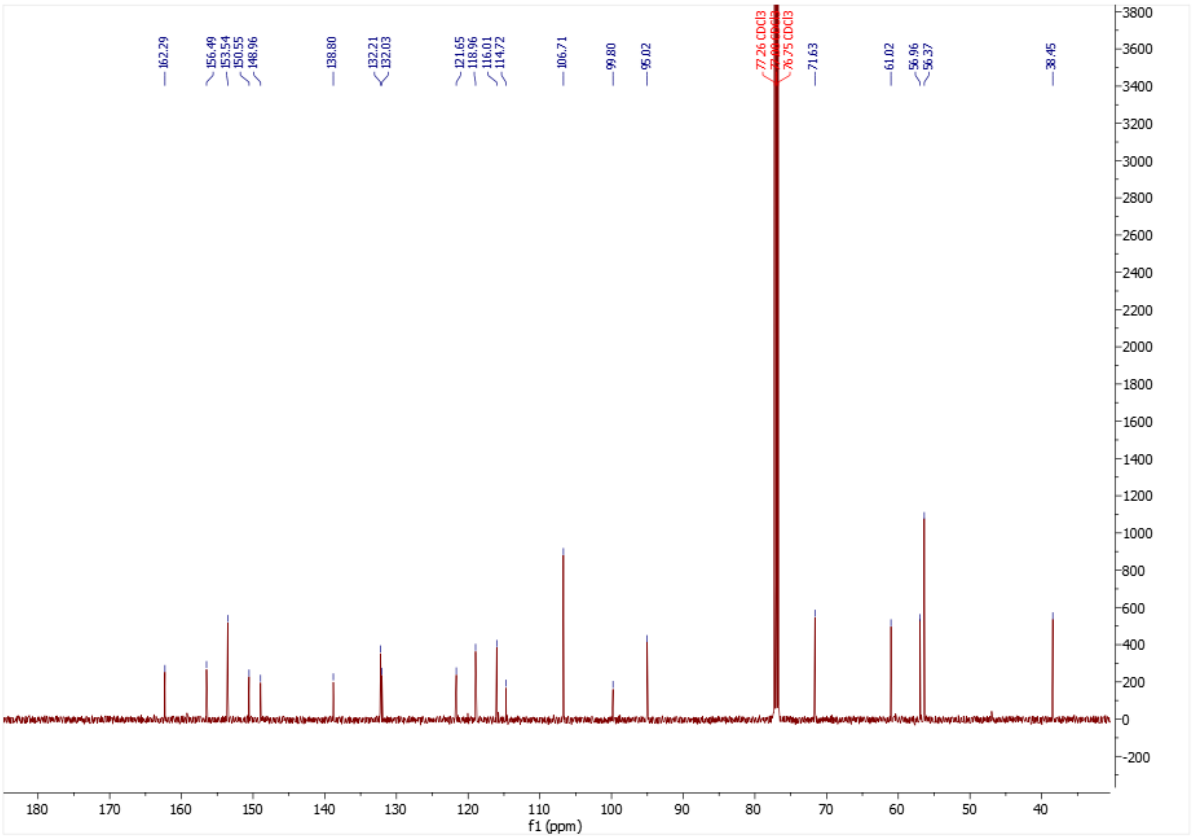


(8-Cyano-7-(methoxymethoxy)-4-(3,4,5-trimethoxyphenyl)quinolin-2-yl)methyl methanesulfonate (**4b**)

^{1}H NMR (500 MHz, chloroform-*d*)

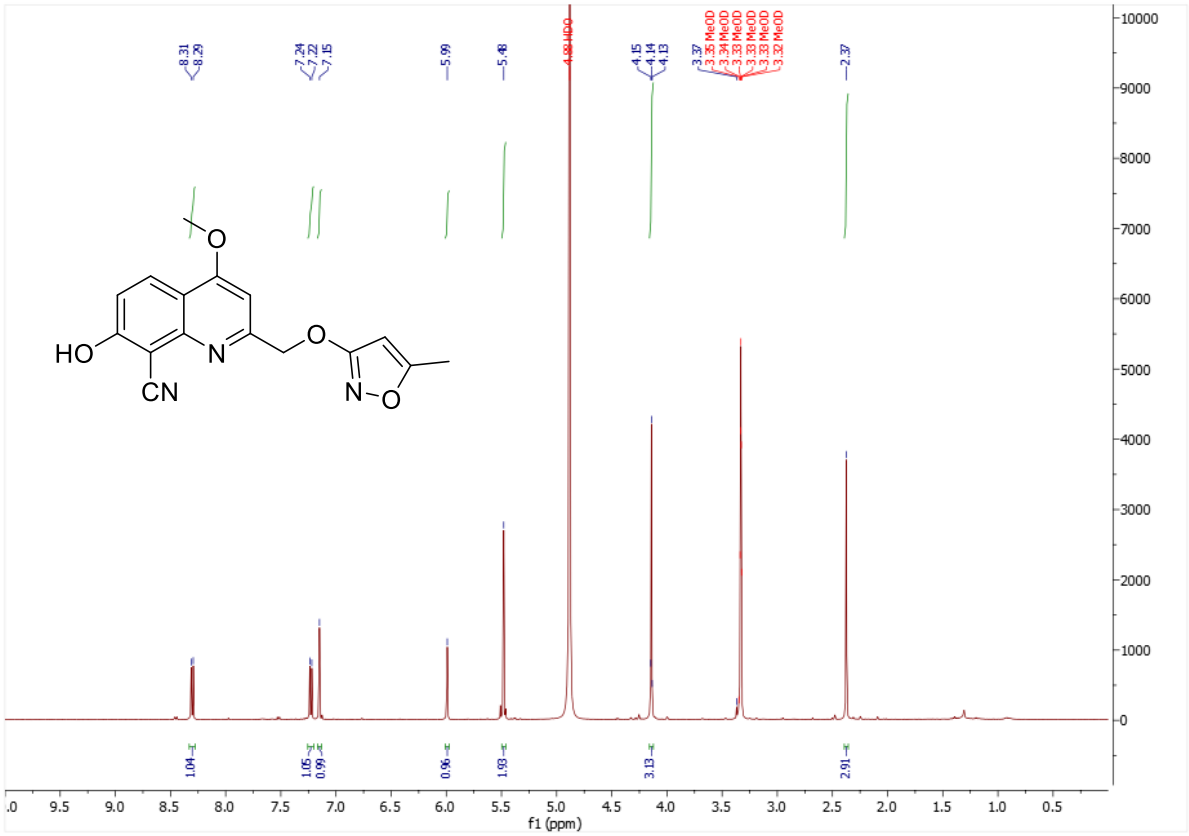


^{13}C NMR (126 MHz, chloroform-*d*)

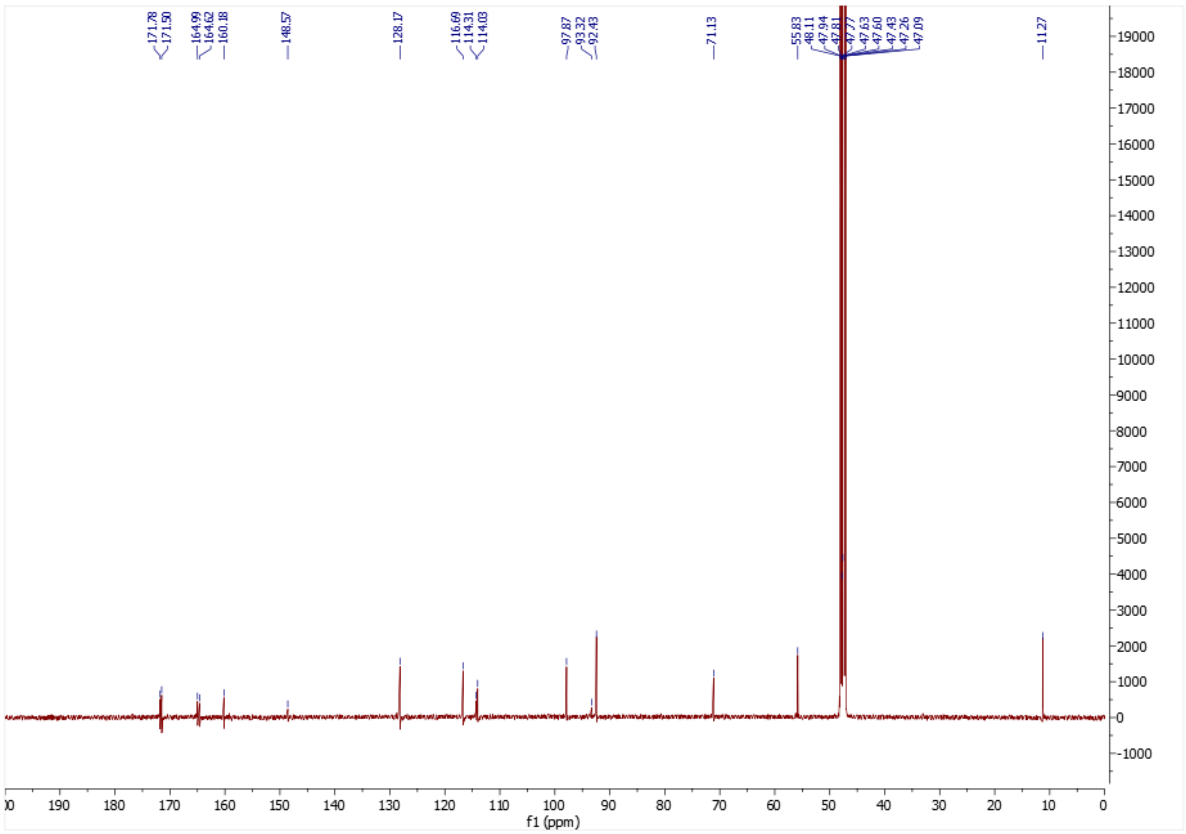


7-Hydroxy-4-methoxy-2-(((5-methylisoxazol-3-yl)oxy)methyl)quinoline-8-carbonitrile (**5a**)

^{1}H NMR (500 MHz, methanol-d_4)

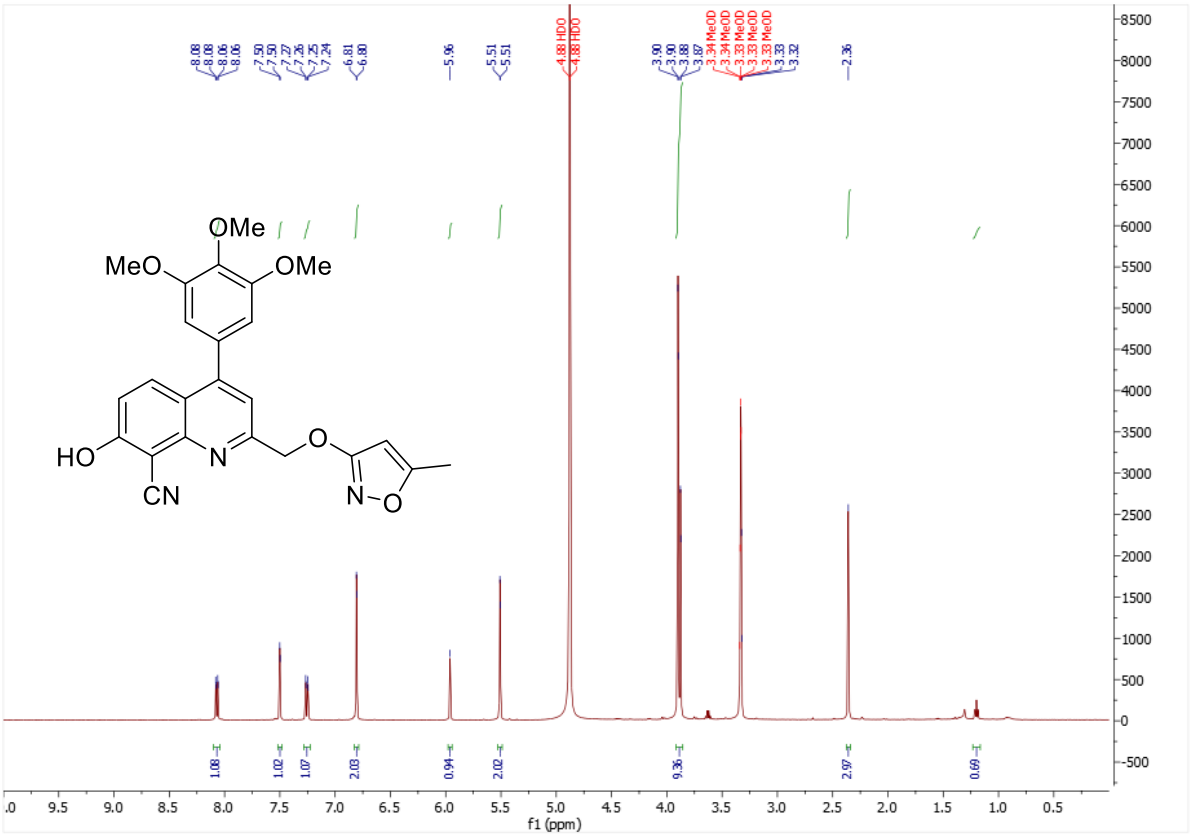


^{13}C NMR (126 MHz, methanol-d_4)

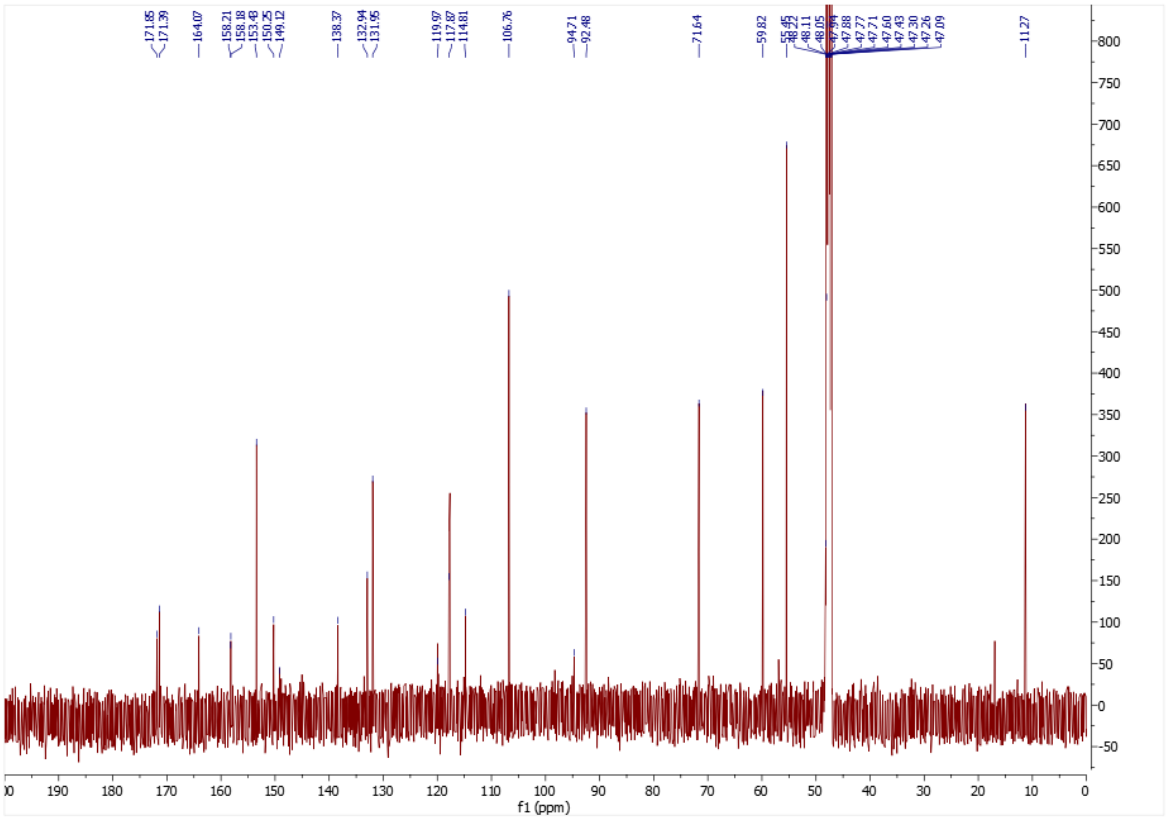


7-Hydroxy-2-(((5-methylisoxazol-3-yl)oxy)methyl)-4-(3,4,5-trimethoxyphenyl)quinoline-8-carbonitrile (**5b**)

^{1}H NMR (500 MHz, methanol-d_4)

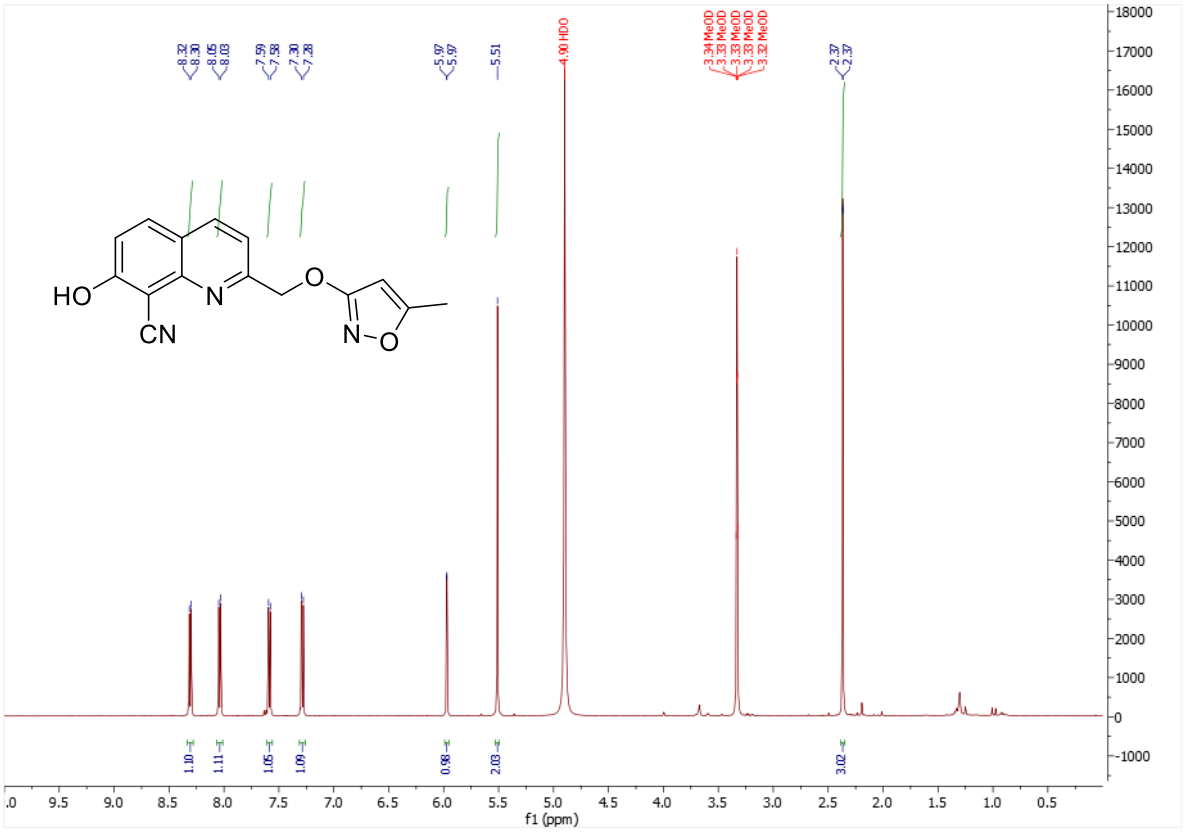


^{13}C NMR (126 MHz, methanol-d_4)

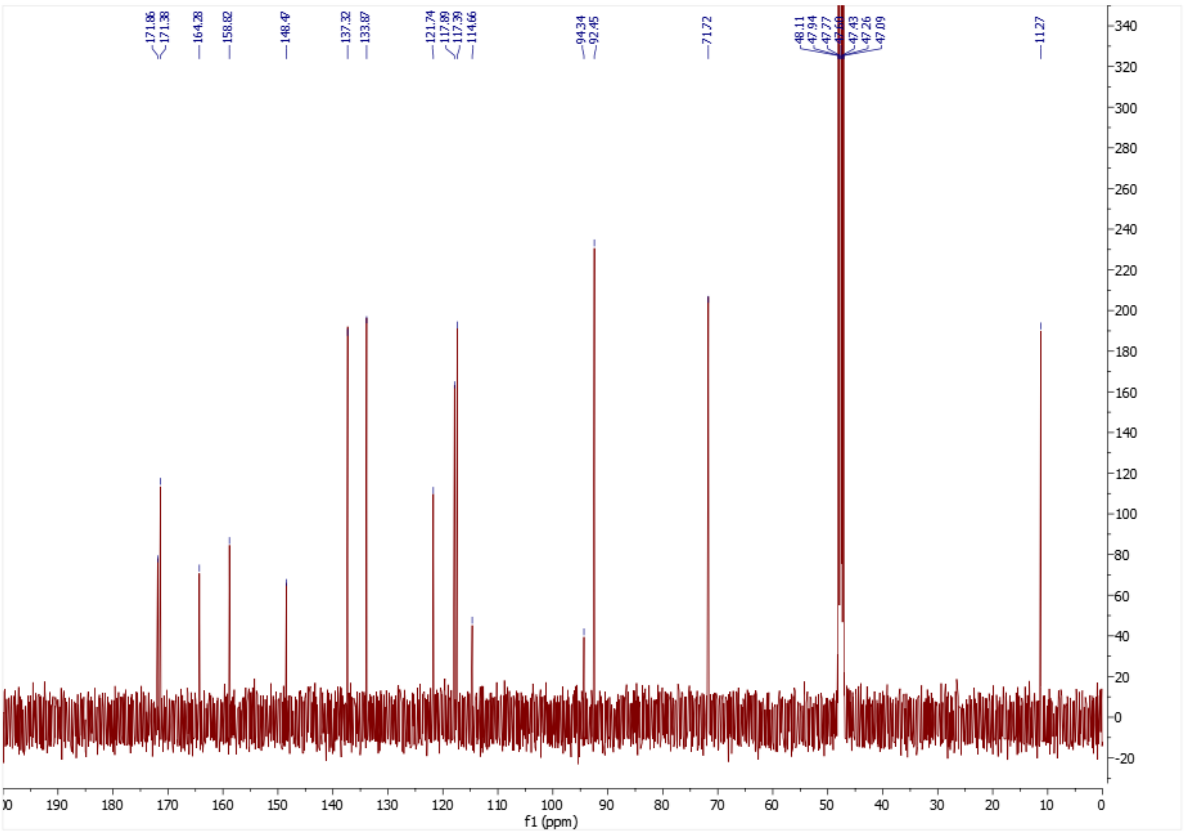


7-Hydroxy-2-(((5-methylisoxazol-3-yl)oxy)methyl)quinoline-8-carbonitrile (**5c**)

^{1}H NMR (500 MHz, methanol-d_4)

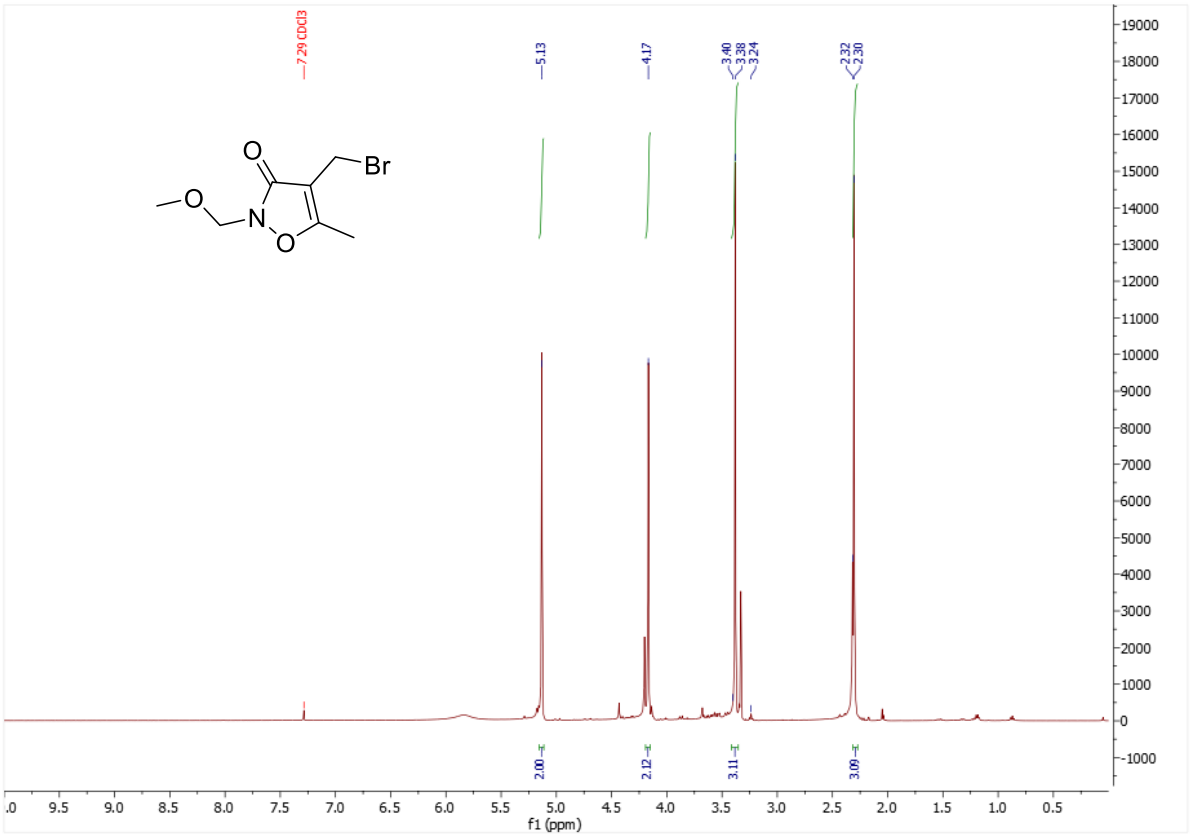


^{13}C NMR (126 MHz, methanol-d_4)

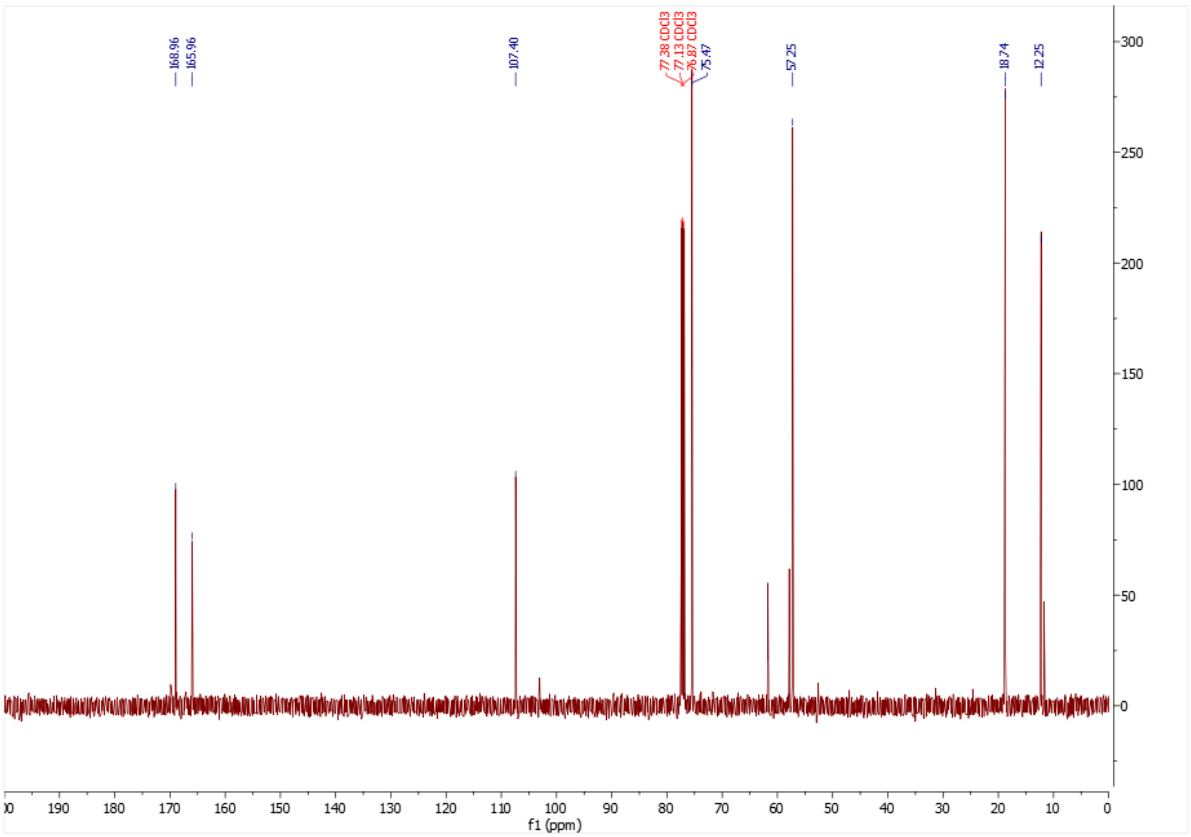


4-(Bromomethyl)-2-(methoxymethyl)-5-methylisoxazol-3(2H)-one (**6**)

^{1}H NMR (500 MHz, chloroform-*d*)

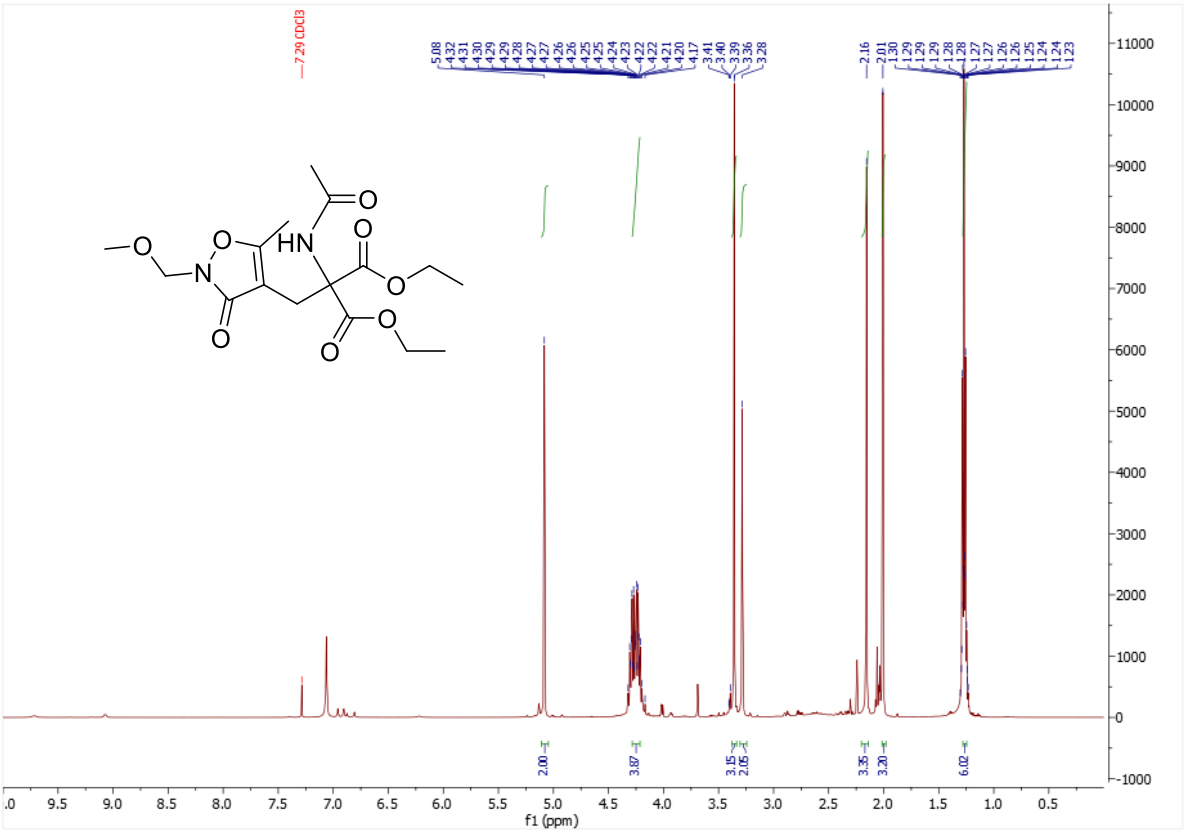


^{13}C NMR (126 MHz, chloroform-*d*)

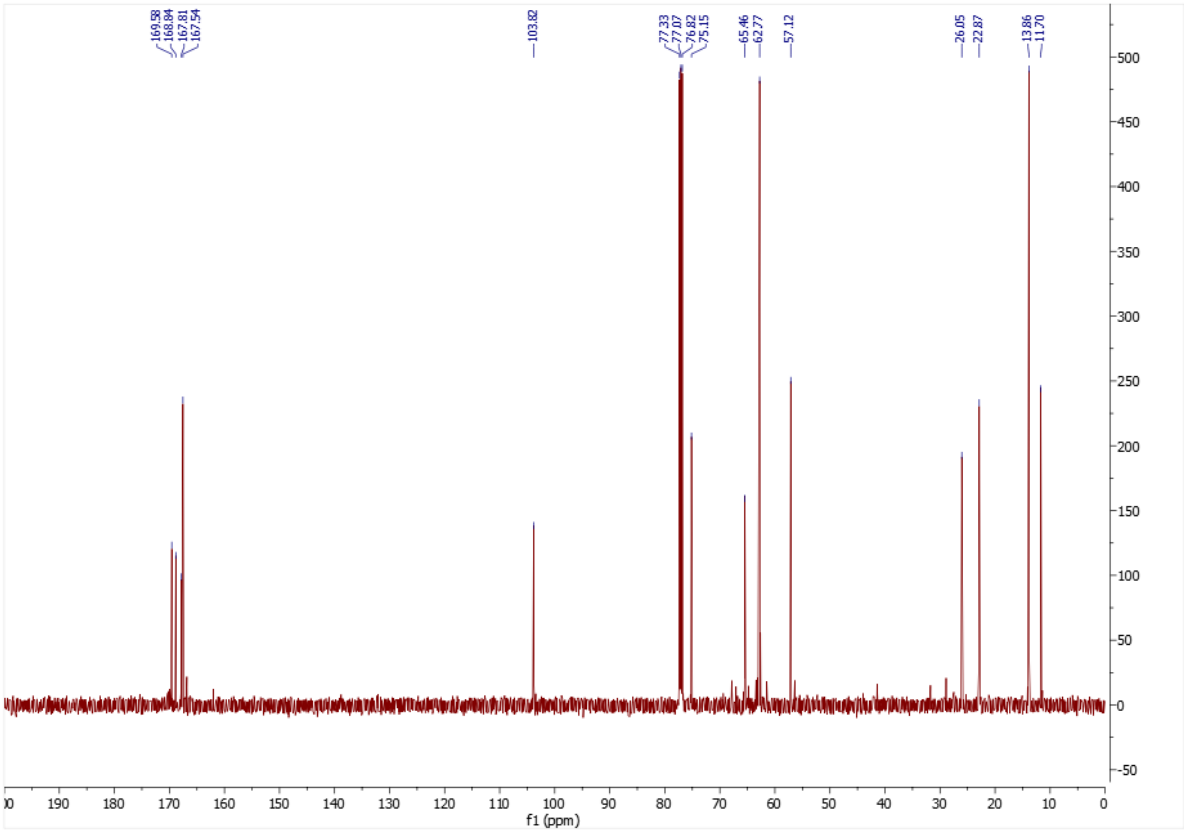


Diethyl 2-acetamido-2-((2-(methoxymethyl)-5-methyl-3-oxo-2,3-dihydroisoxazol-4-yl)methyl)malonate (**7**)

^{1}H NMR (500 MHz, chloroform-*d*)

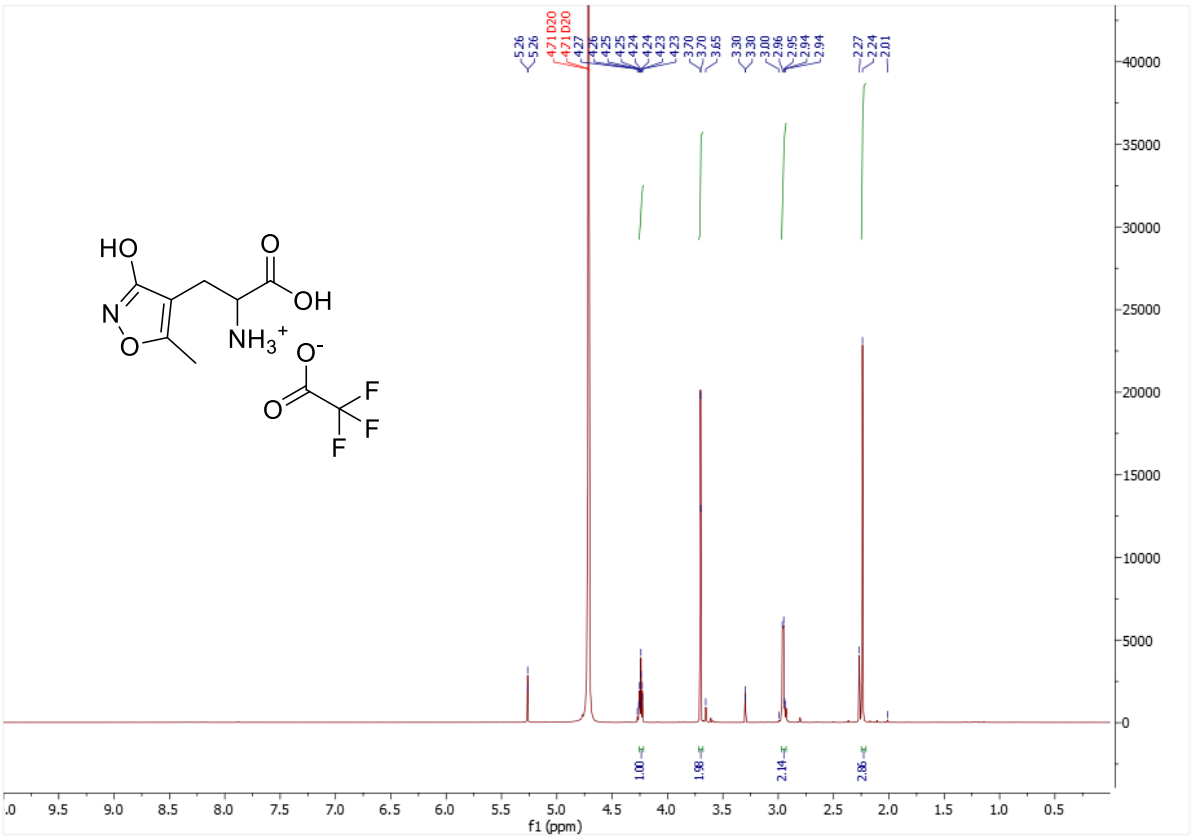


^{13}C NMR (126 MHz, chloroform-*d*)

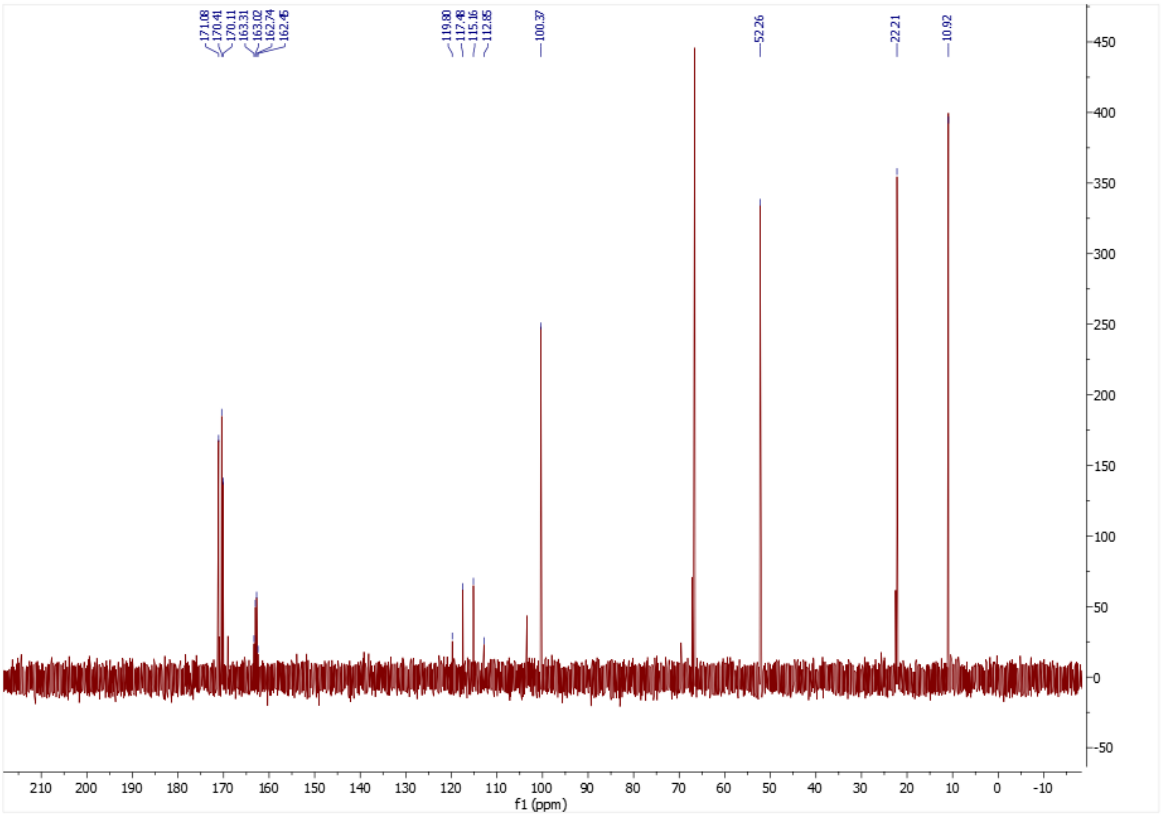


1-Carboxy-2-(3-hydroxy-5-methylisoxazol-4-yl)ethan-1-aminium 2,2,2-trifluoroacetate (± **AMPA**)

^{1}H NMR (500 MHz, deuterium oxide)

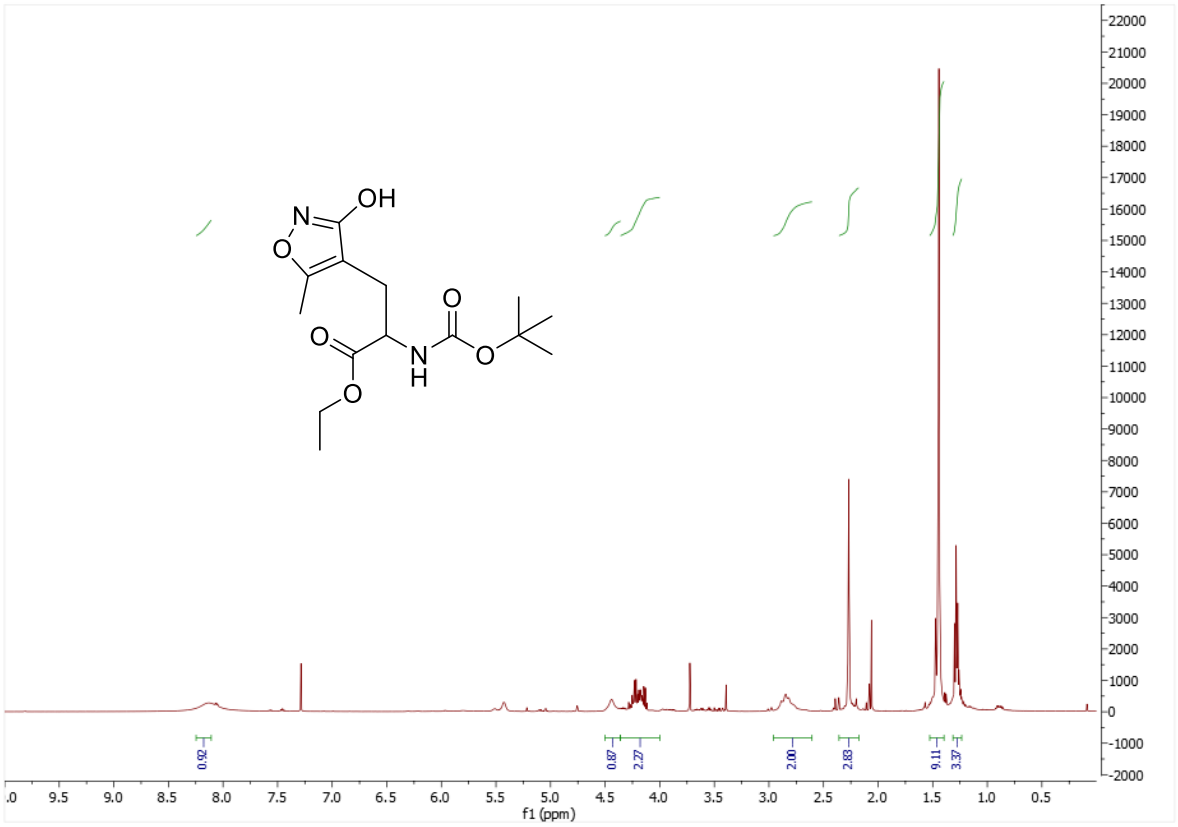


^{13}C NMR (126 MHz, deuterium oxide)

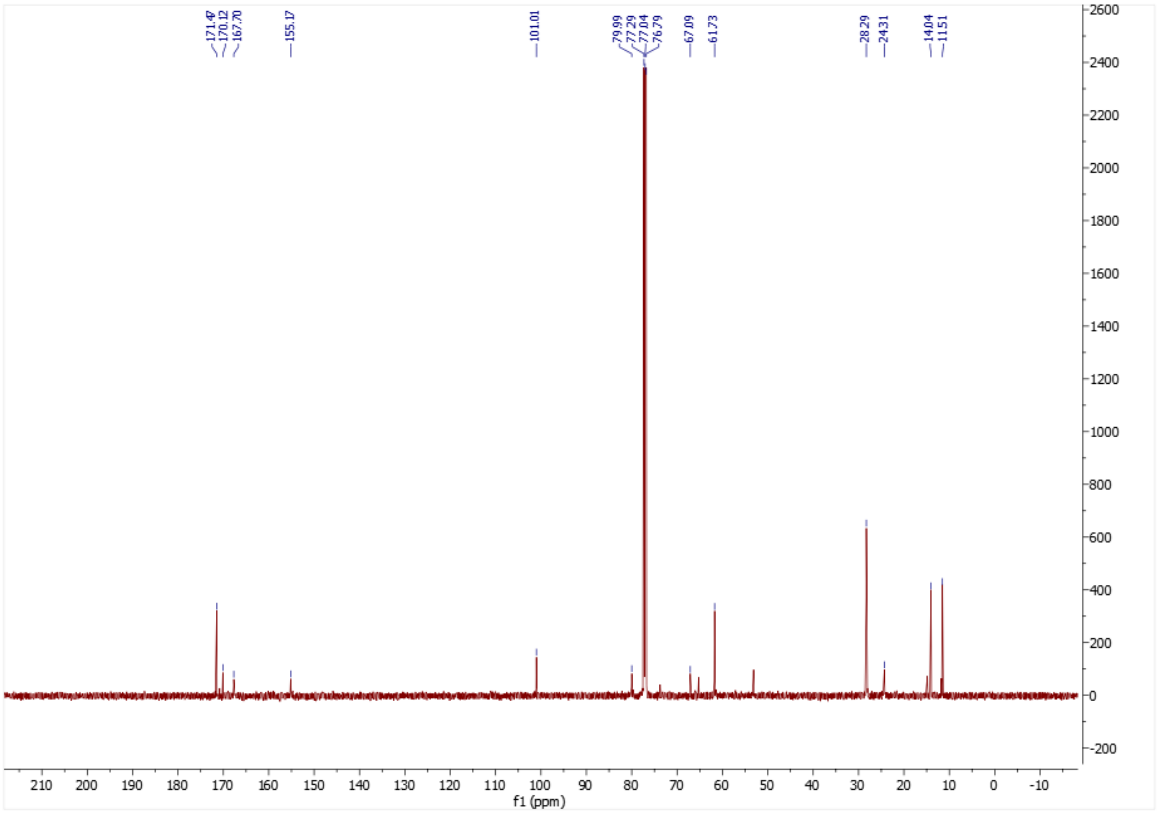


Ethyl 2-((tert-butoxycarbonyl)amino)-3-(3-hydroxy-5-methylisoxazol-4-yl)propanoate (**8**)

^{1}H NMR (500 MHz, chloroform-*d*)

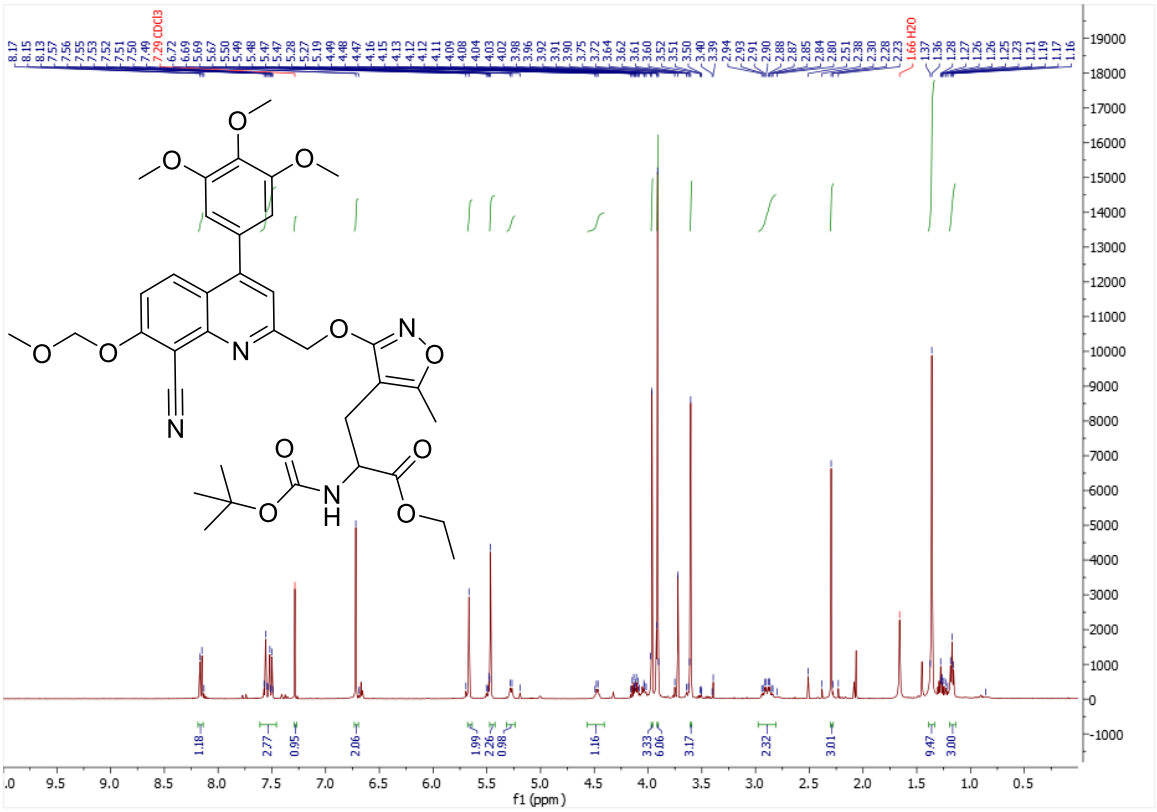


^{13}C NMR (126 MHz, chloroform-*d*)

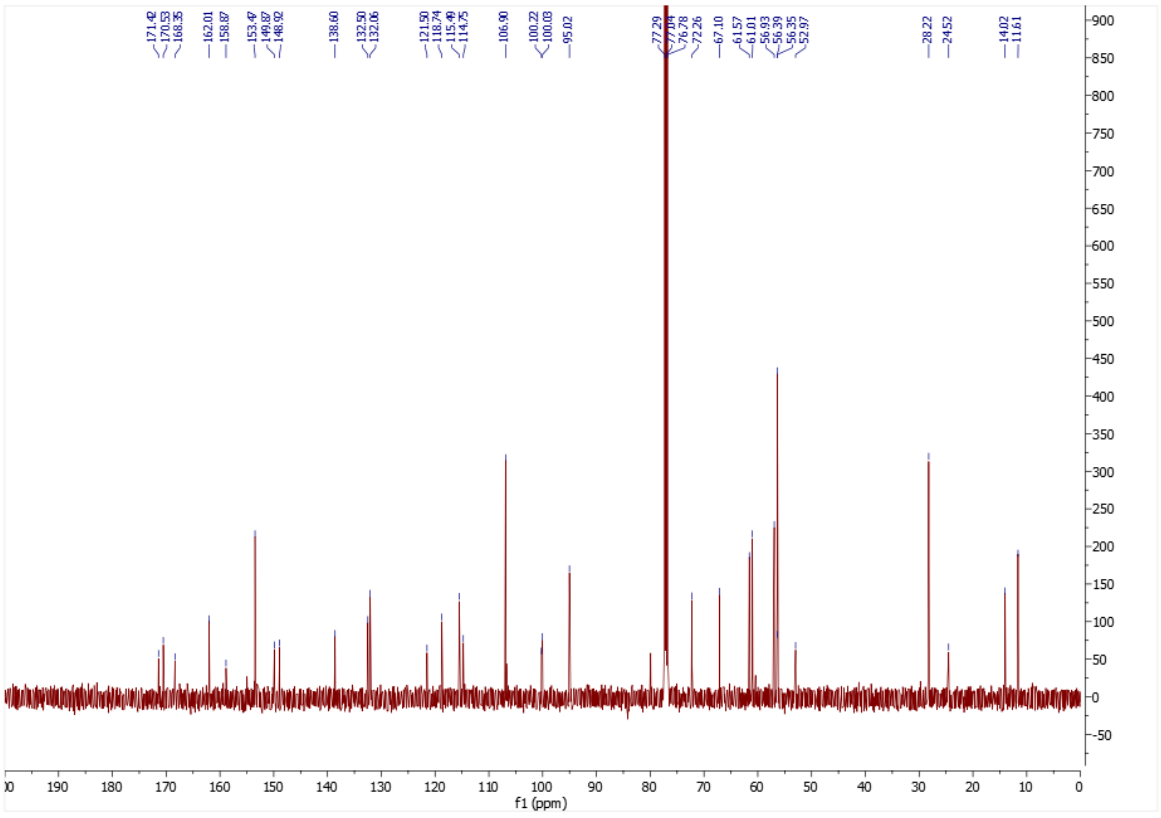


Ethyl 2-((tert-butoxycarbonyl)amino)-3-(3-((8-cyano-7-(methoxymethoxy)-4-(3,4,5-trimethoxyphenyl)quinolin-2-yl)methoxy)-5-methylisoxazol-4-yl)propanoate (**9**)

^{1}H NMR (500 MHz, chloroform-*d*)

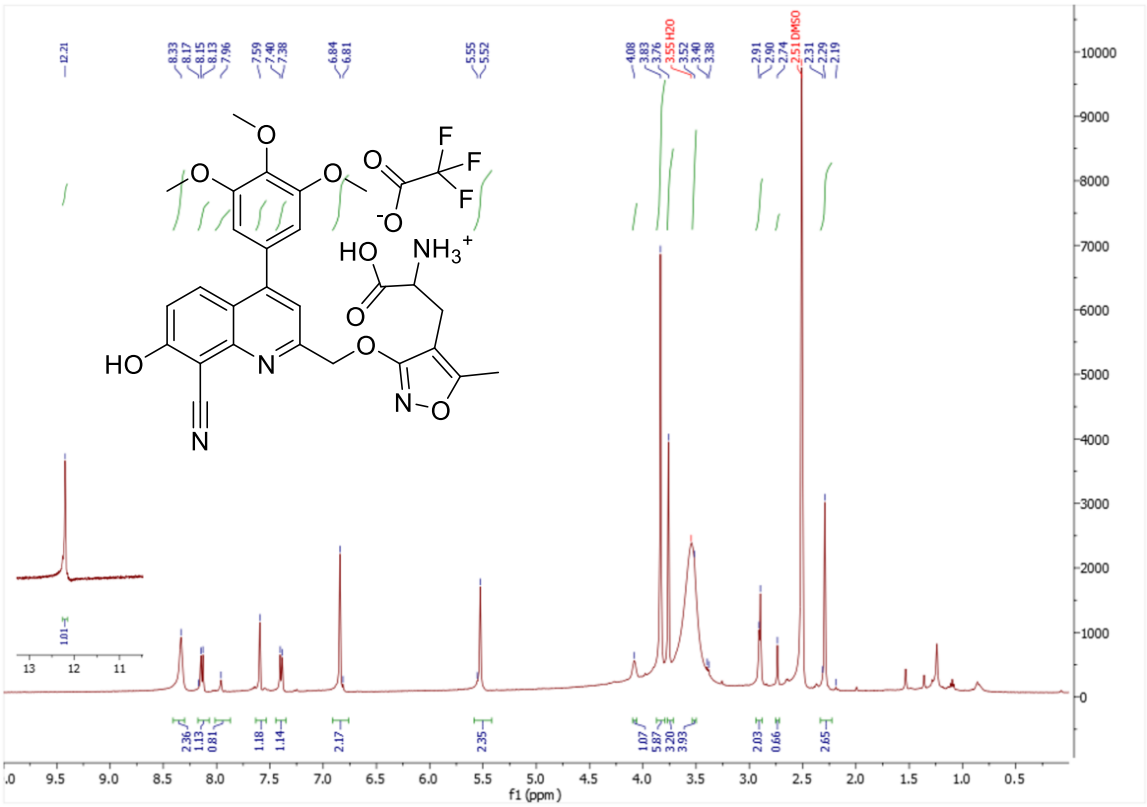


^{13}C NMR (126 MHz, chloroform-*d*)

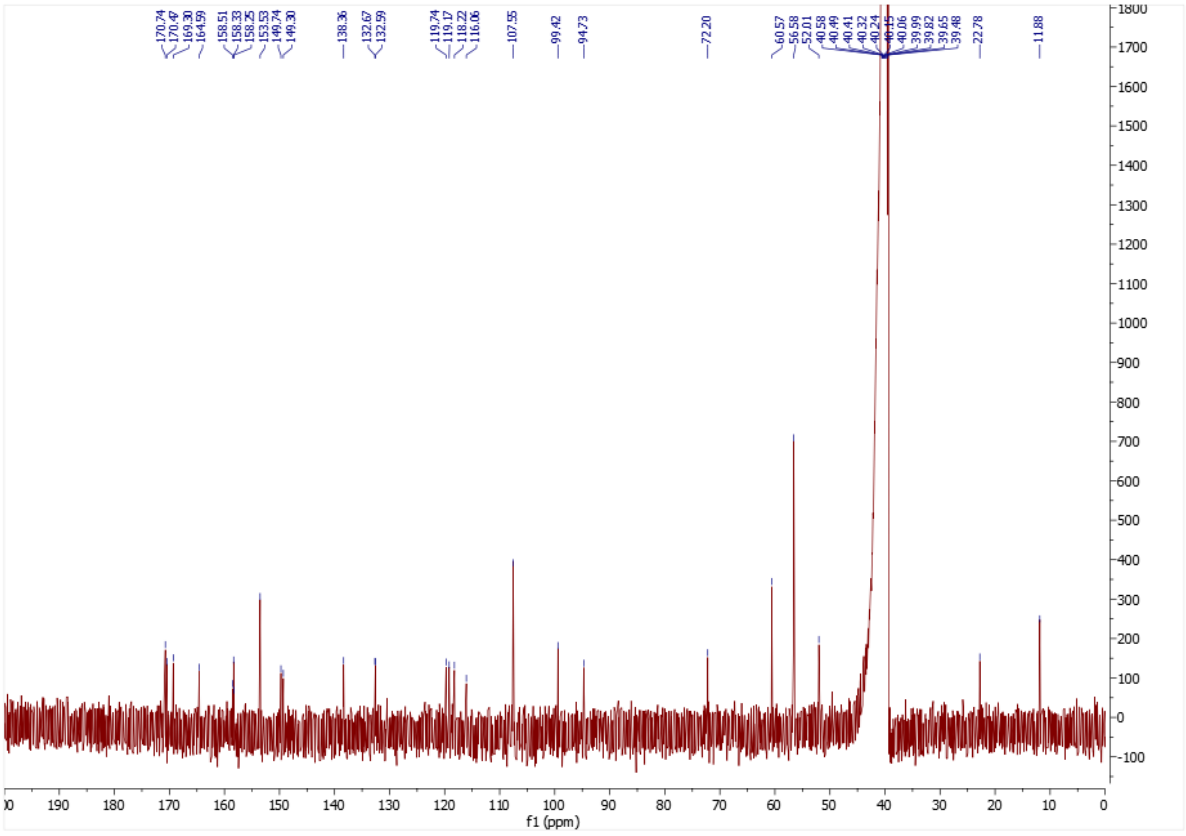


1-Carboxy-2-(3-((8-cyano-7-hydroxy-4-(3,4,5-trimethoxyphenyl)quinolin-2-yl)methoxy)-5-methylisoxazol-4-yl)ethan-1-aminium 2,2,2-trifluoroacetate (**TMP-CyHQ-AMPA**)

^{1}H NMR (500 MHz, dimethylsulfoxide-d_6)



^{13}C NMR (126 MHz, dimethylsulfoxide-d_6)



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