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Design and synthesis of novel phenylaminopyrimidines with antiproliferative activity against colorectal cancer

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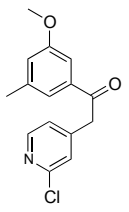
Materials and Methods:

General Synthetic Methods: Reagents and dry solvents purchased from commercial sources were used without further purification. Anhydrous reactions were carried out under an atmosphere of argon, using oven-dried glassware. Reactions were monitored using thin layer chromatography (TLC) on aluminium plates pre-coated with Silica Gel 60 F254 (E. Merck). Developed plates were observed under UV light at 254 nm and then visualized after application of a solution of H_2SO_4 in EtOH (5% v/v) and heating. Flash chromatography was performed on Silica Gel 60 (0.040-0.063mm) using HPLC grade solvents. ^1H and ^{13}C NMR spectra were recorded at 400 and 100 MHz, respectively, on a BrukerAvance 400 MHz spectrometer. Chemical shifts (δ) are reported in parts per million, relative to the residual solvent peak as internal reference [$CDCl_3$: 7.26 (*s*) for ^1H, 77.0 (*t*) for ^{13}C; DMSO: 2.50 (*p*) for ^1H, 39.51 (*hept*) for ^{13}C]. Low-resolution mass spectra (LRMS) were recorded, in electrospray ionization mode, on a BrukerDaltonics Esquire 3000 ESI spectrometer, using positive ionization mode. High-resolution mass spectra (HRMS) were recorded for final derivatives and were carried out on a BrukerDaltonics Apex III 4.7e Fourier transform MS, fitted with an Apollo ESI source. The purities of all final products after chromatographic purification were judged to be ≥95% by ^1H and ^{13}C NMR. The details of synthetic methods used and full characterization data of key intermediates and final products are reported here while copies of ^1H and ^{13}C NMR spectra are available in the supplementary material.

Synthesis of target compounds:

2-(2-Chloropyridin-4-yl)-1-(3-methoxy-5-methylphenyl)ethanone (5).

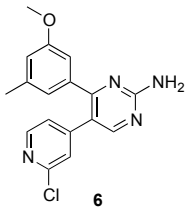


5

To a solution of the ester **4** [1, 2] **(**9.0 g, 50 mmol) and 2-chloro-4-methylpyridine (7.0 g, 55 mmol) in THF (90 mL) was added dropwise lithium bis(trimethylsilyl)amide 1M/THF (75 mL, 75 mmol) at 0

^{o}C under N_2 atmosphere. The mixture was warmed to room temperature and stirred for 18 h. Saturated aqueous ammonium chloride solution (150 mL) was added to the mixture, followed by extraction with ethylacetate (300 mL x 2). The combined organic layers were washed with saturated NaCl, dried over anhydrous $MgSO_4$ and evaporated under vacuum to yield crude **5** (9.92 g, 72%) which was used for the next step without further purification; ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.41 (s, 3H), 3.84 (s, 3H), 4.26 (s, 2H), 6.98 (s, 1H), 7.13 (d, *J* = 4.5 Hz, 1H), 7.25 (s, 1H), 7.30 (s, 1H), 7.38 (s, 1H), 8.34 (d, *J* = 4.6 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 21.36, 21.52, 44.14, 55.41, 55.47, 110.12, 120.79, 121.82, 123.82, 125.45, 137.23, 140.16, 146.86, 149.57, 151.69, 159.97, 195.14; LRMS [$C_{15}H_{14}ClNO_2$] (m/z): (+ve ion mode) 298.8 $[M+Na]^{+}$.

5-(2-Chloropyridin-4-yl)-4-(3-methoxy-5-methylphenyl)pyrimidin-2-amine (6).

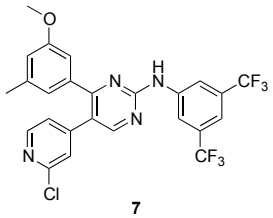


6

A mixture of compound **5** (3.03 g, 11 mmol) and *N,N*-dimethylformamide dimethylacetal (10 mL, 85 mmol) was heated under reflux in an oil bath for 12 h. The excess unreacted *N,N*-dimethylformamide dimethylacetal was removed under vacuum, and the residue was dissolved in absolute ethanol (30 mL) to form portion A. In portion B; an ethanolic solution of sodium ethoxide was prepared by dissolving sodium metal (0.28 g, 12.1 mmol) in absolute ethanol (20 mL), and then guanidine hydrochloride (1.16 g, 12.1 mmol) was added. The mixture was stirred at room temperature for 1 h, then portion A was added. The temperature was raised to reflux, and the mixture was heated for 18 h. The reaction solvent was removed under vacuo, and the residue was diluted with water (100 mL) and extracted with EtOAc (100 mL x 3). The combined organics were dried over anhydrous $MgSO_4$ and evaporated under reduced pressure. The residue was purified by column chromatography (silica gel, ethyl acetate-hexane 1:2 v/v) to give the pure product **6** (2.09 g, 58% over two steps) as off-white powder. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.22 (s, 3H), 3.59 (s, 3H), 6.57 (s, 1H), 6.78 (d, *J* = 11.1

Hz, 2H), 7.05 (dd, *J* = 5.2, 1.5 Hz, 1H), 7.14 (s, 2H), 7.30-7.36 (m, 1H), 8.23 (d, *J* = 5.2 Hz, 1H), 8.39 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.50, 55.41, 112.42, 116.09, 118.45, 122.68, 123.89, 124.09, 138.98, 139.39, 149.51, 149.81, 150.78, 159.14, 160.07, 163.67, 164.87; LRMS [$C_{17}H_{15}ClN_4O$] (m/z): (+ve ion mode) 349.8 $[M+Na]^+$; HRMS (API) (m/z): $[M+1]^+$ calcd for $C_{17}H_{15}ClN_4O$ $[M+1]^+$ 327.1013; found, 327.1015.

***N*-(3,5-Bis(trifluoromethyl)phenyl)-5-(2-chloropyridin-4-yl)-4-(3-methoxy-5-methylphenyl)-pyrimidin-2-amine (7).**

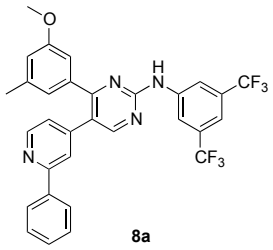


7

A mixture of the amine **6** (2.0 g, 6.12 mmol), 3,5-bis(trifluoromethyl)phenylbromide (1.97 g, 6.73 mmol), dichlorobis-(triphenylphosphine)Pd(II) (0.43 g, 0.61 mmol), Xantphos (0.36 g, 0.61 mmol) and sodium-*tert*-butoxide (0.9 g, 9.18 mmol) was refluxed in toluene (50 mL) under nitrogen atmosphere for 12 h. The reaction solvent was removed under vacuo, and the residue was diluted with water (100 mL) and extracted with EtOAc (100 mL x 3). The combined organics were dried over anhydrous $MgSO_4$ and evaporated under reduced pressure. The residue was purified by column chromatography (silica gel, ethyl acetate-hexane 1:4 v/v) to give the pure product **7** (2.28 g, 69%) as yellowish white powder. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.26 (s, 3H), 3.59 (s, 3H), 6.64 (s, 1H), 6.87 (s, 1H), 6.97 (s, 1H), 7.20 (dd, *J* = 5.3, 1.5 Hz, 1H), 7.51 (s, 1H), 7.65 (s, 1H), 8.33 (d, *J* = 5.1 Hz, 1H), 8.63 (s, 2H), 8.76 (s, 1H), 10.77 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.41, 55.39, 112.67, 114.54, 116.91, 118.74, 122.00, 122.56, 122.84, 124.16, 124.54, 125.27, 131.13 (q, *J* = 32.6 Hz), 138.16, 139.82, 142.64, 148.61, 150.15, 150.98, 159.26, 159.30, 160.36; LRMS [$C_{25}H_{17}ClF_6N_4O$] (m/z): (+ve ion mode) 562.0 $[M+Na]^+$; HRMS (API) (m/z): $[M+1]^+$ calcd for $C_{25}H_{17}ClF_6N_4O$ $[M+1]^+$ 539.1073; found, 539.1079.

General Procedure for the synthesis of compounds 8a-j. A mixture of compound **7** (200 mg, 0.37 mmol), the appropriate aryl boronic acid (0.407 mmol), dichlorobis(triphenylphosphine)Pd(II) (13 mg, 0.019 mmol) and K_2CO_3 (51 mg, 0.37 mmol) was placed in a mixed solvent of THF and water (4:1, 10 mL). N_2 gas was bubbled into this mixture for 10 minutes, and then the mixture was heated at 70 ^oC while stirring under N_2 atmosphere for 20 h. The reaction mixture was left to cool at room temperature, and then poured into ice water (100 mL) and extracted with ethylacetate (100 mL x 2). The combined organic extracts were dried over anhydrous Na_2SO_4 and evaporated under vacuum, and the target products were purified by column chromatography using suitable solvent system.

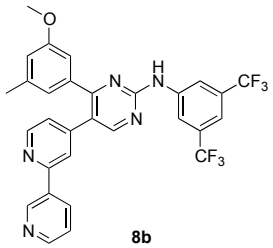
***N*-(3,5-Bis(trifluoromethyl)phenyl)-4-(3-methoxy-5-methylphenyl)-5-(2-phenylpyridin-4-yl)pyrimidin-2-amine (8a).**



8a

Column chromatography (silica, ethyl acetate-hexane 1:3 v/v). Yield: (77%); ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.25 (s, 3H), 3.53 (s, 3H), 6.68 (s, 1H), 6.85 (s, 1H), 7.05 (s, 1H), 7.14 (dd, J = 5.1, 1.6 Hz, 1H), 7.42-7.53 (m, 3H), 7.65 (s, 1H), 7.93 (s, 1H), 7.97-8.07 (m, 2H), 8.57 (d, J = 5.0 Hz, 1H), 8.65 (s, 2H), 8.86 (s, 1H), 10.74 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.41, 55.34, 112.71, 114.31, 116.72, 118.65 (d, J = 4.7 Hz), 121.01, 122.58, 122.79, 123.32, 123.44, 125.29, 127.10, 129.19, 129.66, 131.13 (q, J = 32.5 Hz), 138.54, 139.00, 139.72, 142.79, 145.81, 149.89, 156.90, 159.23, 160.34, 164.31; LRMS [$C_{31}H_{22}F_6N_4O$] (m/z): (+ve ion mode) 603.1 $[M+Na]^+$; HRMS (API) (m/z): $[M+1]^+$ calcd for $C_{31}H_{22}F_6N_4O$ $[M+1]^+$ 581.1776; found, 581.1770.

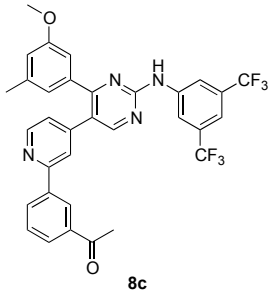
***N*-(3,5-Bis(trifluoromethyl)phenyl)-4-(3-methoxy-5-methylphenyl)-5-(2-(pyridin-3-yl)pyridin-4-yl)pyrimidin-2-amine (8b).**



8b

Column chromatography (silica, ethyl acetate-hexane 1:1 v/v). Yield: (89%); ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.25 (s, 3H), 3.54 (s, 3H), 6.68 (s, 1H), 6.85 (s, 1H), 7.03 (s, 1H), 7.17 (dd, J = 5.1, 1.6 Hz, 1H), 7.52 (ddd, J = 8.0, 4.8, 0.9 Hz, 1H), 7.64 (s, 1H), 8.05 (s, 1H), 8.39 (dt, J = 8.1, 2.0 Hz, 1H), 8.56-8.71 (m, 4H), 8.87 (s, 1H), 9.17 (d, J = 1.8 Hz, 1H), 10.74 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.40, 55.35, 112.73, 114.32, 116.75, 118.65 (d, J = 4.7 Hz), 121.37, 122.83, 123.18, 123.98, 124.28, 125.28, 131.14 (q, J = 33.0, 32.6 Hz), 134.41, 134.52, 138.47, 139.77, 142.75, 146.06, 148.30, 150.15, 150.51, 154.66, 159.21, 159.24, 160.42; LRMS [$C_{30}H_{21}F_6N_5O$] (m/z): (+ve ion mode) 604.5 $[M+Na]^+$; HRMS (API) (m/z): $[M+1]^+$ calcd for $C_{30}H_{21}F_6N_5O$ $[M+1]^+$ 582.1729; found, 582.1732.

1-(3-(4-(2-(3,5-Bis(trifluoromethyl)phenylamino)-4-(3-methoxy-5-methylphenyl)pyrimidin-5-yl)pyridin-2-yl)phenyl)ethanone (8c).

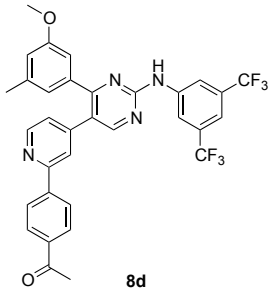


8c

Column chromatography (silica, ethyl acetate-hexane 1:1 v/v). Yield: (85%); ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.25 (s, 3H), 2.64 (s, 3H), 3.53 (s, 3H), 6.68 (s, 1H), 6.85 (s, 1H), 7.04 (s, 1H), 7.19 (dd, J = 5.0, 1.6 Hz, 1H), 7.58-7.70 (m, 2H), 7.98-8.07 (m, 2H), 8.28 (dt, J = 7.9, 1.4 Hz, 1H), 8.53 (s, 1H), 8.61 (d, J = 5.0 Hz, 1H), 8.65 (s, 2H), 8.88 (s, 1H), 10.75 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.41, 27.37, 55.33, 112.75, 114.33, 116.75, 118.65, 121.29, 122.58, 122.82, 123.28, 123.77, 125.29, 126.69, 129.28, 129.70, 130.80 (m), 137.82, 138.48, 139.38, 139.76, 142.77, 146.02,

150.01, 155.93, 159.22, 160.40, 161.55, 164.36, 198.29; LRMS [$C_{33}H_{24}F_6N_4O_2$] (m/z): (+ve ion mode) 645.4 $[M+Na]^+$; HRMS (API) (m/z): $[M+1]^+$ calcd for $C_{33}H_{24}F_6N_4O_2$ $[M+1]^+$ 623.1882; found, 623.1879.

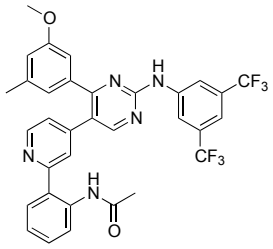
1-(4-(4-(2-(3,5-Bis(trifluoromethyl)phenylamino)-4-(3-methoxy-5-methylphenyl)pyrimidin-5-yl)pyridin-2-yl)phenyl)ethanone (8d).



8d

Column chromatography (silica, ethyl acetate-hexane 1:1 v/v). Yield: (84%); ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.25 (s, 3H), 2.62 (s, 3H), 3.53 (s, 3H), 6.67 (s, 1H), 6.85 (s, 1H), 7.04 (s, 1H), 7.19 (dd, J = 5.2, 1.6 Hz, 1H), 7.64 (s, 1H), 8.05 (d, J = 8.5 Hz, 3H), 8.16 (d, J = 8.5 Hz, 2H), 8.61 (d, J = 5.1 Hz, 1H), 8.65 (s, 2H), 8.87 (s, 1H), 10.75 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.41, 27.34, 55.34, 112.72, 115.60, 116.75, 118.67, 121.75, 122.58, 122.82, 123.21, 124.10, 125.29, 127.29, 129.14, 130.97, 131.24 (m), 137.47, 138.47, 139.77, 142.77, 143.03, 146.06, 150.08, 155.66, 159.22, 159.24, 160.41, 198.10; LRMS [$C_{33}H_{24}F_6N_4O_2$] (m/z): (+ve ion mode) 645.5 $[M+Na]^+$; HRMS (API) (m/z): $[M+1]^+$ calcd for $C_{33}H_{24}F_6N_4O_2$ $[M+1]^+$ 623.1882; found, 623.1879.

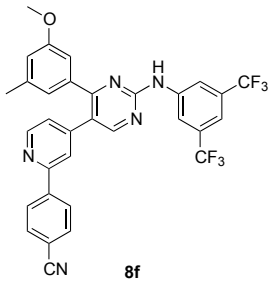
***N*-(2-(4-(2-(3,5-Bis(trifluoromethyl)phenylamino)-4-(3-methoxy-5-methylphenyl)pyrimidin-5-yl)pyridin-2-yl)phenyl)acetamide (8e).**



Column chromatography (silica, ethyl acetate-hexane 2:1 v/v). Yield: (79%); ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.06 (s, 3H), 2.26 (s, 3H), 3.55 (s, 3H), 6.69 (s, 1H), 6.87 (s, 1H), 7.03 (s, 1H), 7.17 (t,

J = 7.6 Hz, 1H), 7.22 (dd, *J* = 5.2, 1.6 Hz, 1H), 7.40 (td, *J* = 7.8, 1.5 Hz, 1H), 7.55-7.61 (m, 1H), 7.65 (s, 1H), 7.82 (s, 1H), 8.22 (d, *J* = 8.2 Hz, 1H), 8.63 (d, *J* = 9.9 Hz, 3H), 8.86 (s, 1H), 10.76 (s, 1H), 11.65 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.37, 24.97, 55.33, 112.66, 114.40, 116.89, 118.72, 122.58, 122.89, 123.10, 123.18, 123.97, 124.17, 125.29, 127.26, 129.92, 130.13, 131.14 (q, *J* = 32.4 Hz), 137.42, 138.45, 139.83, 142.74, 146.62, 148.39, 157.76, 159.25, 159.31, 160.35, 168.43, 198.66; LRMS [$C_{33}H_{25}F_6N_5O_2$] (m/z): (+ve ion mode) 660.6 $[M+Na]^+$; HRMS (API) (m/z): $[M+1]^+$ calcd for $C_{33}H_{25}F_6N_5O_2$ $[M+1]^+$ 638.1991; found, 638.1979.

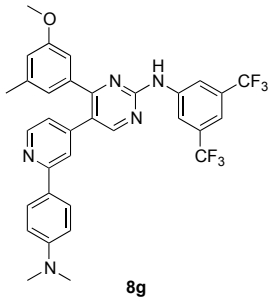
4-(4-(2-(3,5-Bis(trifluoromethyl)phenylamino)-4-(3-methoxy-5-methylphenyl)pyrimidin-5-yl)pyridin-2-yl)benzonitrile (8f).



8f

Column chromatography (silica, ethyl acetate-hexane 2:3 v/v). Yield: (91%); ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.25 (s, 3H), 3.53 (s, 3H), 6.66 (s, 1H), 6.84 (s, 1H), 7.02 (s, 1H), 7.17 (dd, *J* = 5.0, 1.5 Hz, 1H), 7.64 (s, 1H), 7.97 (d, *J* = 8.3 Hz, 2H), 8.11 (s, 1H), 8.24 (d, *J* = 8.4 Hz, 2H), 8.62 (d, *J* = 18.4 Hz, 3H), 8.88 (s, 1H), 10.76 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.41, 55.35, 112.10, 112.76, 116.74, 116.87, 118.68, 119.21, 121.86, 122.58, 122.82, 123.11, 124.53, 125.29, 127.86, 128.00, 131.14 (q, *J* = 32.7 Hz), 133.24, 134.72, 138.44, 139.78, 142.75, 143.15, 146.19, 150.11, 154.98, 159.24; LRMS [$C_{32}H_{22}F_6N_5O$] (m/z): (+ve ion mode) 638.3 $[M+Na]^+$; HRMS (API) (m/z): $[M+1]^+$ calcd for $C_{32}H_{22}F_6N_5O$ $[M+1]^+$ 606.1729; found, 606.1731.

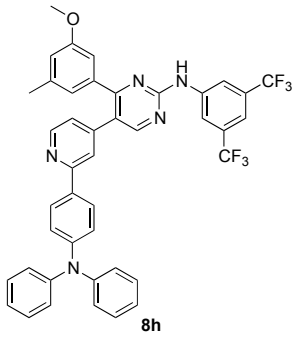
***N*-(3,5-Bis(trifluoromethyl)phenyl)-5-(2-(4-(dimethylamino)phenyl)pyridin-4-yl)-4-(3-methoxy-5-methylphenyl)pyrimidin-2-amine (8g).**



8g

Column chromatography (silica, ethyl acetate-hexane 1:2 v/v). Yield: (79%); ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.25 (s, 3H), 2.96 (s, 6H), 3.53 (s, 3H), 6.68 (s, 1H), 6.76 (d, J = 9.0 Hz, 2H), 6.83 (s, 1H), 6.97 (dd, J = 5.1, 1.3 Hz, 1H), 7.06 (s, 1H), 7.64 (s, 1H), 7.75 (s, 1H), 7.87 (d, J = 8.9 Hz, 2H), 8.46 (d, J = 5.1 Hz, 1H), 8.65 (d, J = 1.7 Hz, 2H), 8.80 (s, 1H), 10.71 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.43, 55.30, 112.35, 112.70, 114.25, 116.68, 118.60, 119.21, 121.58, 122.59, 122.75, 123.75, 125.30, 126.36, 127.91, 131.12 (q, J = 33.4, 32.6 Hz), 138.59, 139.66, 142.84, 145.39, 149.60, 151.44, 157.26, 159.11, 159.17, 160.28; LRMS [$C_{33}H_{27}F_6N_5O$] (m/z): (+ve ion mode) 646.5 $[M+Na]^+$; HRMS (API) (m/z): $[M+1]^+$ calcd for $C_{33}H_{27}F_6N_5O$ $[M+1]^+$ 624.2198; found, 624.2203.

5-(2-(4-(Diphenylamino)phenyl)pyridin-4-yl)-N-(3,5-bis(trifluoromethyl)phenyl)-4-(3-methoxy-5-methylphenyl)pyrimidin-2-amine (8h).

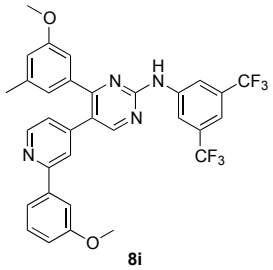


8h

Column chromatography (silica, ethyl acetate-hexane 1:3 v/v). Yield: (80%); ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.24 (s, 3H), 3.53 (s, 3H), 6.67 (s, 1H), 6.83 (s, 1H), 6.97-7.14 (m, 10H), 7.29-7.39 (m, 4H), 7.63 (s, 1H), 7.86 (s, 1H), 7.94 (d, J = 8.7 Hz, 2H), 8.50 (d, J = 5.1 Hz, 1H), 8.64 (s, 2H), 8.81 (s, 1H), 10.72 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.42, 55.34, 112.71, 114.29, 116.74, 118.63, 120.15, 122.56, 122.70, 122.79, 123.56, 124.12, 125.06, 125.28, 127.99, 128.23, 130.14, 131.12 (q, J = 32.5 Hz), 132.48, 138.52, 139.69, 142.79, 145.74, 147.27, 148.78, 149.69, 156.53,

159.15, 159.20, 160.32, 164.17; LRMS [$C_{43}H_{31}F_6N_5O$] (m/z): (+ve ion mode) 770.9 [M+Na]$^+$; HRMS (API) (m/z): [M+1]$^+$ calcd for $C_{43}H_{31}F_6N_5O$ [M+1]$^+$ 748.2511; found, 748.2515.

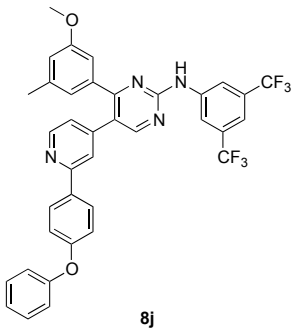
***N*-(3,5-Bis(trifluoromethyl)phenyl)-4-(3-methoxy-5-methylphenyl)-5-(2-(3-methoxyphenyl)-pyridin-4-yl)pyrimidin-2-amine (8i).**



8i

Column chromatography (silica, ethyl acetate-hexane 1:2 v/v). Yield: (84%); ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.25 (s, 3H), 3.54 (s, 3H), 3.81 (s, 3H), 6.68 (s, 1H), 6.85 (s, 1H), 6.95-7.08 (m, 2H), 7.17 (d, J = 5.1 Hz, 1H), 7.38 (t, J = 7.8 Hz, 1H), 7.51 (s, 1H), 7.58 (d, J = 7.8 Hz, 1H), 7.64 (s, 1H), 7.90 (s, 1H), 8.58 (d, J = 5.0 Hz, 1H), 8.65 (s, 2H), 8.86 (s, 1H), 10.74 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.40, 55.33, 55.62, 112.03, 112.70, 114.35 (d, J = 5.6 Hz), 115.65, 116.72, 118.65, 119.46, 121.23, 122.58, 122.81, 123.39, 125.29, 129.16, 130.28, 131.13 (d, J = 32.5 Hz), 138.57, 139.73, 140.48, 142.79, 145.78, 149.86, 156.61, 159.18, 159.23, 160.13, 160.36; LRMS [$C_{32}H_{24}F_6N_4O_2$] (m/z): (+ve ion mode) 633.5 [M+Na]$^+$; HRMS (API) (m/z): [M+1]$^+$ calcd for $C_{32}H_{24}F_6N_4O_2$ [M+1]$^+$ 611.1882; found, 611.1880.

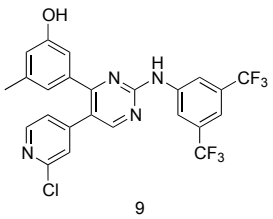
***N*-(3,5-Bis(trifluoromethyl)phenyl)-4-(3-methoxy-5-methylphenyl)-5-(2-(4-phenoxyphenyl)-pyridin-4-yl)pyrimidin-2-amine (8j).**



8j

Column chromatography (silica, ethyl acetate-hexane 1:3 v/v). Yield: (80%); ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.25 (s, 3H), 3.53 (s, 3H), 6.67 (s, 1H), 6.84 (s, 1H), 7.02-7.13 (m, 6H), 7.16-7.23 (m, 1H), 7.43 (dd, J = 8.6, 7.4 Hz, 2H), 7.64 (s, 1H), 7.92 (s, 1H), 8.06 (d, J = 8.8 Hz, 2H), 8.54 (d, J = 5.1 Hz, 1H), 8.65 (s, 2H), 8.84 (s, 1H), 10.74 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.42, 55.33, 112.73, 114.27, 116.73, 118.64 (d, J = 3.8 Hz), 118.77, 119.65, 120.53, 122.59, 122.80, 123.04, 123.46, 124.42, 125.29, 128.93, 130.65, 131.13 (d, J = 32.5 Hz), 134.03, 138.52, 139.73, 142.79, 145.85, 149.79, 156.32, 156.57, 158.41, 159.17, 159.21, 160.37; LRMS [$C_{37}H_{26}F_6N_4O_2$] (m/z): (+ve ion mode) 695.2 [M+Na]$^+$; HRMS (API) (m/z): [M+1]$^+$ calcd for $C_{37}H_{26}F_6N_4O_2$ [M+1]$^+$ 672.2038; found, 672.2047.

***N*-(3,5-Bis(trifluoromethyl)phenyl)-5-(2-chloropyridin-4-yl)-4-(3-hydroxy-5-methylphenyl) pyrimidin-2-amine (9).**



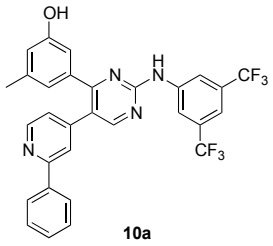
9

To a solution of compound **7** (100 mg, 0.185 mmol), in anhydrous chloroform (10 mL) was added borontrifluoride-methyl sulfide complex (0.39 mL, 3.7 mmol) dropwise at room temperature and under N_2 atmosphere. The resulting suspension was stirred for 48 h, and then the mixture was concentrated under vacuum. The residue was partitioned between ethylacetate (100 mL) and saturated Na_2CO_3 (50 mL). The organic layer was separated and dried over anhydrous Na_2SO_4, then evaporated under vacuum. The residue was then purified by column chromatography (silica, ethyl acetate-hexane 1:3 v/v), to yield the pure hydroxyl product **9** (81 mg, 81%) as buff powder. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.23 (s, 3H), 6.44 (s, 1H), 6.66 (s, 1H), 6.90 (s, 1H), 7.19 (dd, J = 5.2, 1.5 Hz, 1H), 7.52 (s, 1H), 7.65 (s, 1H), 8.27-8.38 (m, 1H), 8.63 (s, 2H), 8.74 (s, 1H), 9.47 (s, 1H), 10.75 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.37, 114.22, 114.43 (d, J = 2.7 Hz), 118.09, 118.71 (d, J = 3.4 Hz), 121.14, 121.79, 122.56, 124.06, 124.36, 125.27, 131.13 (q, J = 32.4 Hz), 138.12,

139.67, 142.67, 148.60, 150.11, 151.04, 157.37, 159.26, 160.43; LRMS [$C_{24}H_{15}ClF_6N_4O$] (m/z): (+ve ion mode) 547.9 $[M+Na]^+$; HRMS (API) (m/z): $[M+1]^+$ calcd for $C_{24}H_{15}ClF_6N_4O$ $[M+1]^+$ 525.0917; found, 525.0920.

General Procedure for the synthesis of compounds 10a-j. To a solution of the starting methoxy compound **8a-j** (0.15 mmol) in anhydrous chloroform (10 mL) was added borontrifluoride-methyl sulfide complex (0.315 mL, 3.0 mmol) dropwise at room temperature and under N_2 atmosphere. The resulting suspension was stirred for 48 h, and then the mixture was concentrated under vacuum. The residue was partitioned between ethylacetate (100 mL) and saturated Na_2CO_3 (50 mL). The organic layer was separated and dried over anhydrous Na_2SO_4, then evaporated under vacuum. The residue was then purified by column chromatography using suitable solvent system to yield the pure hydroxyl product.

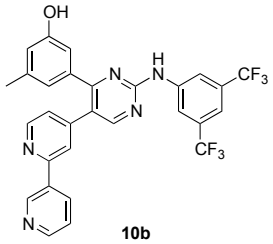
3-(2-(3,5-Bis(trifluoromethyl)phenylamino)-5-(2-phenylpyridin-4-yl)pyrimidin-4-yl)-5-methylphenol (10a).



10a

Column chromatography (silica, ethyl acetate-hexane 1:3 v/v). Yield: (84%); ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.22 (s, 3H), 6.50 (s, 1H), 6.65 (s, 1H), 6.94 (s, 1H), 7.13 (dd, J = 5.0, 1.6 Hz, 1H), 7.40-7.55 (m, 3H), 7.64 (s, 1H), 7.94 (s, 1H), 7.98-8.13 (m, 2H), 8.57 (d, J = 5.1 Hz, 1H), 8.65 (s, 2H), 8.84 (s, 1H), 9.43 (s, 1H), 10.72 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.38, 114.17, 117.84, 118.63, 119.89, 120.80, 121.14, 122.59, 123.23, 125.30, 127.12, 129.19, 129.66, 131.14 (d, J = 32.5 Hz), 138.60, 139.04, 139.55, 142.83, 145.75, 149.85, 156.85, 157.37, 158.27, 159.14, 160.41; LRMS [$C_{30}H_{20}F_6N_4O$] (m/z): (+ve ion mode) 589.6 $[M+Na]^+$; HRMS (API) (m/z): $[M+1]^+$ calcd for $C_{30}H_{20}F_6N_4O$ $[M+1]^+$ 567.1620; found, 567.1616.

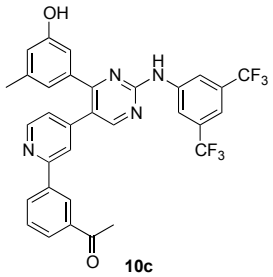
3-(2-(3,5-Bis(trifluoromethyl)phenylamino)-5-(2-(pyridin-3-yl)pyridin-4-yl)pyrimidin-4-yl)-5-methylphenol (10b).



10b

Column chromatography (silica, ethyl acetate-hexane 1:1 v/v). Yield: (85%); ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.22 (s, 3H), 6.49 (s, 1H), 6.66 (s, 1H), 6.94 (s, 1H), 7.16 (dd, J = 5.0, 1.6 Hz, 1H), 7.53 (dd, J = 8.0, 4.8 Hz, 1H), 7.64 (s, 1H), 8.07 (d, J = 1.6 Hz, 1H), 8.41 (dt, J = 8.2, 1.9 Hz, 1H), 8.60 (d, J = 5.1 Hz, 1H), 8.62-8.72 (m, 3H), 8.87 (s, 1H), 9.16-9.25 (m, 1H), 9.43 (s, 1H), 10.74 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.37, 114.20, 114.31, 117.89, 118.64, 121.16, 122.58, 122.96, 123.89, 124.26, 125.30, 131.14 (q, J = 32.1 Hz), 134.44, 134.50, 138.51, 139.60, 142.79, 145.99, 148.34, 150.11, 150.49, 154.65, 157.36, 159.17, 160.48, 164.78; LRMS [$C_{29}H_{19}F_6N_5O$] (m/z): (+ve ion mode) 590.5 $[M+Na]^+$; HRMS (API) (m/z): $[M+1]^+$ calcd for $C_{29}H_{19}F_6N_5O$ $[M+1]^+$ 568.1572; found, 568.1575.

1-(3-(4-(2-(3,5-Bis(trifluoromethyl)phenylamino)-4-(3-hydroxy-5-methylphenyl)pyrimidin-5-yl)pyridin-2-yl)phenyl)ethanone (10c).

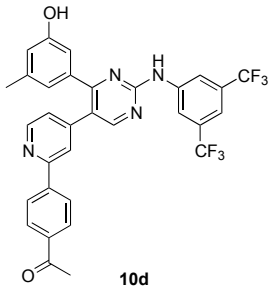


10c

Column chromatography (silica, ethyl acetate-hexane 1:1 v/v). Yield: (80%); ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.23 (s, 3H), 2.65 (s, 3H), 6.49 (s, 1H), 6.65 (s, 1H), 6.96 (s, 1H), 7.18 (dd, J = 5.0, 1.5 Hz, 1H), 7.59-7.71 (m, 2H), 7.99-8.10 (m, 2H), 8.31 (dt, J = 7.9, 1.3 Hz, 1H), 8.56 (s, 1H), 8.61 (d, J = 4.8 Hz, 1H), 8.65 (s, 2H), 8.87 (s, 1H), 9.42 (s, 1H), 10.73 (s, 1H); ^{13}C NMR (100 MHz, DMSO-

d_6): δ 21.38, 27.39, 114.20, 117.90, 118.66, 121.08, 121.17, 122.58, 123.08, 123.71, 125.29, 126.73, 129.23, 129.49, 129.70, 131.14 (d, J = 32.7 Hz), 131.65, 137.83, 138.54, 139.45, 139.59, 142.81, 145.99, 149.98, 155.91, 157.35, 159.17, 160.45, 198.33; LRMS [$C_{32}H_{22}F_6N_4O_2$] (m/z): (+ve ion mode) 631.6 $[M+Na]^+$; HRMS (API) (m/z): $[M+1]^+$ calcd for $C_{32}H_{22}F_6N_4O_2$ $[M+1]^+$ 609.1725; found, 609.1722.

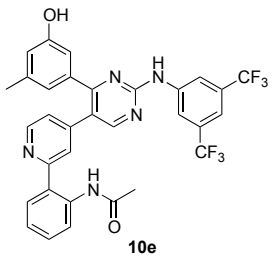
1-(4-(4-(2-(3,5-Bis(trifluoromethyl)phenylamino)-4-(3-hydroxy-5-methylphenyl)pyrimidin-5-yl)pyridin-2-yl)phenyl)ethanone (10d).



10d

Column chromatography (silica, ethyl acetate-hexane 1:1 v/v). Yield: (77%); ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.22 (s, 3H), 2.63 (s, 3H), 6.49 (s, 1H), 6.66 (s, 1H), 6.94 (s, 1H), 7.18 (dd, J = 5.1, 1.6 Hz, 1H), 7.64 (s, 1H), 7.99-8.11 (m, 3H), 8.19 (d, J = 8.4 Hz, 2H), 8.57-8.71 (m, 3H), 8.86 (s, 1H), 9.43 (s, 1H), 10.73 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.37, 27.34, 114.18, 115.60, 117.87, 118.64, 121.15, 121.52, 122.58, 122.99, 124.00, 125.29, 127.28, 129.12, 131.14 (q, J = 31.9, 31.5 Hz), 137.43, 138.52, 139.60, 142.80, 143.10, 145.96, 150.06, 155.63, 157.37, 159.17, 160.43, 198.11; LRMS [$C_{32}H_{22}F_6N_4O_2$] (m/z): (+ve ion mode) 631.5 $[M+Na]^+$; HRMS (API) (m/z): $[M+1]^+$ calcd for $C_{32}H_{22}F_6N_4O_2$ $[M+1]^+$ 609.1725; found, 609.1721.

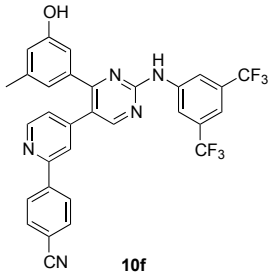
***N*-(2-(4-(2-(3,5-Bis(trifluoromethyl)phenylamino)-4-(3-hydroxy-5-methylphenyl)pyrimidin-5-yl)pyridin-2-yl)phenyl)acetamide (10e).**



10e

Column chromatography (silica, ethyl acetate-hexane 2:1 v/v). Yield: (64%); ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.07 (s, 3H), 2.23 (s, 3H), 6.51 (s, 1H), 6.67 (s, 1H), 6.93 (s, 1H), 7.17 (t, J = 7.6 Hz, 1H), 7.22 (d, J = 5.2 Hz, 1H), 7.41 (t, J = 7.8 Hz, 1H), 7.58-7.70 (m, 2H), 7.85 (s, 1H), 8.26 (d, J = 8.2 Hz, 1H), 8.63 (d, J = 10.1 Hz, 3H), 8.85 (s, 1H), 9.47 (s, 1H), 10.75 (s, 1H), 11.79 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.36, 25.06, 114.23, 117.87, 118.69, 121.16, 122.32, 122.32, 122.57, 122.95, 122.99, 123.72, 124.07, 125.28, 129.90, 130.11, 131.14 (q, J = 32.4 Hz), 137.54, 138.47, 139.63, 142.77, 146.56, 148.34, 157.45, 157.77, 159.19, 160.32, 164.82, 168.48; carboxylate LRMS [$C_{32}H_{23}F_6N_5O_2$] (m/z): (+ve ion mode) 646.7 [M+Na]$^+$; HRMS (API) (m/z): [M+1]$^+$ calcd for $C_{32}H_{23}F_6N_5O_2$ [M+1]$^+$ 624.1834; found, 624.1822.

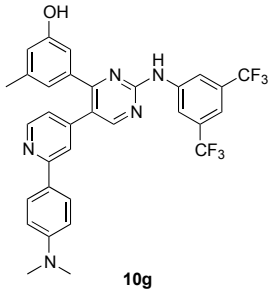
4-(4-(2-(3,5-Bis(trifluoromethyl)phenylamino)-4-(3-hydroxy-5-methylphenyl)pyrimidin-5-yl)pyridin-2-yl)benzonitrile (10f).



10f

Column chromatography (silica, ethyl acetate-hexane 2:3 v/v). Yield: (78%); ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.22 (s, 3H), 6.47 (s, 1H), 6.65 (s, 1H), 6.94 (s, 1H), 7.11-7.23 (m, 1H), 7.64 (s, 1H), 7.97 (d, J = 8.1 Hz, 2H), 8.13 (s, 1H), 8.27 (d, J = 8.1 Hz, 2H), 8.54-8.76 (m, 3H), 8.86 (s, 1H), 9.43 (s, 1H), 10.74 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.37, 112.07, 114.20, 117.92, 118.65, 119.24, 121.15, 121.64, 122.48, 122.58, 122.89, 124.46, 125.29, 127.85, 131.14 (q, J = 32.8 Hz), 133.23, 138.45, 139.61, 142.78, 143.19, 146.16, 150.07, 154.95, 157.35, 159.18, 160.49; LRMS [$C_{31}H_{19}F_6N_5O$] (m/z): (+ve ion mode) 614.8 [M+Na]$^+$; HRMS (API) (m/z): [M+1]$^+$ calcd for $C_{31}H_{19}F_6N_5O$ [M+1]$^+$ 592.1572; found, 592.1579.

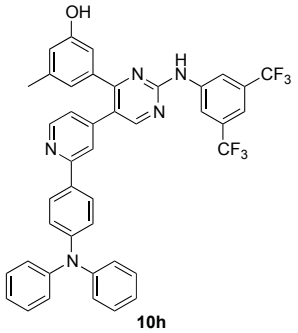
3-(2-(3,5-Bis(trifluoromethyl)phenylamino)-5-(2-(4-(dimethylamino)phenyl)pyridin-4-yl)pyrimidin-4-yl)-5-methylphenol (10g).



10g

Column chromatography (silica, ethyl acetate-hexane 1:2 v/v). Yield: (69%); ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.22 (s, 3H), 2.97 (s, 6H), 6.51 (s, 1H), 6.65 (s, 1H), 6.77 (d, J = 9.0 Hz, 2H), 6.89-7.02 (m, 2H), 7.63 (s, 1H), 7.77 (s, 1H), 7.89 (d, J = 8.9 Hz, 2H), 8.45 (d, J = 5.1 Hz, 1H), 8.65 (s, 2H), 8.80 (s, 1H), 9.41 (s, 1H), 10.69 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.39, 112.34, 114.14, 117.80, 118.59, 119.01, 121.10, 121.49, 122.60, 123.53, 125.31, 126.43, 127.93, 131.13 (d, J = 32.0 Hz), 138.69, 139.47, 142.87, 145.29, 147.74, 149.53, 151.43, 157.23, 157.32, 159.06, 162.35; LRMS [$C_{32}H_{25}F_6N_5O$] (m/z): (+ve ion mode) 632.2 $[M+Na]^+$; HRMS (API) (m/z): $[M+1]^+$ calcd for $C_{32}H_{25}F_6N_5O$ $[M+1]^+$ 610.2042; found, 610.2055.

3-(5-(2-(4-(Diphenylamino)phenyl)pyridin-4-yl)-2-(3,5-bis(trifluoromethyl)phenylamino)-pyrimidin-4-yl)-5-methylphenol (10h).

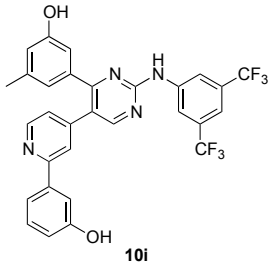


10h

Column chromatography (silica, ethyl acetate-hexane 1:3 v/v). Yield: (76%); ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.21 (s, 3H), 6.50 (s, 1H), 6.64 (s, 1H), 6.93 (d, J = 2.0 Hz, 1H), 7.01 (d, J = 8.7 Hz, 2H), 7.04-7.13 (m, 6H), 7.29-7.42 (m, 4H), 7.63 (s, 1H), 7.87 (d, J = 1.5 Hz, 1H), 7.96 (d, J = 8.8 Hz, 2H), 8.51 (d, J = 5.1 Hz, 1H), 8.64 (d, J = 1.7 Hz, 2H), 8.81 (s, 1H), 9.39 (s, 1H), 10.68 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.39, 114.19, 117.84, 118.61, 119.99, 121.13, 122.59, 123.37, 124.11, 125.05, 125.29, 128.23, 130.15, 131.13 (d, J = 32.3 Hz), 132.58, 138.59, 139.49, 142.82,

145.65, 147.28, 148.75, 149.69, 156.00, 156.46, 157.33, 159.10, 160.30; LRMS [$C_{42}H_{29}F_6N_5O$] (m/z): (+ve ion mode) 756.6 $[M+Na]^+$; HRMS (API) (m/z): $[M+1]^+$ calcd for $C_{42}H_{29}F_6N_5O$ $[M+1]^+$ 734.2355; found, 734.2359.

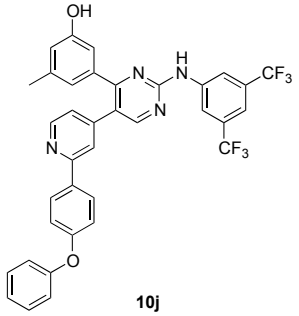
3-(2-(3,5-Bis(trifluoromethyl)phenylamino)-5-(2-(3-hydroxyphenyl)pyridin-4-yl)pyrimidin-4-yl)-5-methylphenol (10i).



10i

Column chromatography (silica, ethyl acetate-hexane 1:1 v/v). Yield: (83%); ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.22 (s, 3H), 6.49 (s, 1H), 6.65 (s, 1H), 6.83 (dd, J = 8.0, 1.8 Hz, 1H), 6.94 (s, 1H), 7.12 (dd, J = 5.1, 1.6 Hz, 1H), 7.25 (t, J = 7.9 Hz, 1H), 7.40 (dt, J = 7.9, 1.2 Hz, 1H), 7.49 (s, 1H), 7.63 (s, 1H), 7.84 (s, 1H), 8.55 (d, J = 5.1 Hz, 1H), 8.60-8.71 (m, 2H), 8.82 (s, 1H), 9.42 (s, 1H), 9.56 (s, 1H), 10.71 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.38, 113.96, 114.17, 114.25, 116.64, 117.85, 118.63, 120.75, 121.13, 122.59, 123.19, 123.25, 125.30, 129.60, 130.14, 131.14 (q, J = 32.6 Hz), 138.59, 139.53, 140.42, 142.84, 145.66, 149.76, 156.86, 157.34, 158.20, 159.12, 160.38; LRMS [$C_{30}H_{20}F_6N_4O_2$] (m/z): (+ve ion mode) 605.4 $[M+Na]^+$; HRMS (API) (m/z): $[M+1]^+$ calcd for $C_{30}H_{20}F_6N_4O_2$ $[M+1]^+$ 583.1569; found, 583.1555.

3-(2-(3,5-Bis(trifluoromethyl)phenylamino)-5-(2-(4-phenoxyphenyl)pyridin-4-yl)pyrimidin-4-yl)-5-methylphenol (10j).



10j

Column chromatography (silica, ethyl acetate-hexane 1:3 v/v). Yield: (84%); ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.22 (s, 3H), 6.49 (s, 1H), 6.65 (s, 1H), 6.94 (s, 1H), 7.03-7.15 (m, 5H), 7.15-7.26 (m, 1H), 7.43 (dd, J = 8.5, 7.3 Hz, 2H), 7.64 (s, 1H), 7.93 (s, 1H), 8.07 (d, J = 8.7 Hz, 2H), 8.53 (d, J = 5.1 Hz, 1H), 8.65 (s, 2H), 8.83 (s, 1H), 9.42 (s, 1H), 10.72 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.37, 114.18, 114.26, 117.84, 118.62, 118.77, 119.61, 120.31, 121.12, 122.57, 122.91, 123.24, 124.39, 125.28, 128.91, 130.63, 131.29 (q, J = 32.4 Hz), 134.09, 138.57, 139.52, 142.81, 145.76, 149.74, 156.25, 156.59, 157.34, 158.36, 159.11, 160.34; LRMS [$C_{36}H_{24}F_6N_4O_2$] (m/z): (+ve ion mode) 681.6 $[M+Na]^+$; HRMS (API) (m/z): $[M+1]^+$ calcd for $C_{36}H_{24}F_6N_4O_2$ $[M+1]^+$ 659.1882; found, 659.1889.

Tyrosine kinase inhibition assay

Compounds under investigation were assessed for their tyrosine kinase inhibiting activity using universal tyrosine kinase assay kit (Takara Bio Inc., Kusatsu-Shiga, Japan). Briefly, a mixture of protein tyrosine kinases were extracted from HT-29 (ATCC® HTB-38) cell line using RIPA buffer. Tyrosine kinase cellular extract was incubated with test compounds (10 μM) along with immobilized tyrosine kinase peptide substrate according to the manufacturer protocol. Phosphorylated substrate was detected using HRP-labelled anti-phosphorylated antibody and percent activity was measured relative to control uninhibited enzyme mixture. Similarly, purified Src-kinase enzyme purchased from Takara Bio Inc., Kusatsu-Shiga, Japan, was incubated with test compounds along with tyrosine kinase substrate to measure specific Src-kinase inhibiting activity of compounds under investigation.

Molecular Modelling Simulations

Docking studies were performed using 'Molecular Operating Environment (MOE) version 2008.10', Chemical Computing Group Inc., 1010 Sherbrooke Street West, Suite 910, Montreal, H3A 2R7, Canada. Compounds **8b** was built using the builder interface of the MOE program and

subjected to energy minimization using the included Forcefield MMFF94x calculations. The X-ray crystallographic structure of src kinase complexed with Bosutinib (PDB ID: 4MXO) was obtained from Protein Data Bank.[3] The enzyme was prepared for docking studies as follows: (i) Hydrogen atoms were added to the structure with their standard geometry. (iii) The whole protein structure containing the ligand inside was subjected to energy minimization to relax any abnormally strained parts of the structure. (iii) The inhibitor **8b** was docked into the binding site containing the Bosutinib ligand, setting the receptor to 'receptor + solvent', the site to 'ligand atoms', and the pharmacophore to 'none'. Placement method was set to default 'Triangle Matcher', the first scoring function, 'Rescoring 1' was set to the default 'London dG' and the Retain dropdown was set to 10. Refinement inside the binding site was allowed using forcefield calculations so as to allow energy minimization of the docking poses inside the binding pocket. The refinement scoring function 'Rescoring 2' was set to none and the retain dropdown was set to 10. The final refined poses were ranked by the MM/GBVI binding free energy estimation, and the pose showing the lowest free energy (the most stable poses) was selected.

Cytotoxicity assessment

Cell Culture

The human colorectal cancer cell lines HCT-116 (ATCC® CCL-247), HT-29 (ATCC® HTB-38) and LS-174T (ATCC® CL-188) were obtained from NAWAH Scientific Inc., (Mokkatam, Cairo, Egypt). All cell lines were cultured in RPMI-1640 media supplemented with 10% FBS; 100 U/mL penicillin and 100 µg/mL streptomycin and kept in humidified incubator at 37°C with 5% CO_2. Cells were passaged at 80-90% confluence by trypsinization based on standard procedures.

Screening test compounds for cell killing effect

Compounds under investigation were tested preliminarily for their cell killing effects at two concentrations 10 and 100 µM. Briefly, cells were seeded in 96-well plate ($5x10^3$ cells/well) for 24 h;

and were then exposed to test compounds (at 10 and 100 μM) for further 72 h. At the end of the incubation period, cells were fixed by adding 10% (w/v) trichloroacetic acid (TCA) for 1 h at 4°C, followed by cell washing with distilled water. After that, cells were stained with 0.4% (w/v) sulfarhodamine-B (SRB) for 10 min at room temperature in dark place, washed with 1% (v/v) glacial acetic acid and left to dry overnight. After drying, protein-bound SRB dye was solubilized by adding Tris-HCl and absorbance was measured at 540 nm with ELISA microplate reader. The percentages of growth inhibition of test compounds were determined by comparison to control untreated cells (drug concentration of zero μM). Remaining DMSO concentration did not exceed 0.1% in all treatment conditions to avoid viability interference (DMSO interfere with cell viability at concentrations higher than 2%).

Dose-response curve for compounds with promising cytotoxic profile

Compounds that showed significant potential growth inhibitory/cell killing effect were further tested for detailed dose response relationship and their detailed cytotoxic parameters were determined [4]. Briefly, cells were seeded in 96-well plate and treated with serial concentrations of test compounds (0.01-100 μM). Remaining DMSO concentration did not exceed 0.1% in all treatment concentrations to avoid viability interference (DMSO interfere with cell viability at concentrations higher than 2%).. After 72 h, cell viability was assessed using SRB-assay (as previously mentioned) and detailed cytotoxic parameters were calculated as follows:

Cytotoxicity data analysis

Cell viability and the dose-response curves were calculated by using the E_{max} model as previously described [5]

$$\%\ Cell\ Viability = (100 - R) \times \left(1 - \frac{[D]^m}{K_d^m + [D]^m}\right) \quad \ldots\ldots\ldots\ldots\ldots\ldots \text{ Equation 1}$$

Where "R" is the resistance fraction, "D" is the drug concentration used, "K_d" is the drug concentration for which 50% of the maximum effect is obtained and "m" is a Hill-type coefficient. The IC_{50} represents the concentration of drug that is needed to inhibit population cell growth by half. (i.e., K_d = IC_{50} when $R \geq 0$ and E_{max} = 100-R).

Analysis of Cell Cycle Distribution

To assess the effect of test compounds on cell cycle distribution, HCT-116 and HT-29 cells were subjected to 10 μM of compounds under investigation or drug free media for 48 h. After treatment, cells were collected by trypsinization and washed twice with ice-cold PBS and re-suspended in 0.5 mL of PBS. Two milliliters of 60% ice-cold ethanol were added gently while vortexing and cells were incubated at 4ºC for 1 h for fixation. Upon analysis, fixed cells were washed and re-suspended in 1 mL of PBS containing 50 μg/mL RNAase-A and 10 μg/mL propidium iodide (PI). After 20 min of incubation in dark at 37°C, cells were analyzed for DNA contents using flow cytometry analysis FL2 ($\lambda_{ex/em}$ 535/617 nm) signal detector (ACEA Novocyte™ flowcytometer, ACEA Biosciences Inc., San Diego, CA, USA). For each sample, at least 12,000 events were acquired. Cell cycle distribution was calculated using ACEA NovoExpress™ software (ACEA Biosciences Inc., San Diego, CA, USA) [6]. Remaining DMSO concentration did not exceed 0.1% in all treatment conditions to avoid viability interference

Apoptosis assay

To elucidate the method of cell death by which test compounds induced cell kill, apoptosis and necrosis cell populations were determined using Annexin V-FITC detection kit (Abcam Inc., Cambridge Science Park, Cambridge, UK). Briefly, the cells were exposed to 10 μM of compounds under investigation or drug free media for 48 h. Cells were harvested and washed twice with PBS, and incubated in dark with 0.5 ml of Annexin V-FITC/PI solution for 30 min at room temperature according to manufacturer protocol. After staining, cells were injected via ACEA Novocyte™ flowcytometer (ACEA

Biosciences Inc., San Diego, CA, USA) and analyzed for FITC and PI fluorescent signals using FL1 and FL2 signal detector, respectively ($\lambda_{ex/em}$ 488/530 nm for FITC and $\lambda_{ex/em}$ 535/617 nm for PI). For each sample, 12,000 events were acquired and positive FITC and/or PI cells were quantified by quadrant analysis and calculated using ACEA NovoExpress™ software (ACEA Biosciences Inc., San Diego, CA, USA) [7]. Remaining DMSO concentration did not exceed 0.1% in all treatment conditions to avoid viability interference

Autophagy assay

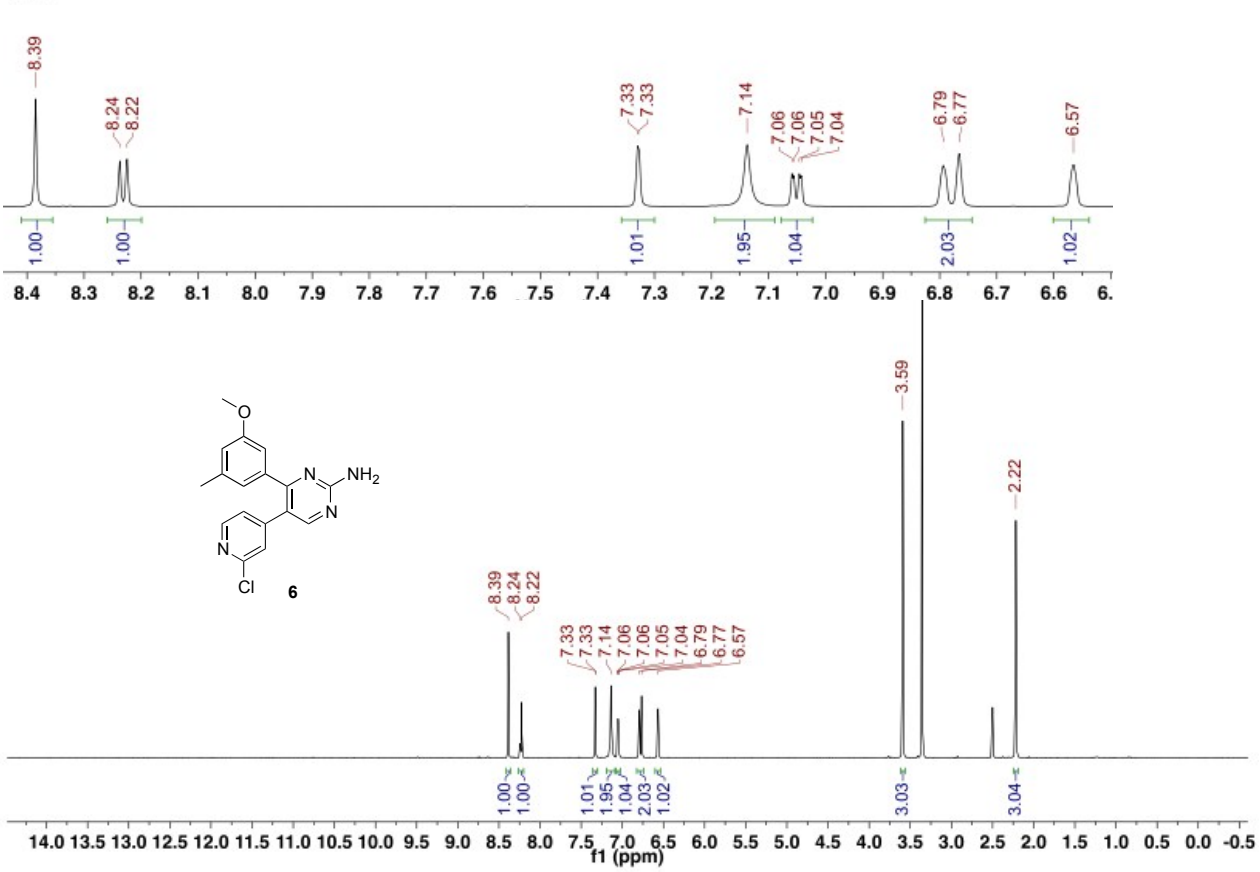
To further elucidate the method of cell death by which test compounds induced cell kill, autophagic cell death was quantitatively assessed using Cyto-ID Autophagy Detection Kit (Abcam Inc., Cambridge Science Park, Cambridge, UK). In brief, cells were exposed to 10 μM of test compounds; simultaneously, cells were exposed to 10 μM chloroquine (CQ) as a positive control (autophagy inducing agent), and drug free media (negative control group) for 48h. After treatment, cells were collected and washed twice with PBS. Cells were stained with Cyto-ID Green and incubated in a dark place at 37°C for 30 minutes according to manufacturer protocol. After staining, cells were injected via ACEA Novocyte™ flowcytometer (ACEA Biosciences Inc., San Diego, CA, USA) and analyzed for Cyto-ID differential green/orange fluorescent signals using FL1 and FL2 signal detector, respectively ($\lambda_{ex/em}$ 488/530 nm for FITC and $\lambda_{ex/em}$ 535/617 nm for acridine orange). For each sample, at least 12,000 events were acquired and mean green fluorescent intensities (NFI) were quantified using ACEA NovoExpress™ software (ACEA Biosciences Inc., San Diego, CA, USA) [6]. Remaining DMSO concentration did not exceed 0.1% in all treatment conditions to avoid viability interference

Statistical analysis

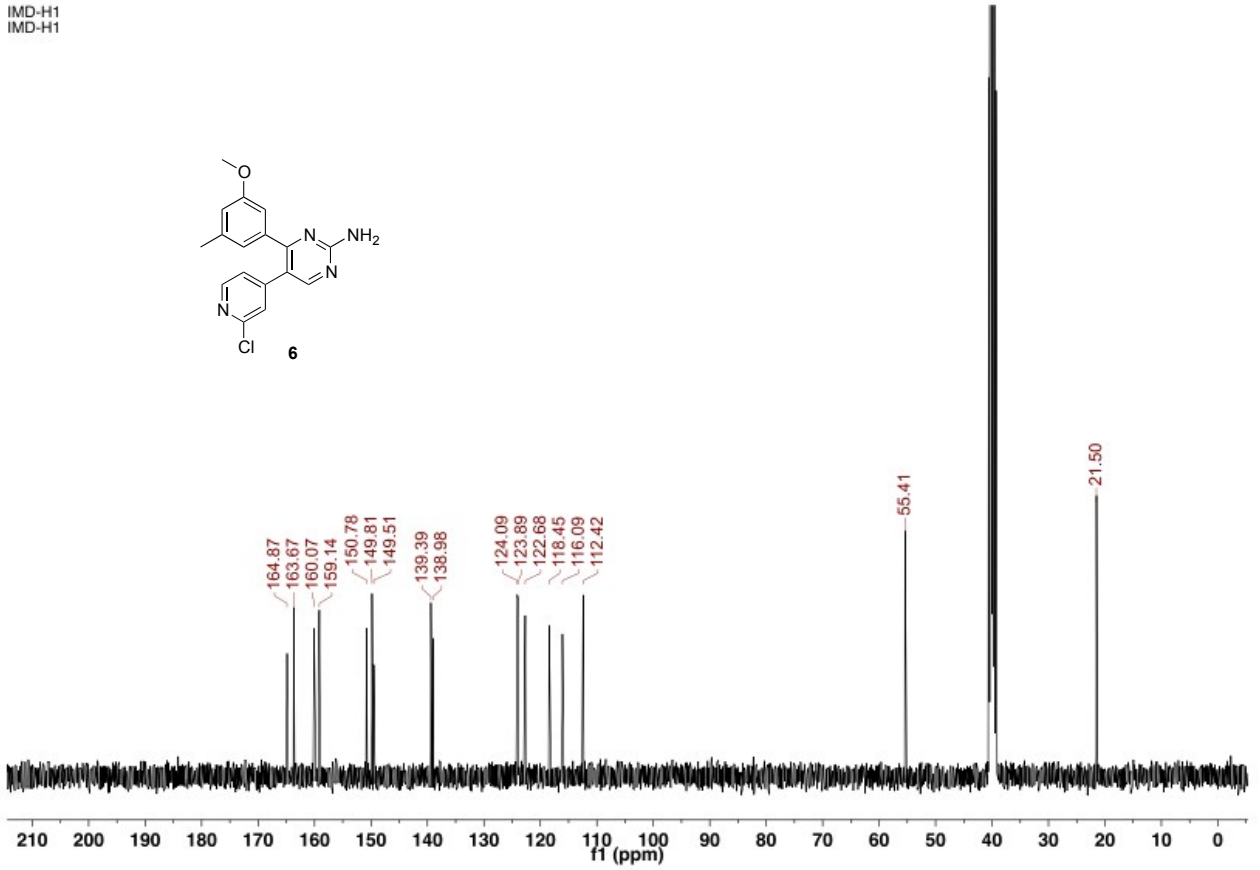
Data are presented as mean ± SD using Prism® for Windows, ver. 5.00 (GraphPad Software Inc., La Jolla, CA, USA). Analysis of variance (ANOVA) with Tukey post hoc test was used for testing the

significance using SPSS® for windows, version 17.0.0. $p<0.05$ was taken as a cut off value for significance.

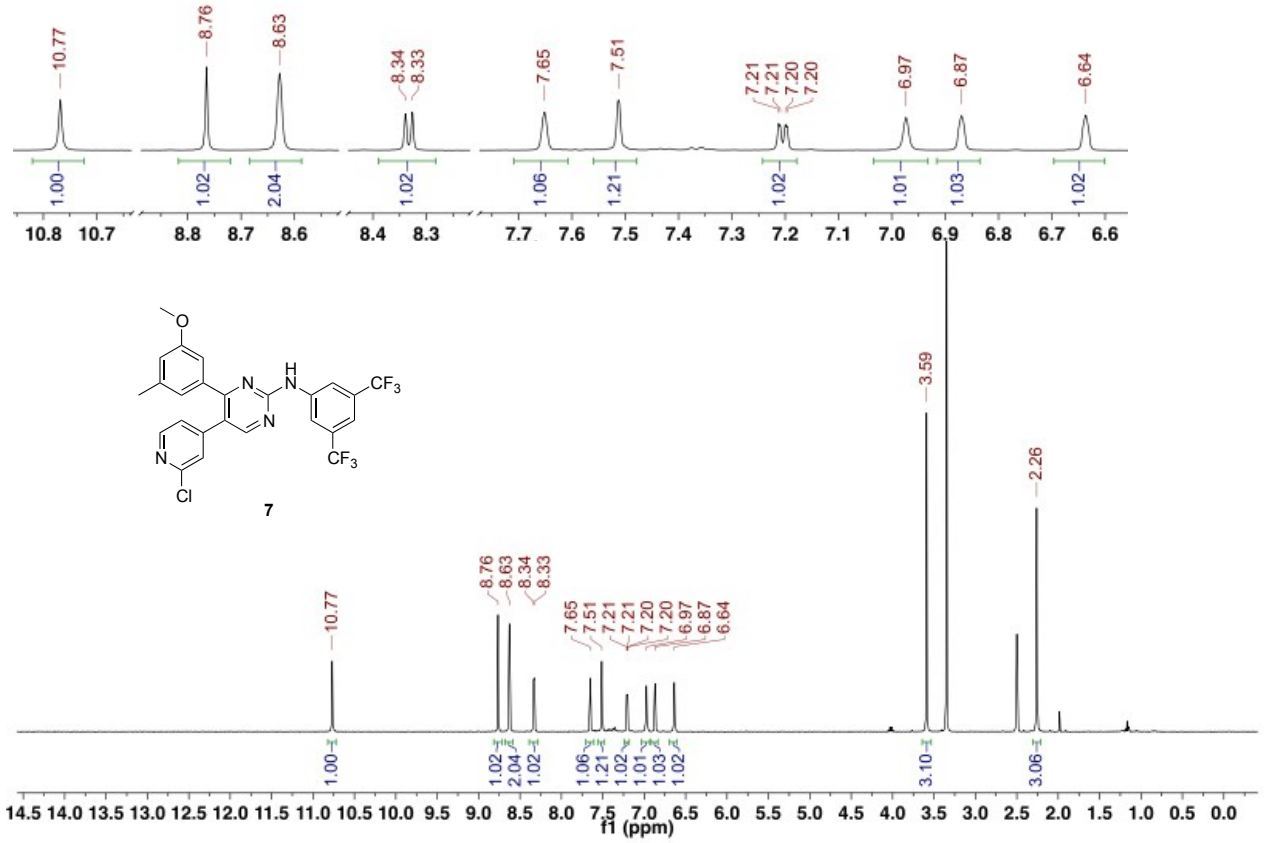
^{1}H and ^{13}C NMR spectra



^{1}H NMR spectrum of 6 (400 MHz, DMSO)



^{13}C NMR spectrum of 6 (100 MHz, DMSO)



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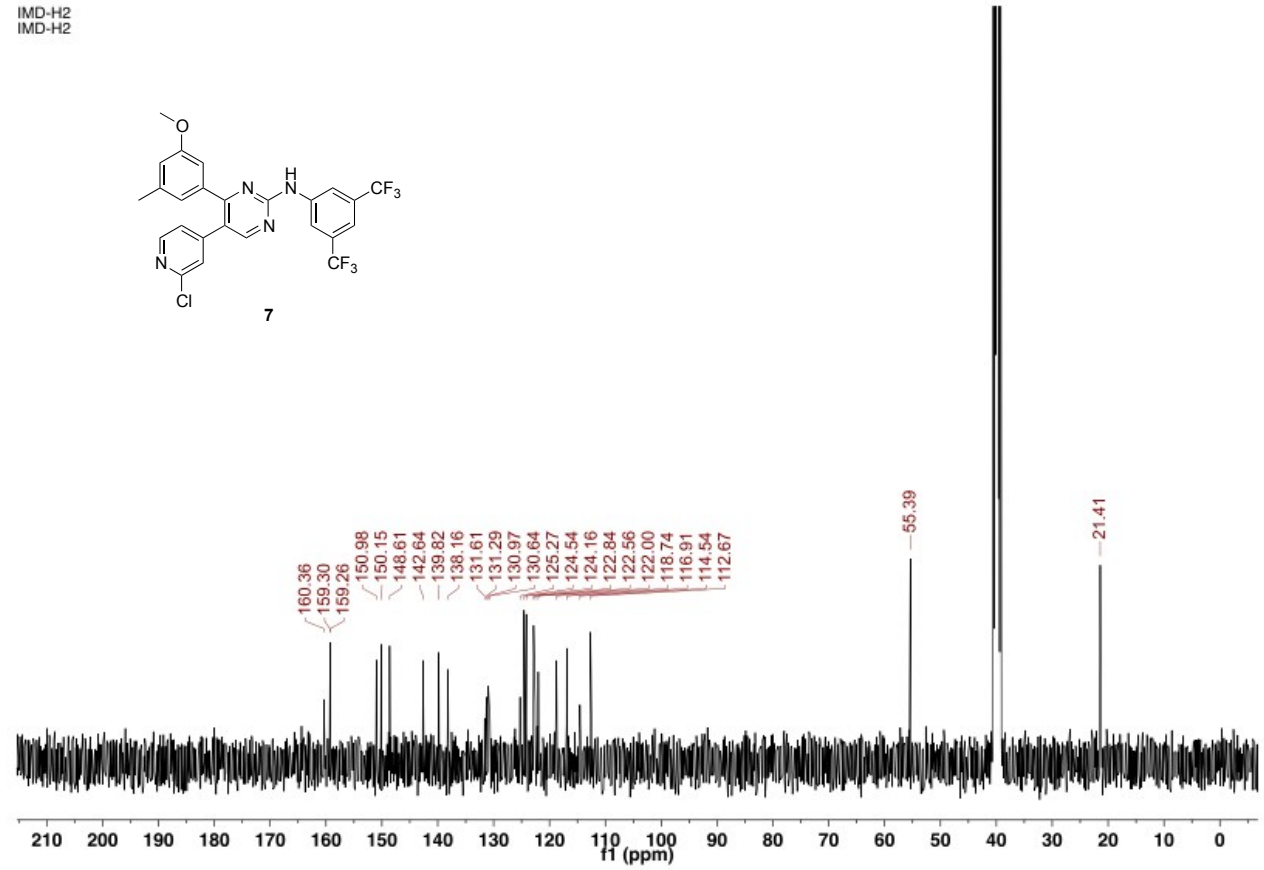
^{1}H NMR spectrum of 7 (400 MHz, DMSO)



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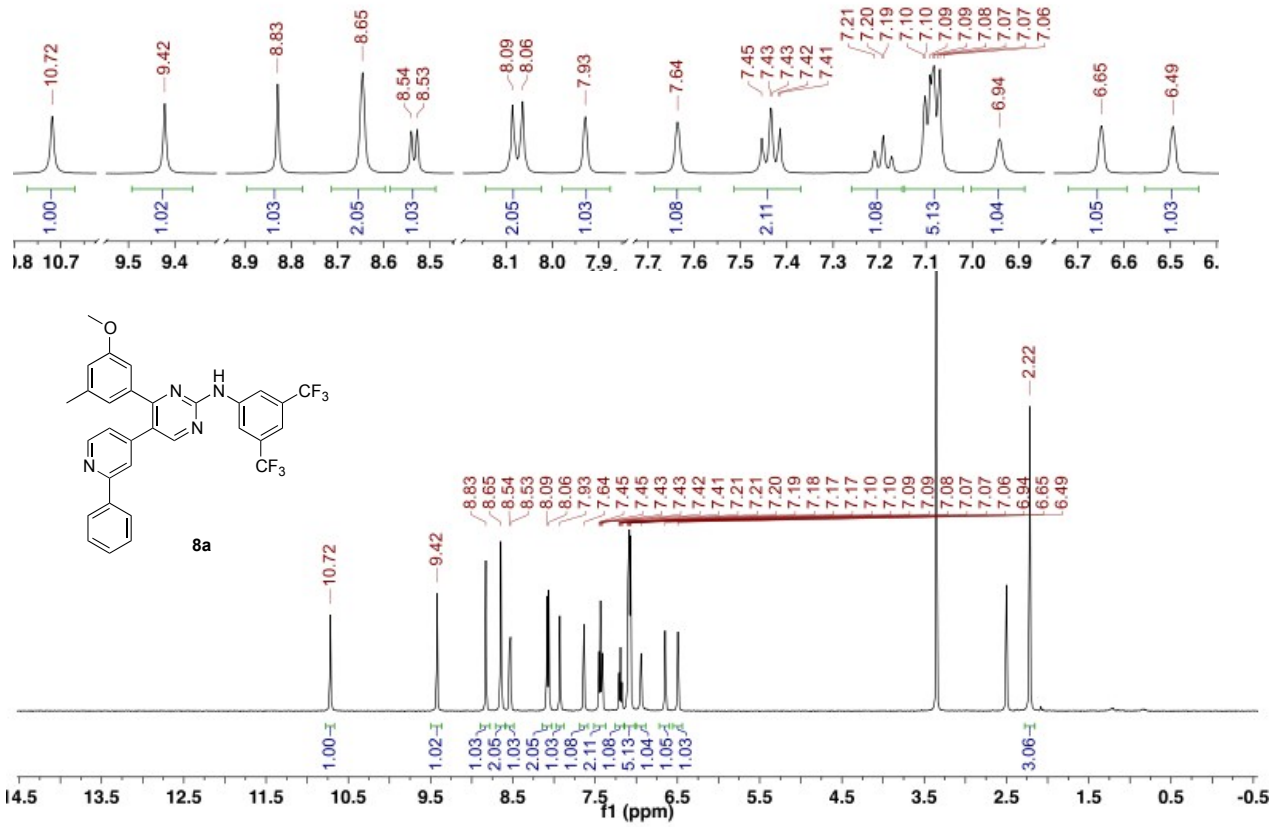
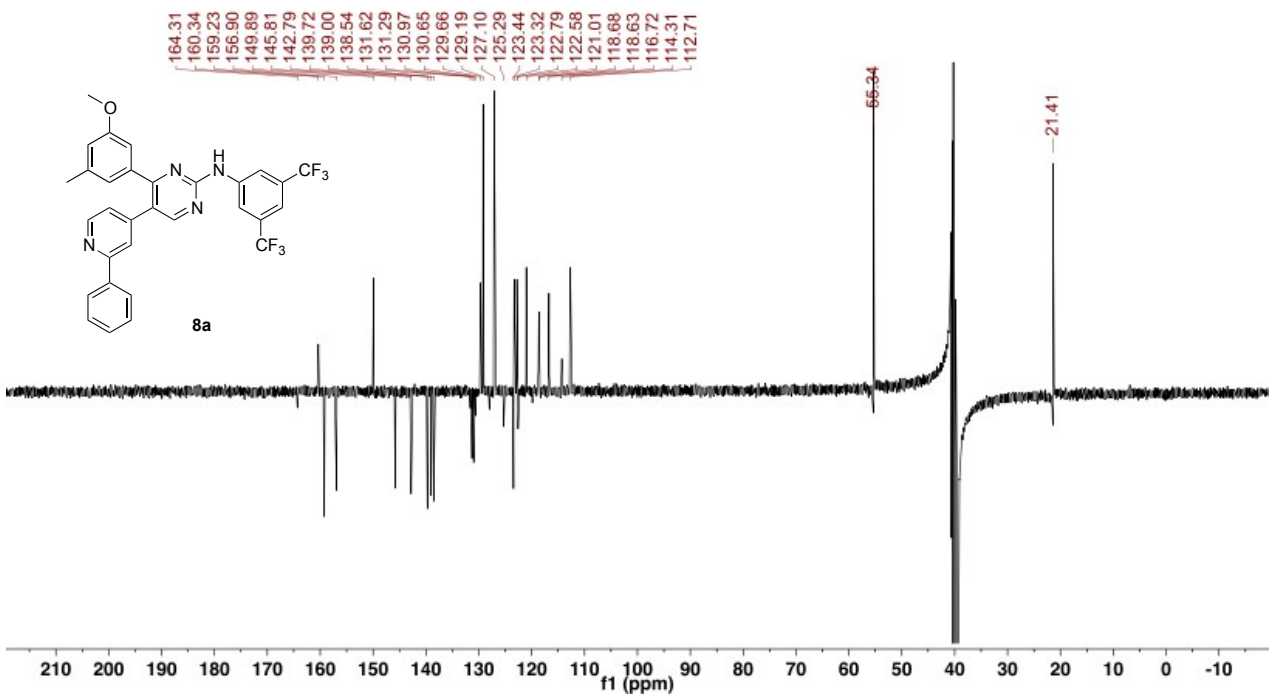
^{13}C NMR spectrum of 7 (100 MHz, DMSO)

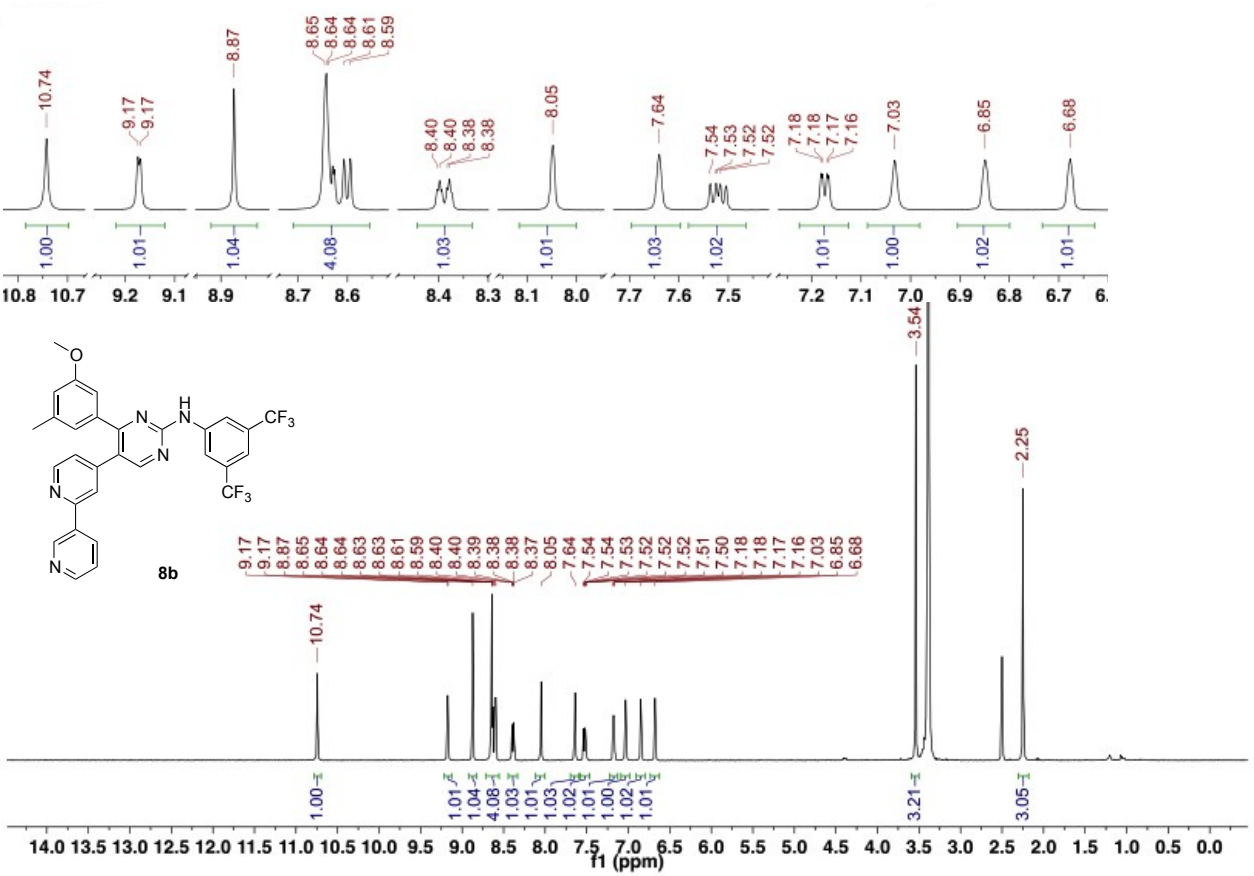


^{1}H NMR spectrum of 8a (400 MHz, DMSO)

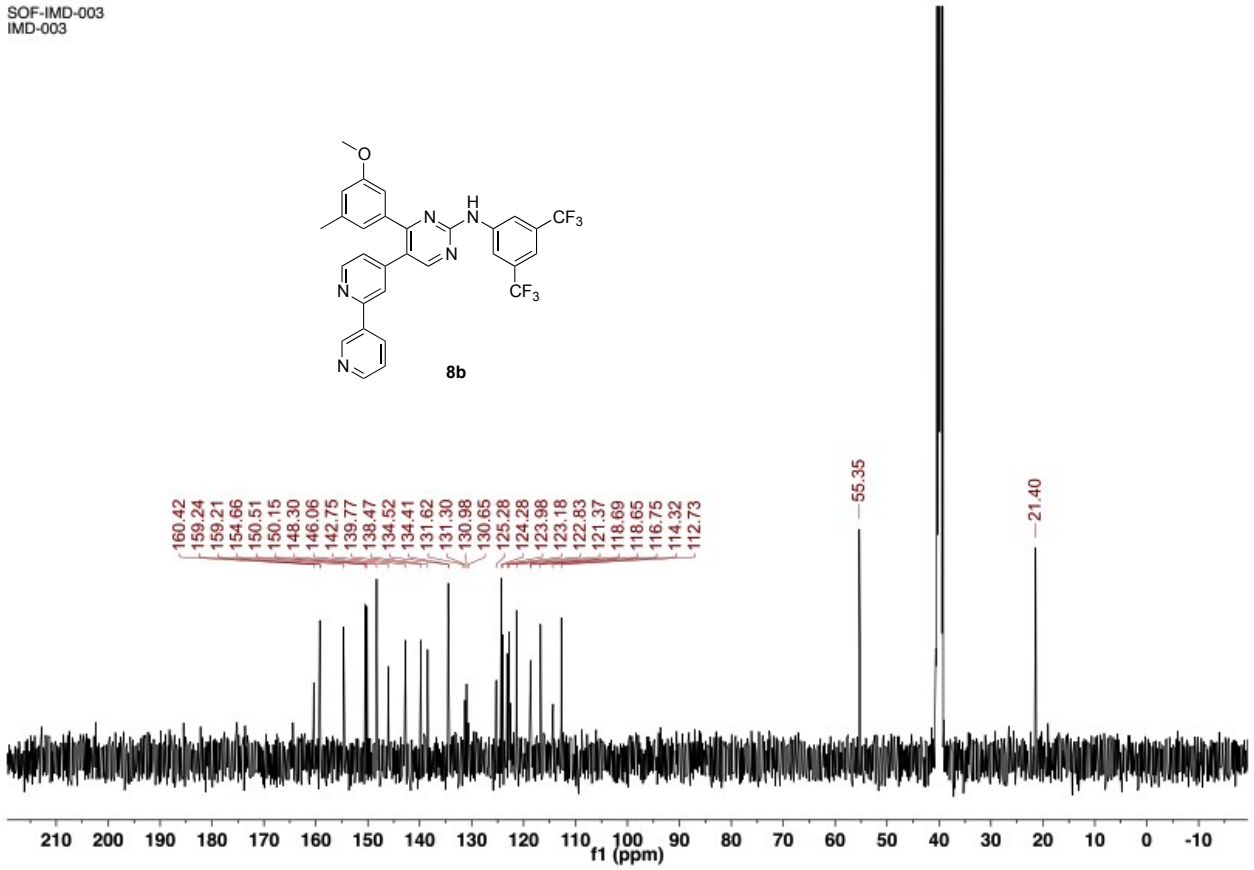
IE1041.99.fid
IE1041-3
DMSO
#97-99



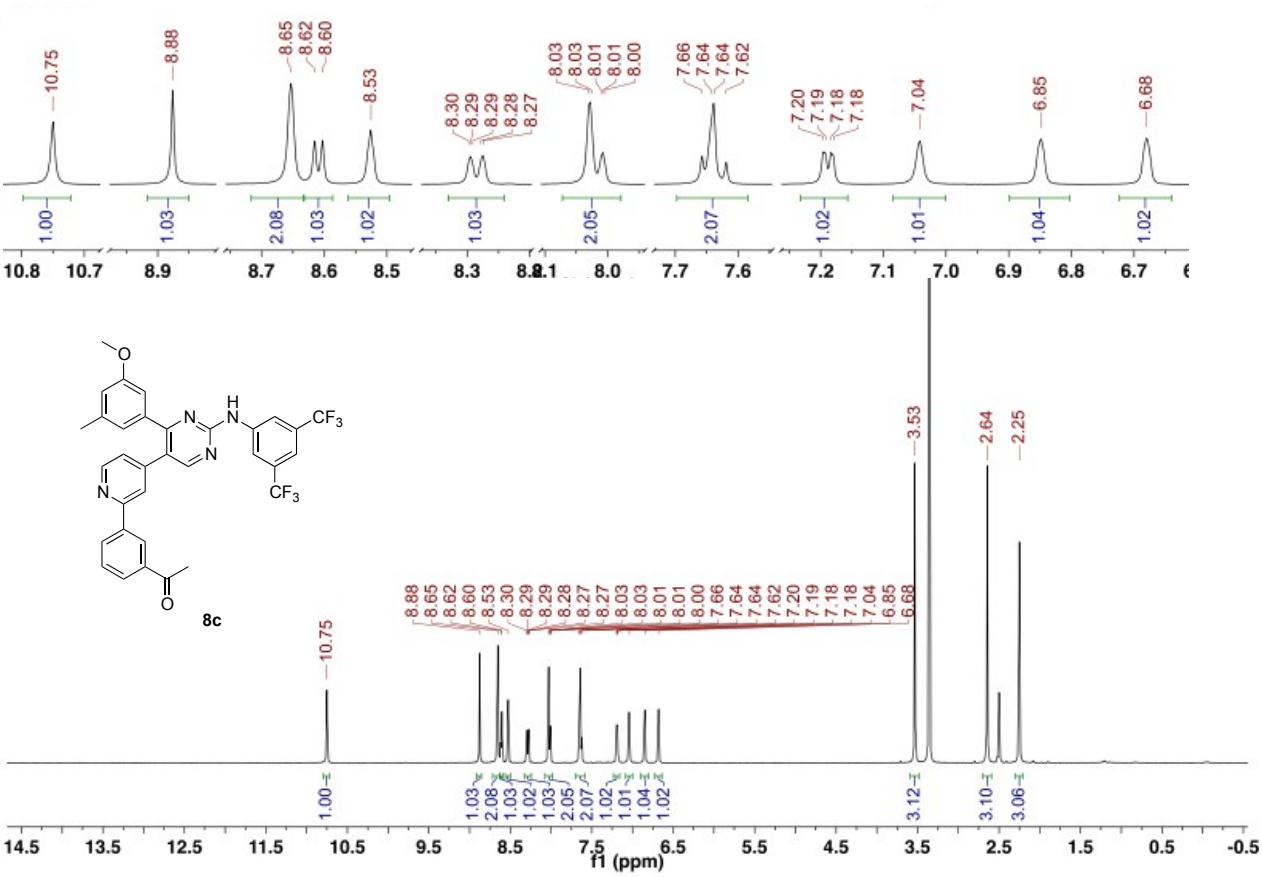
^{13}C NMR spectrum of 8a (100 MHz, DMSO)



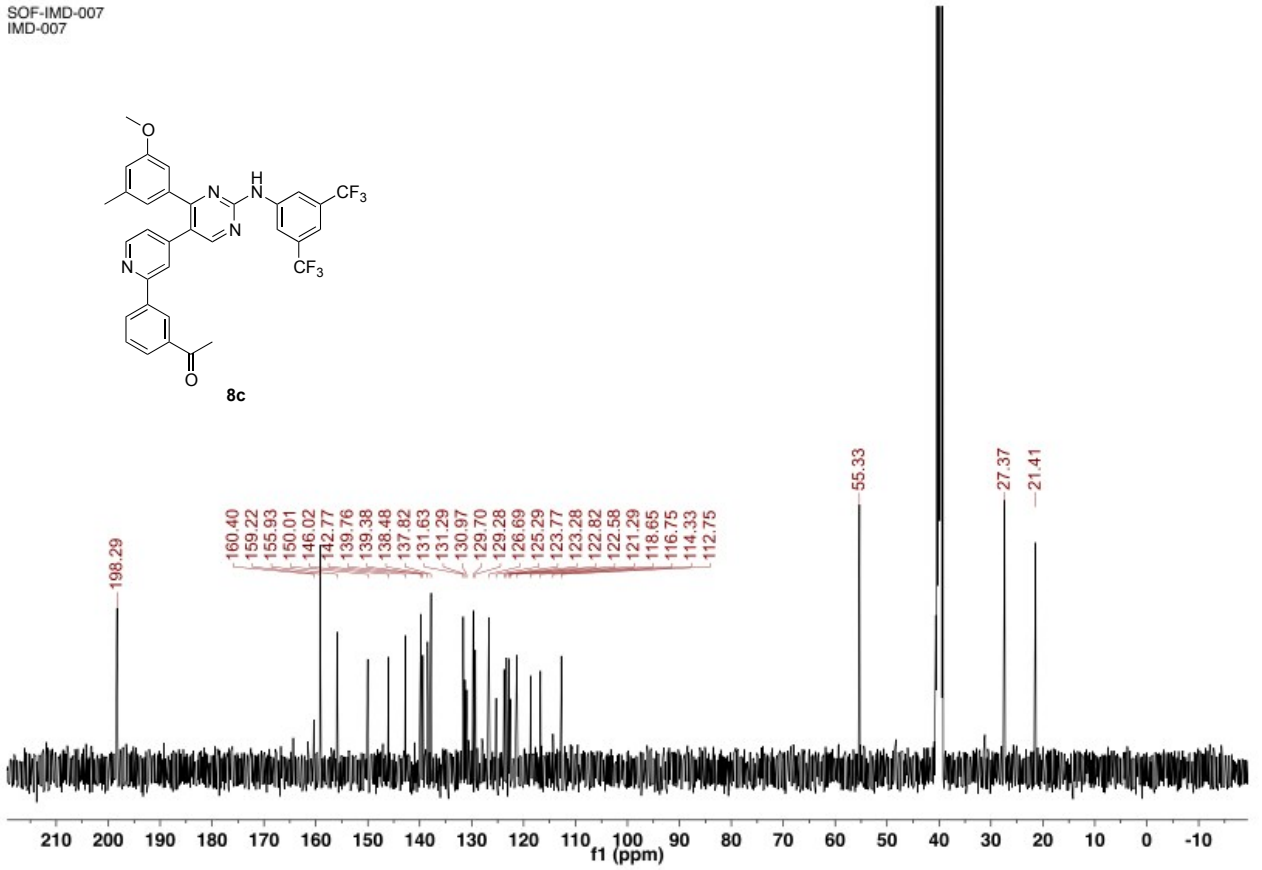
^{1}H NMR spectrum of 8b (400 MHz, DMSO)



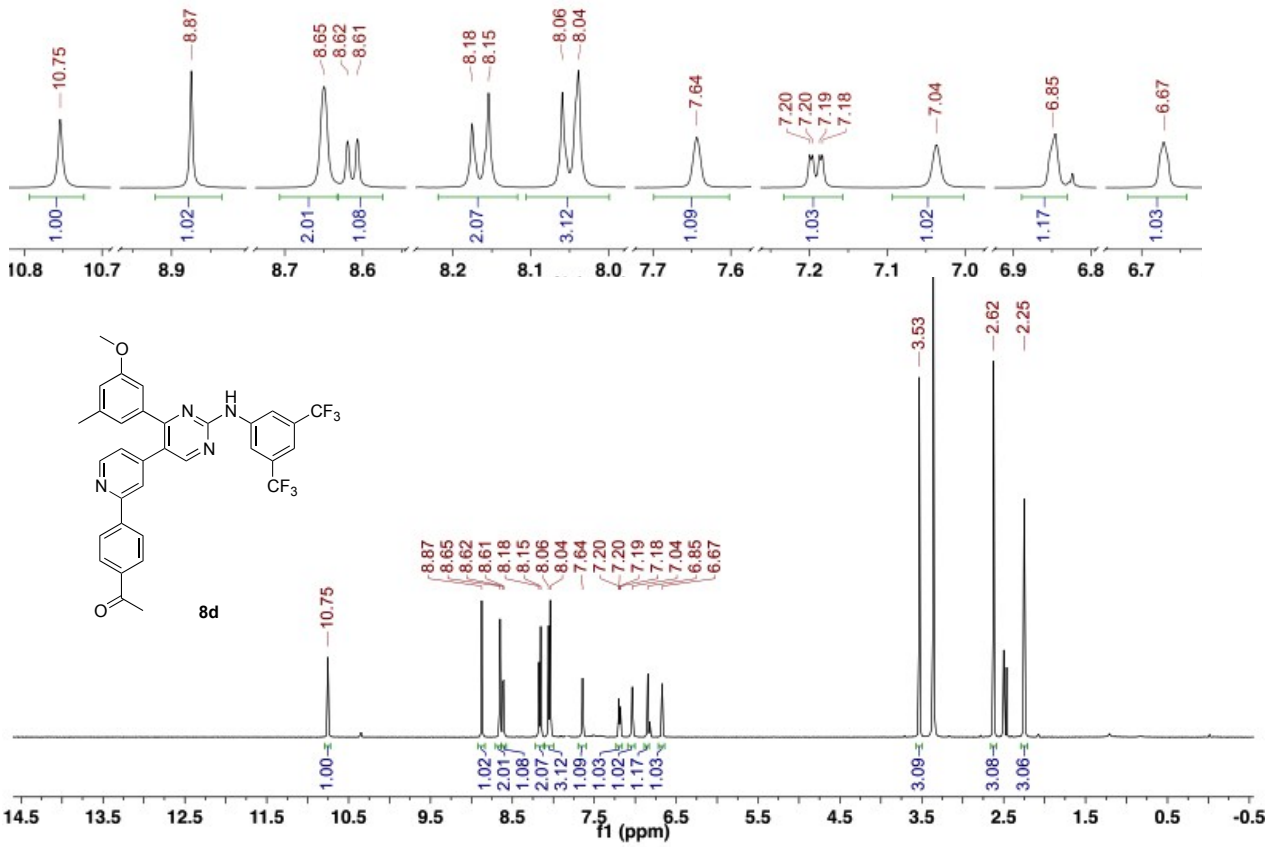
^{13}C NMR spectrum of 8b (100 MHz, DMSO)



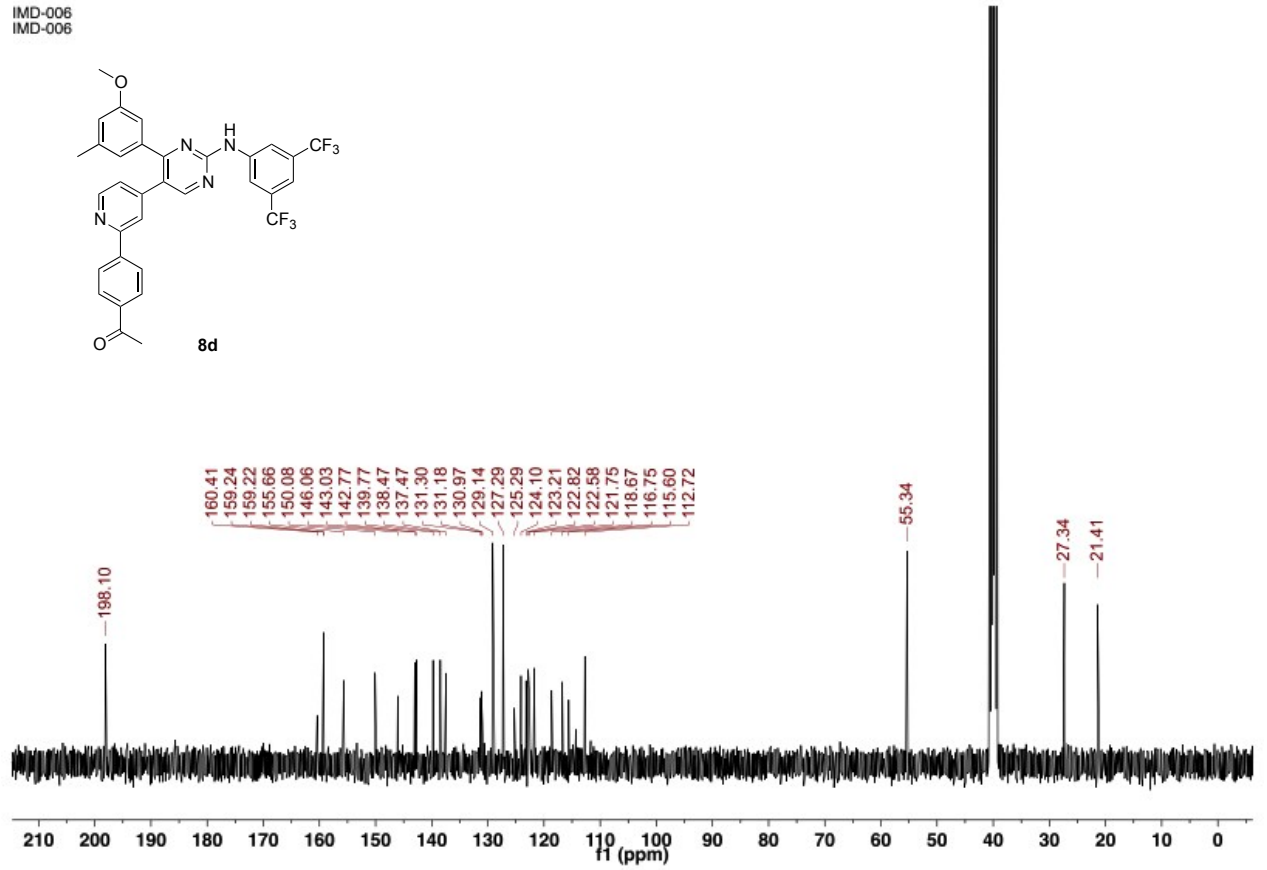
^{1}H NMR spectrum of 8c (400 MHz, DMSO)



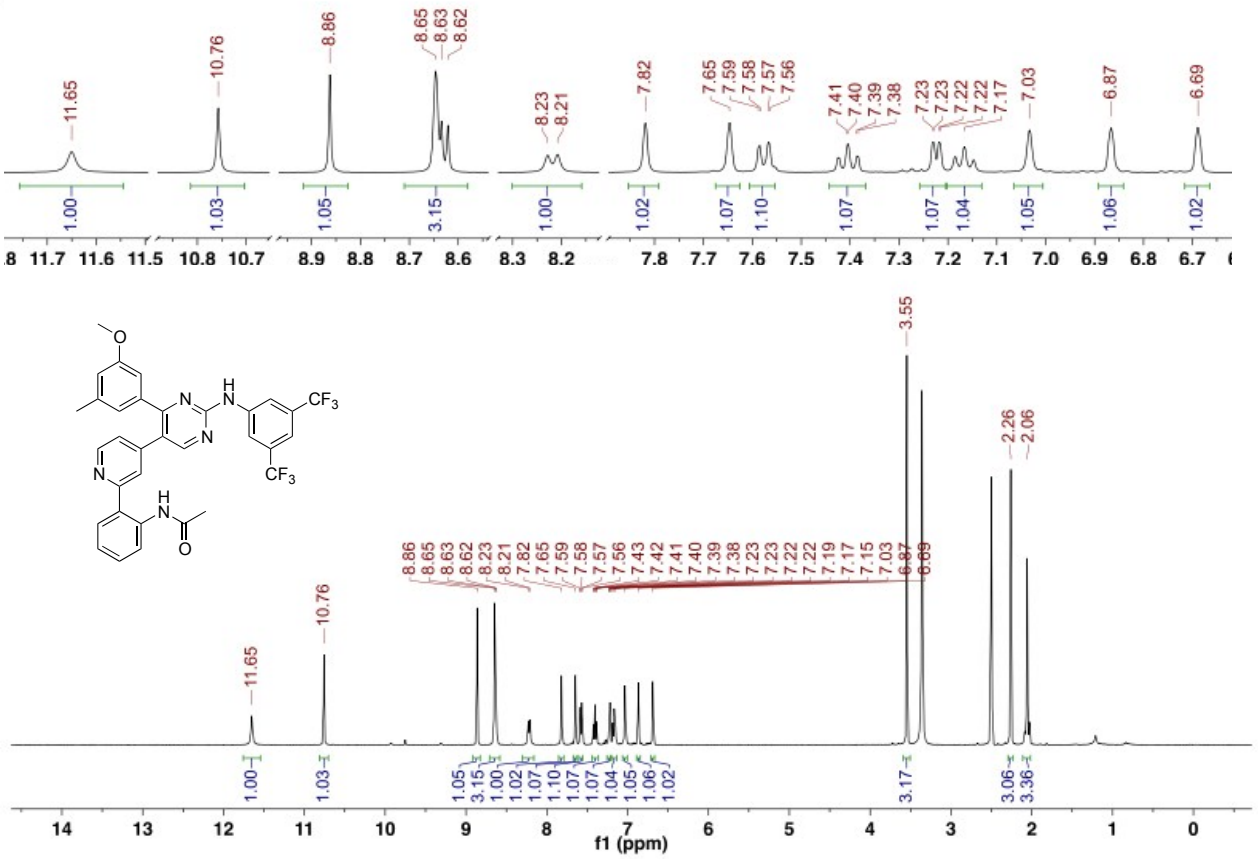
^{13}C NMR spectrum of 8c (100 MHz, DMSO)



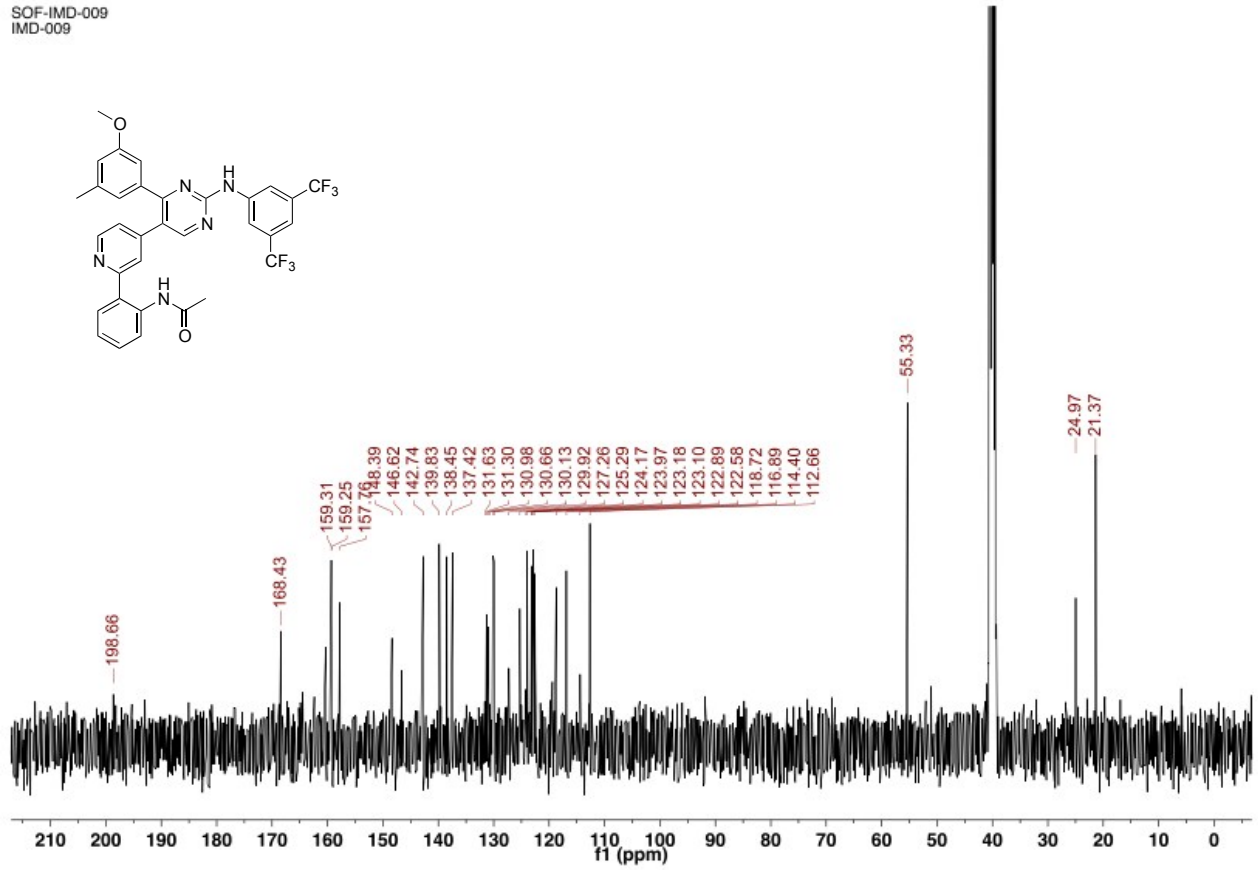
^{1}H NMR spectrum of 8d (400 MHz, DMSO)



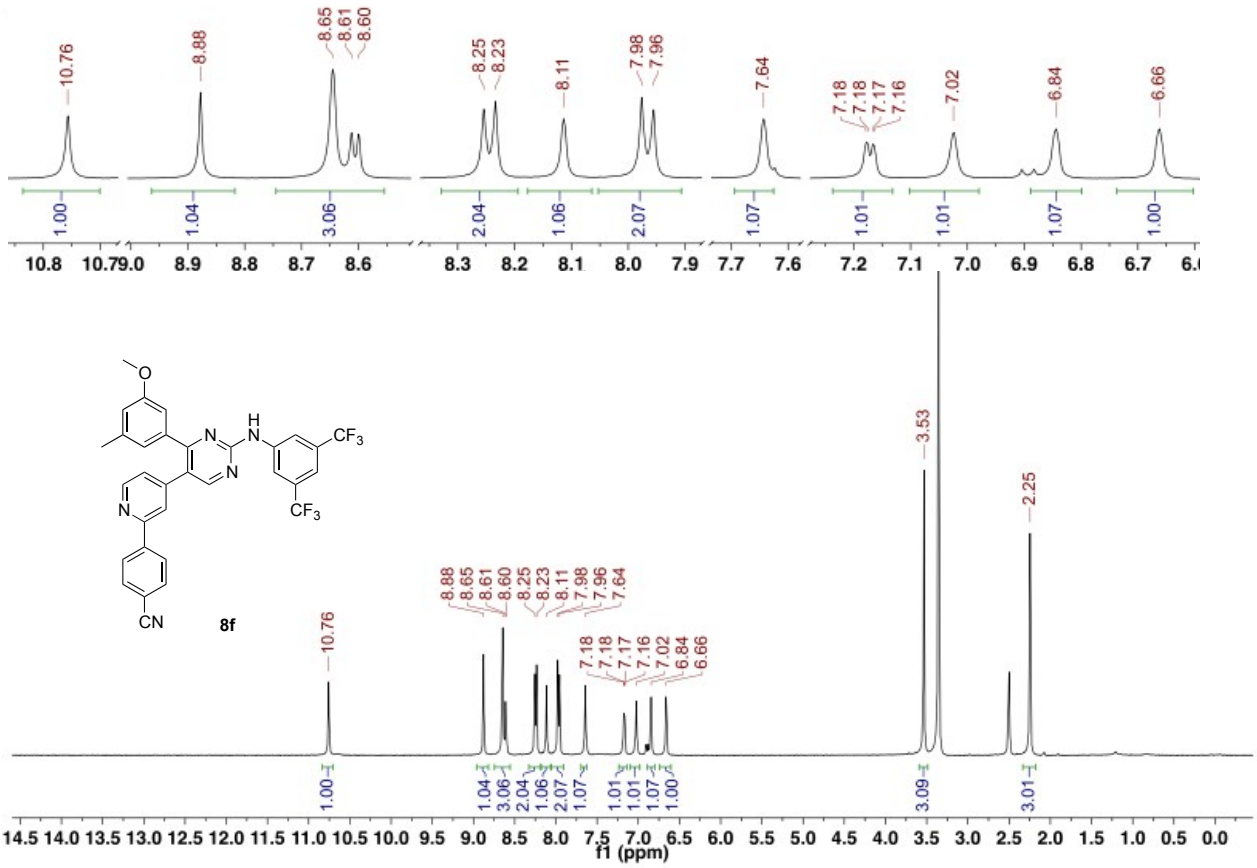
^{13}C NMR spectrum of 8d (100 MHz, DMSO)



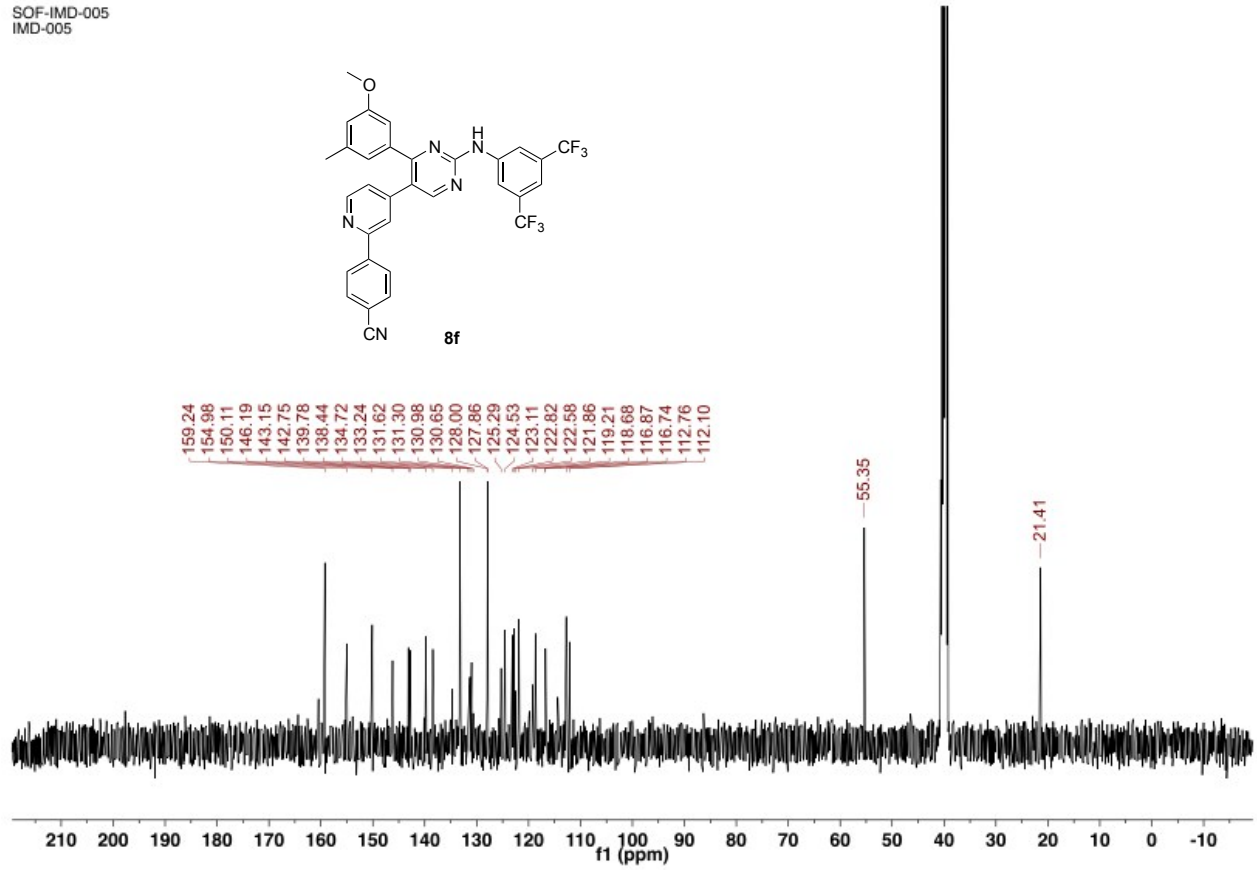
^{1}H NMR spectrum of 8e (400 MHz, DMSO)



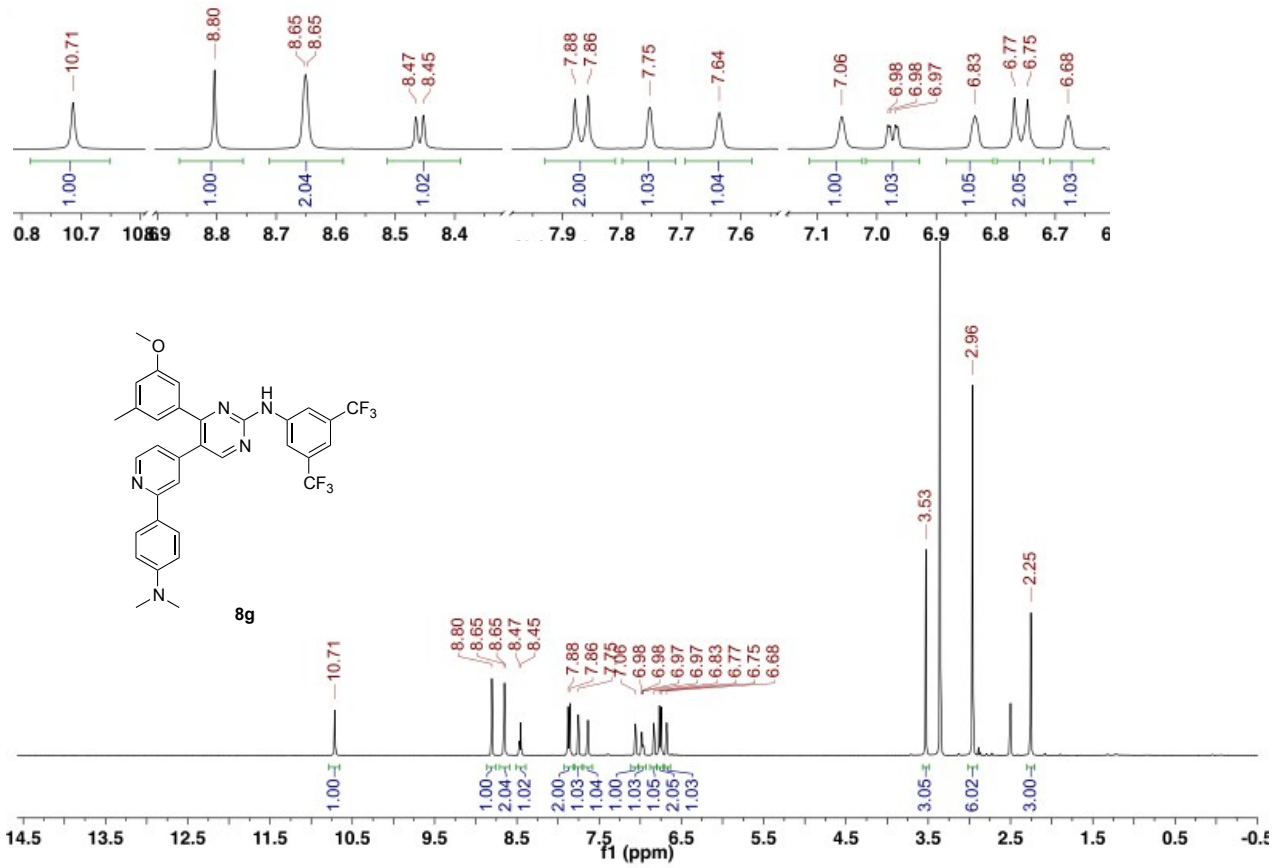
^{13}C NMR spectrum of 8e (100 MHz, DMSO)



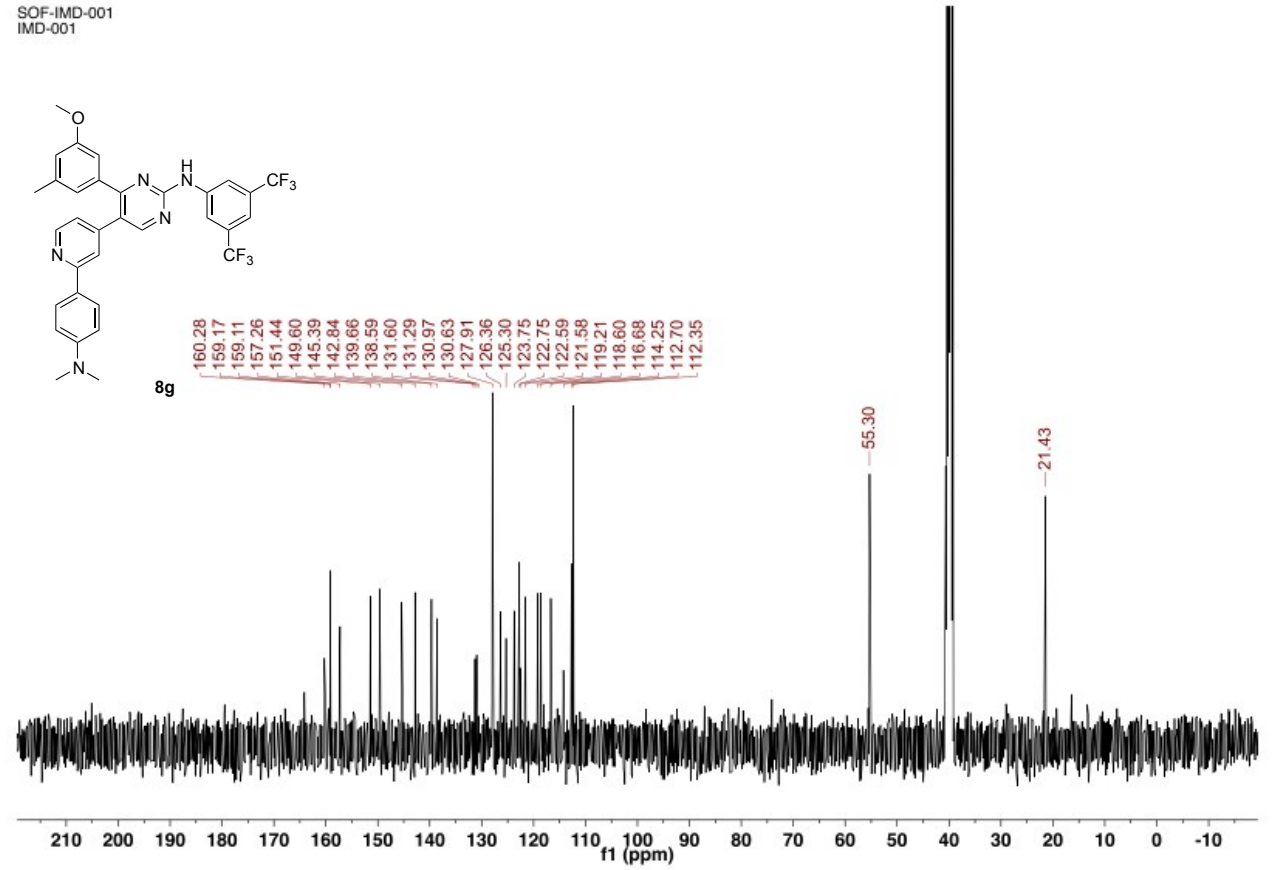
^{1}H NMR spectrum of 8f (400 MHz, DMSO)



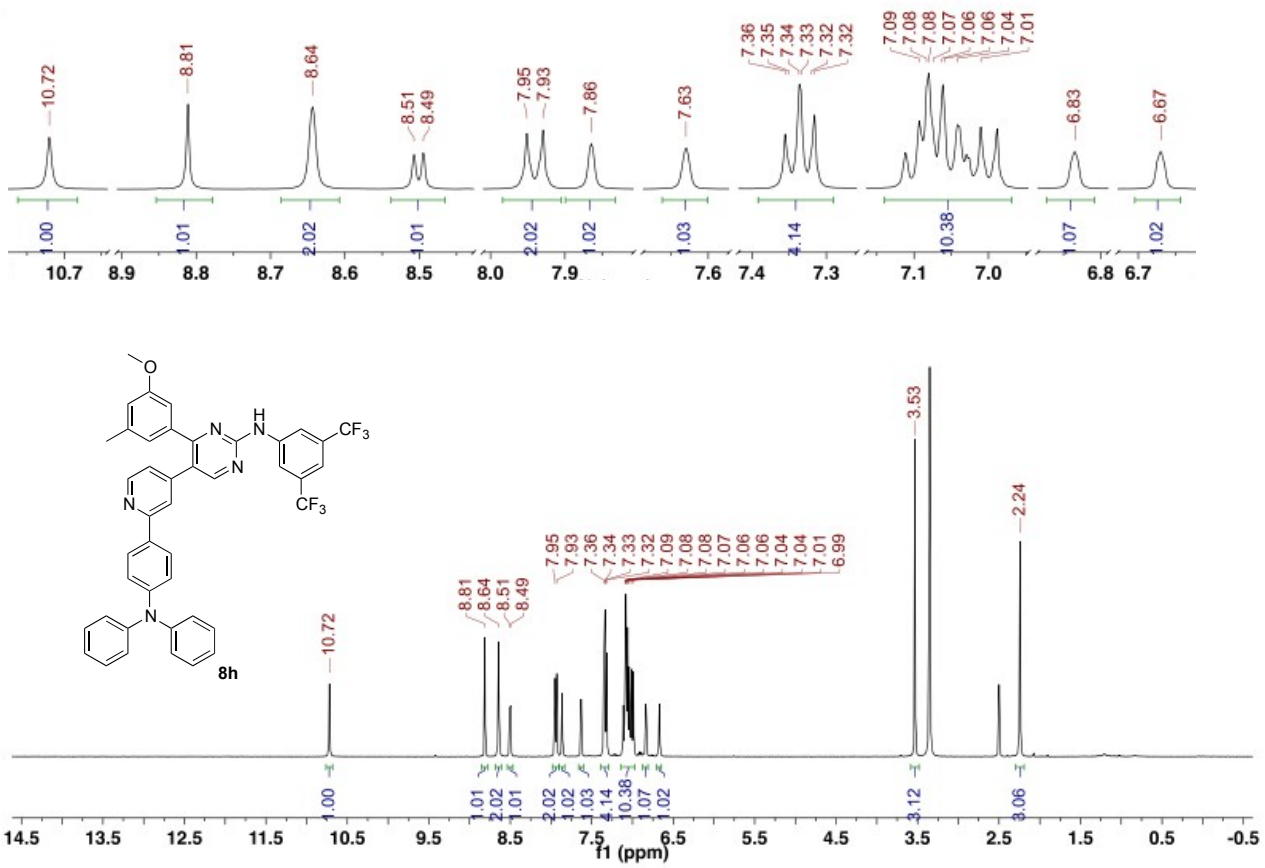
^{13}C NMR spectrum of 8f (100 MHz, DMSO)



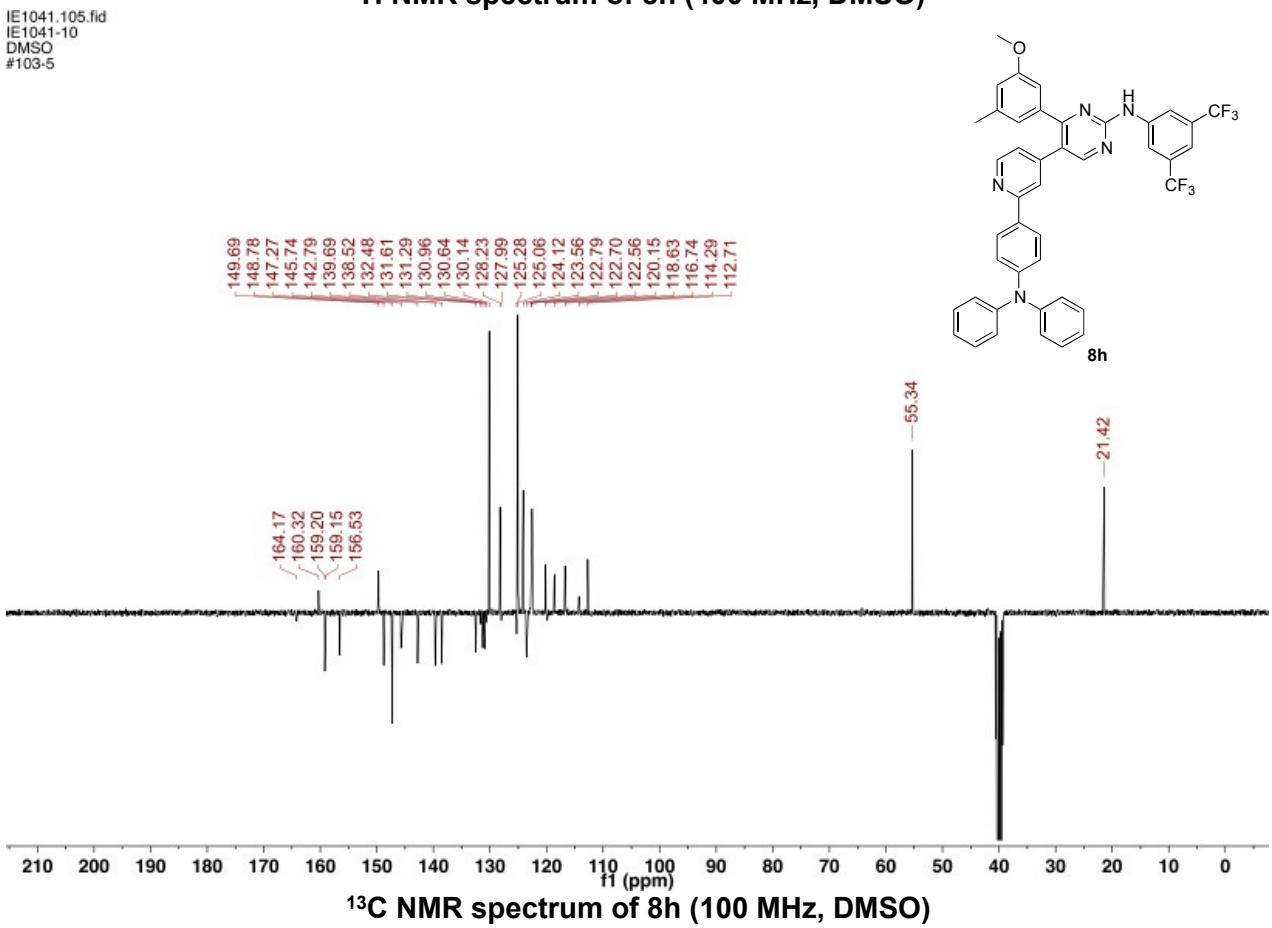
^{1}H NMR spectrum of 8g (400 MHz, DMSO)



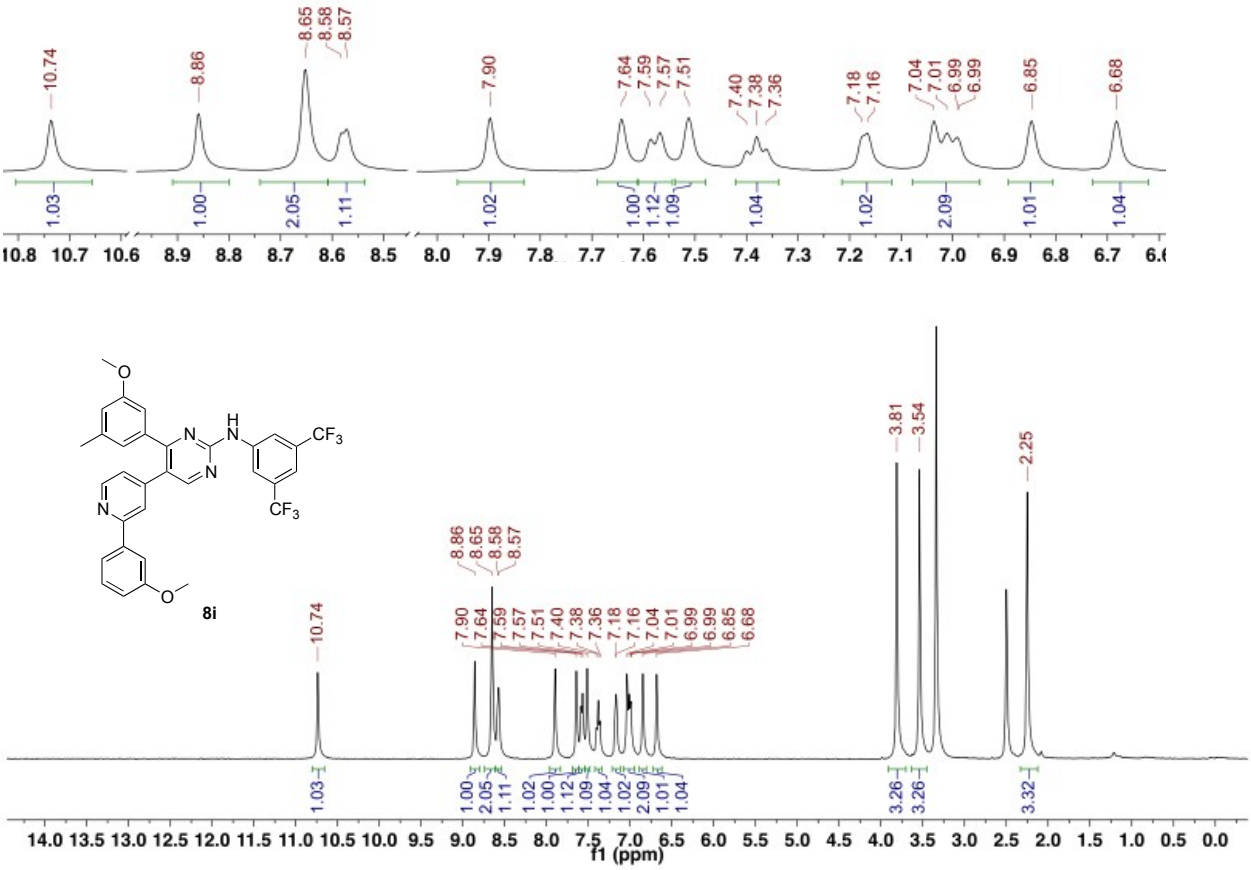
^{13}C NMR spectrum of 8g (100 MHz, DMSO)



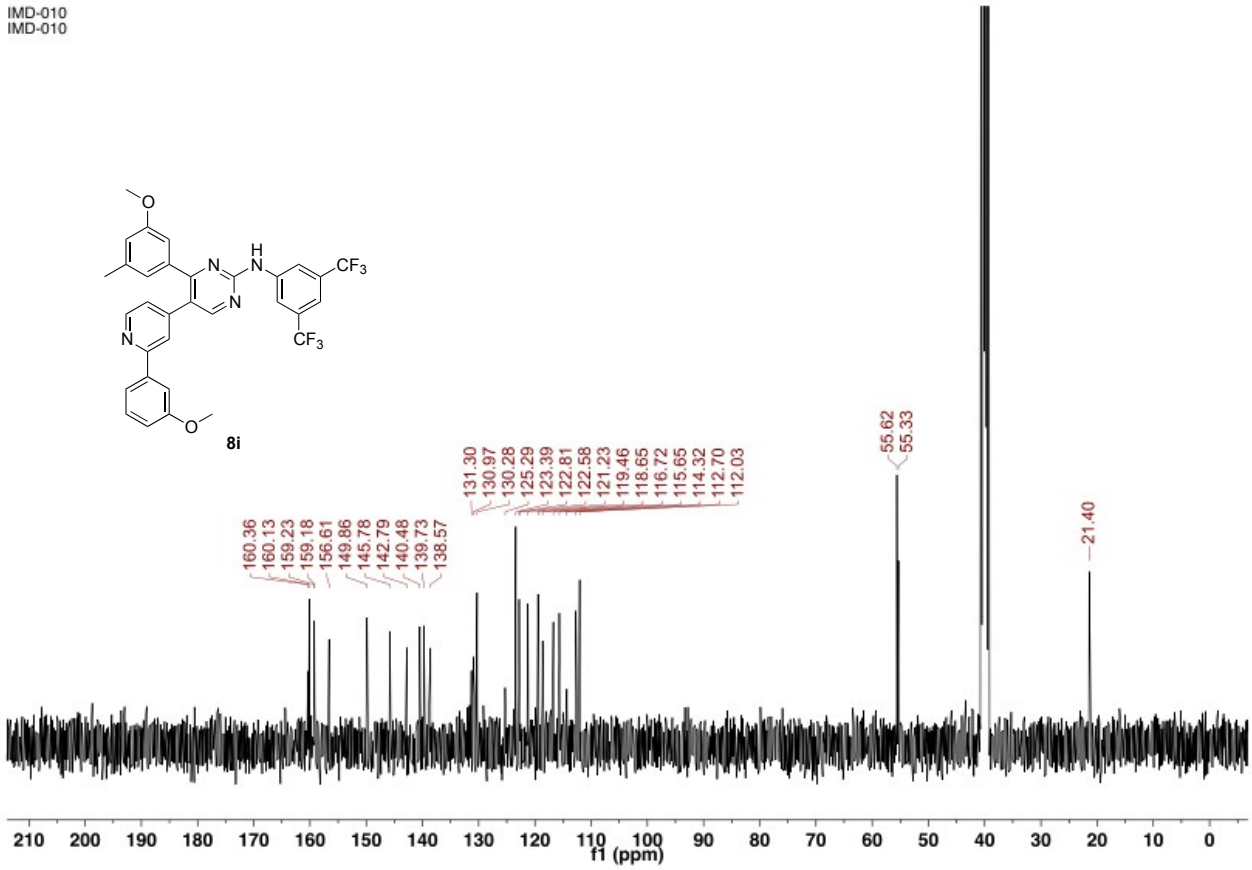
^{1}H NMR spectrum of 8h (400 MHz, DMSO)



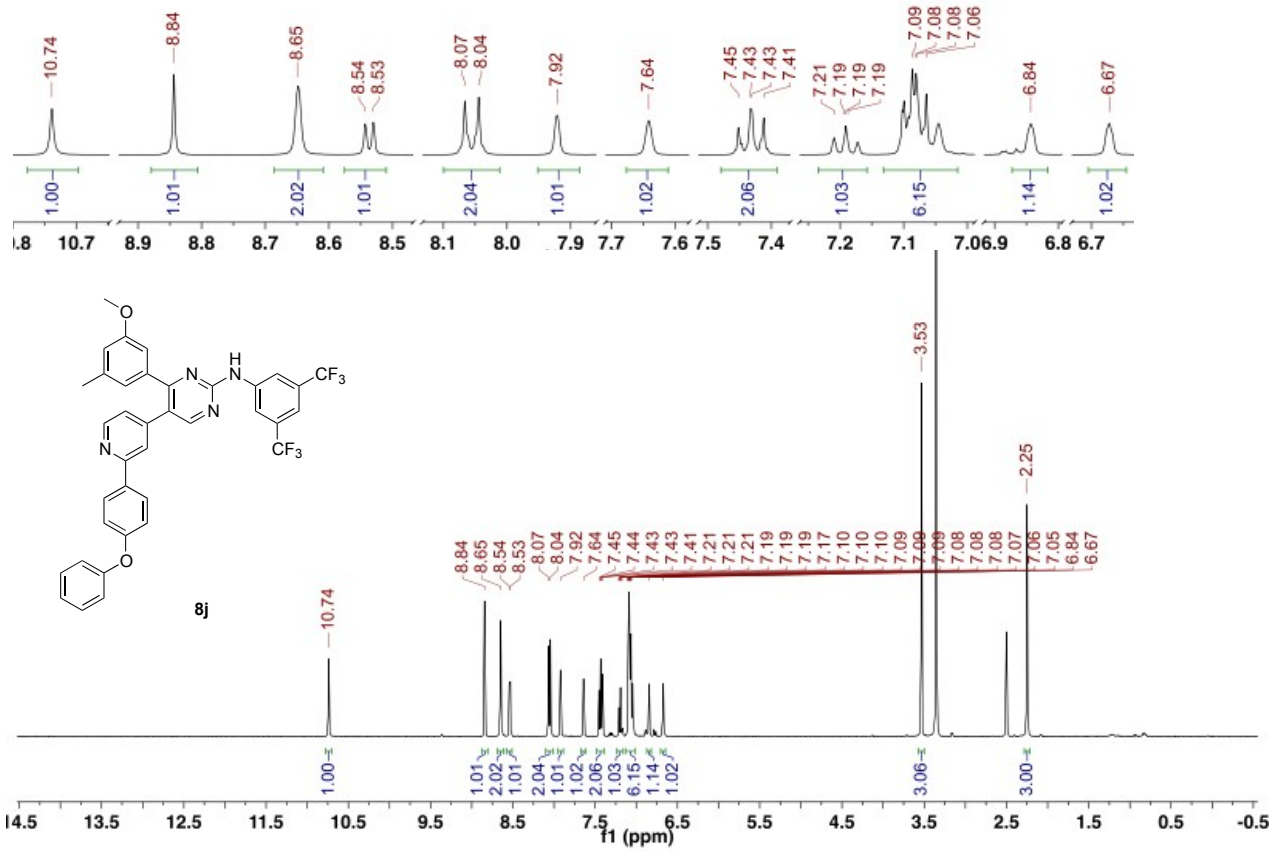
^{13}C NMR spectrum of 8h (100 MHz, DMSO)



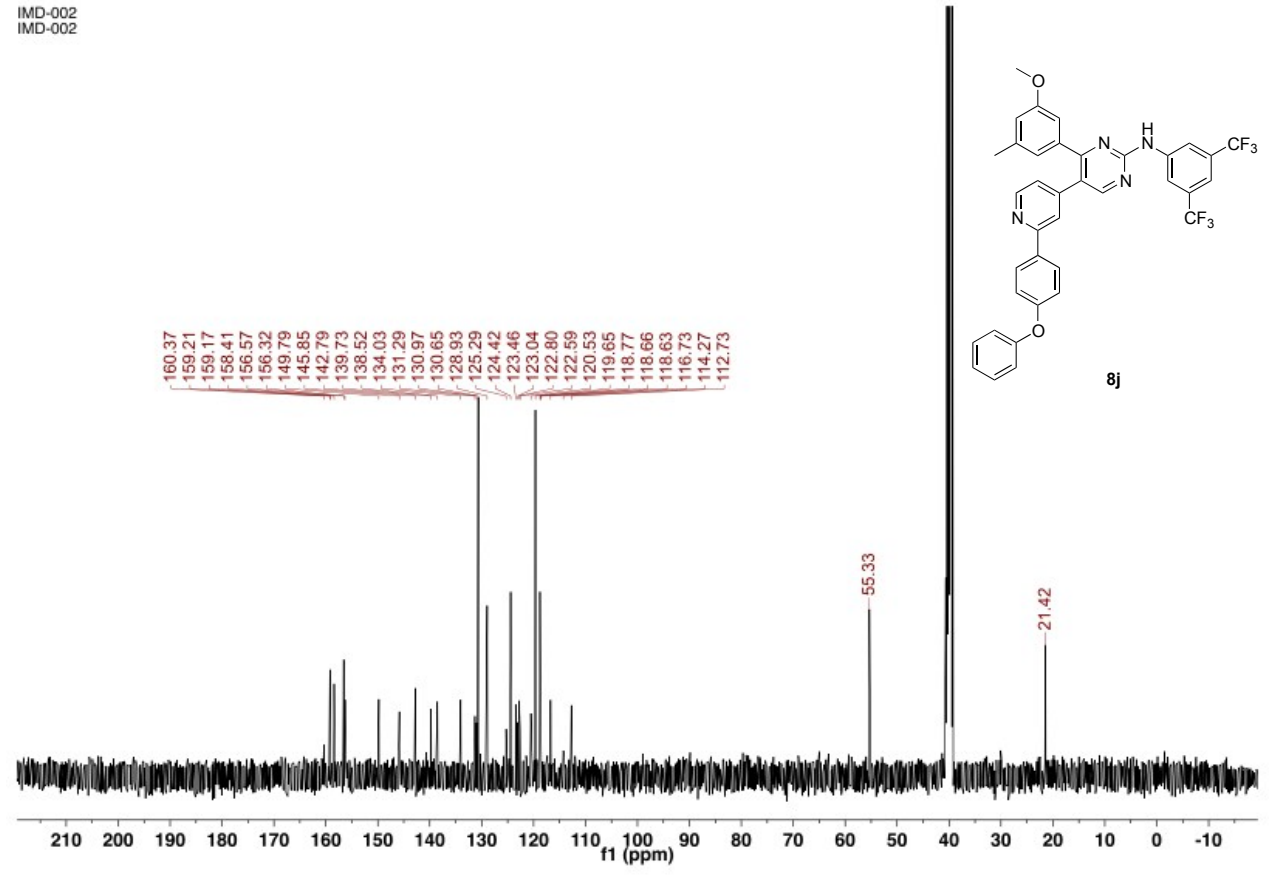
^{1}H NMR spectrum of 8i (400 MHz, DMSO)



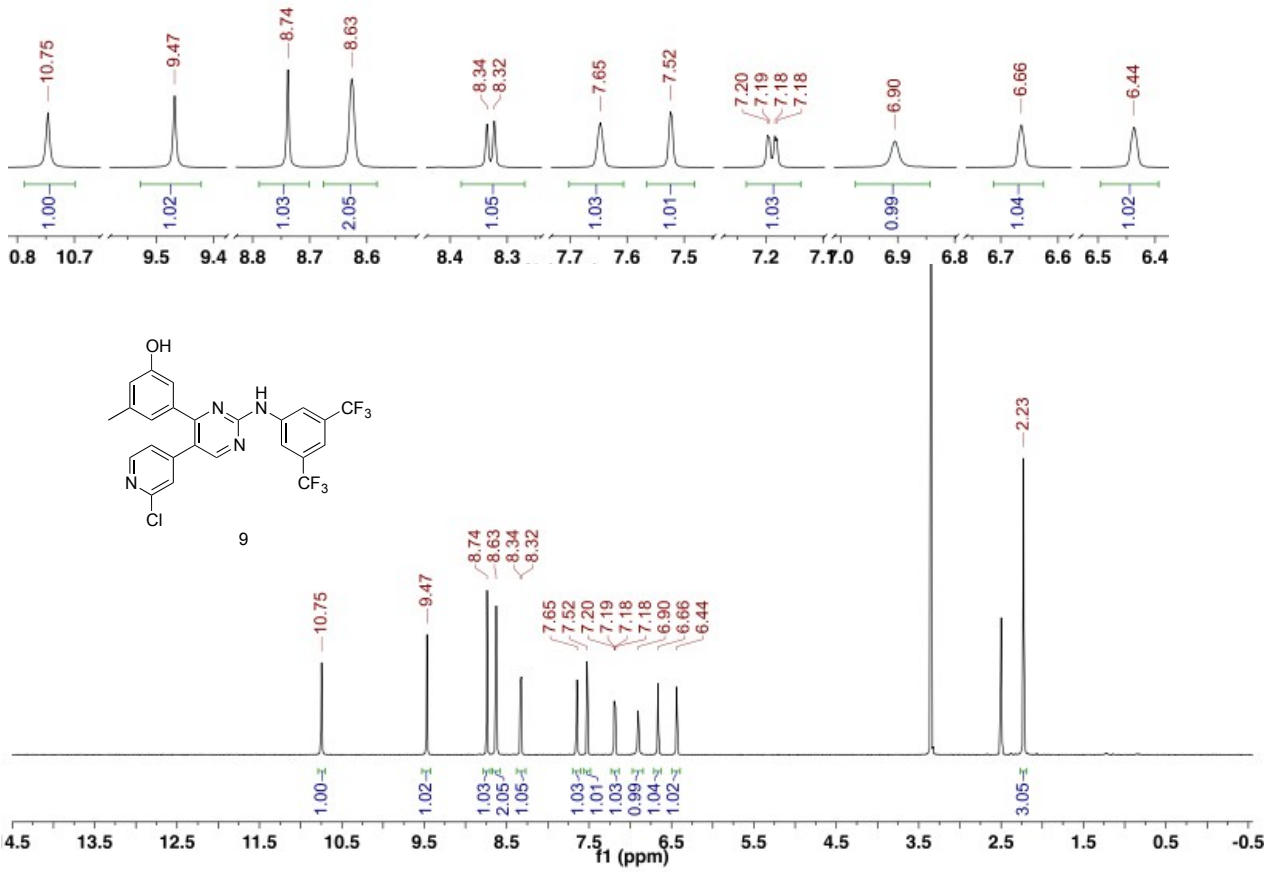
^{13}C NMR spectrum of 8i (100 MHz, DMSO)



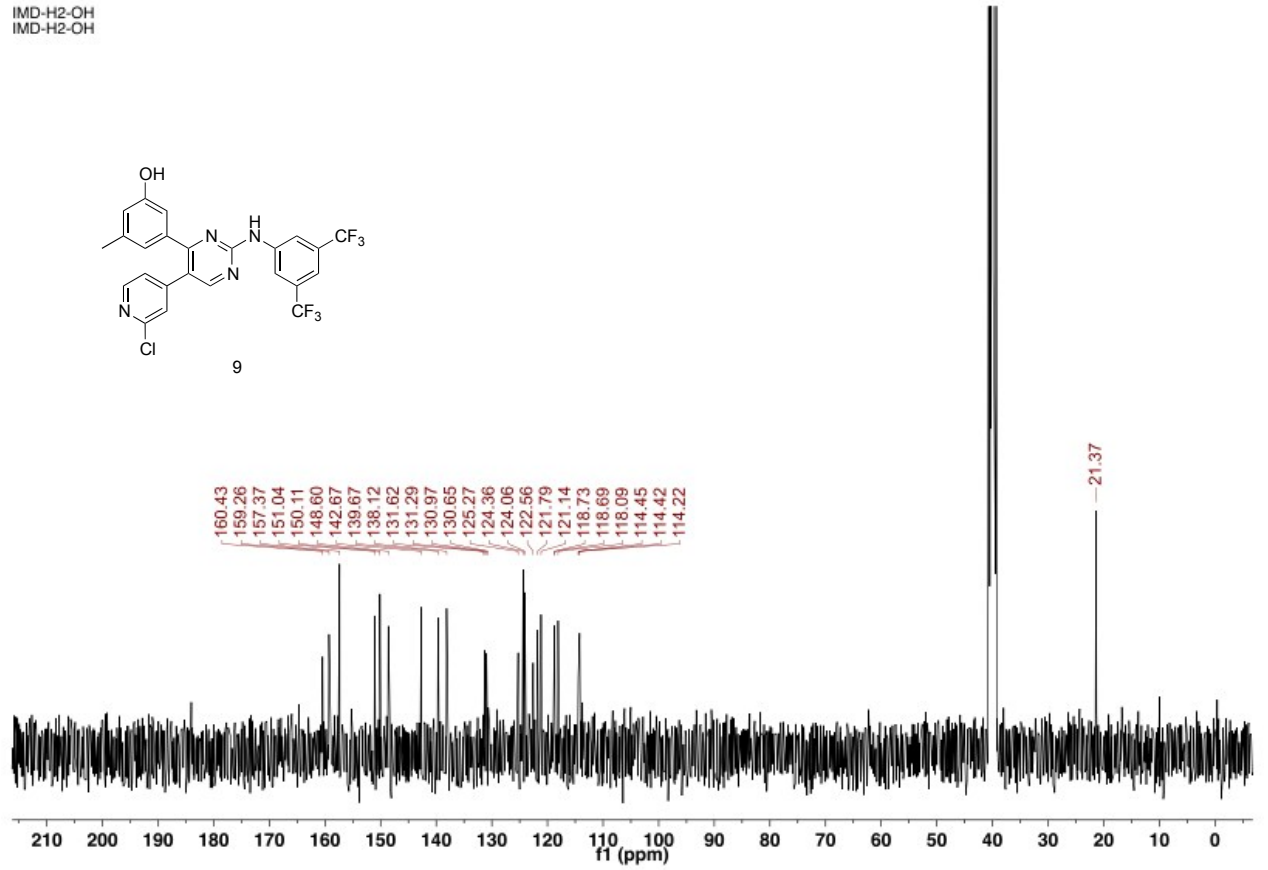
^{1}H NMR spectrum of 8j (400 MHz, DMSO)



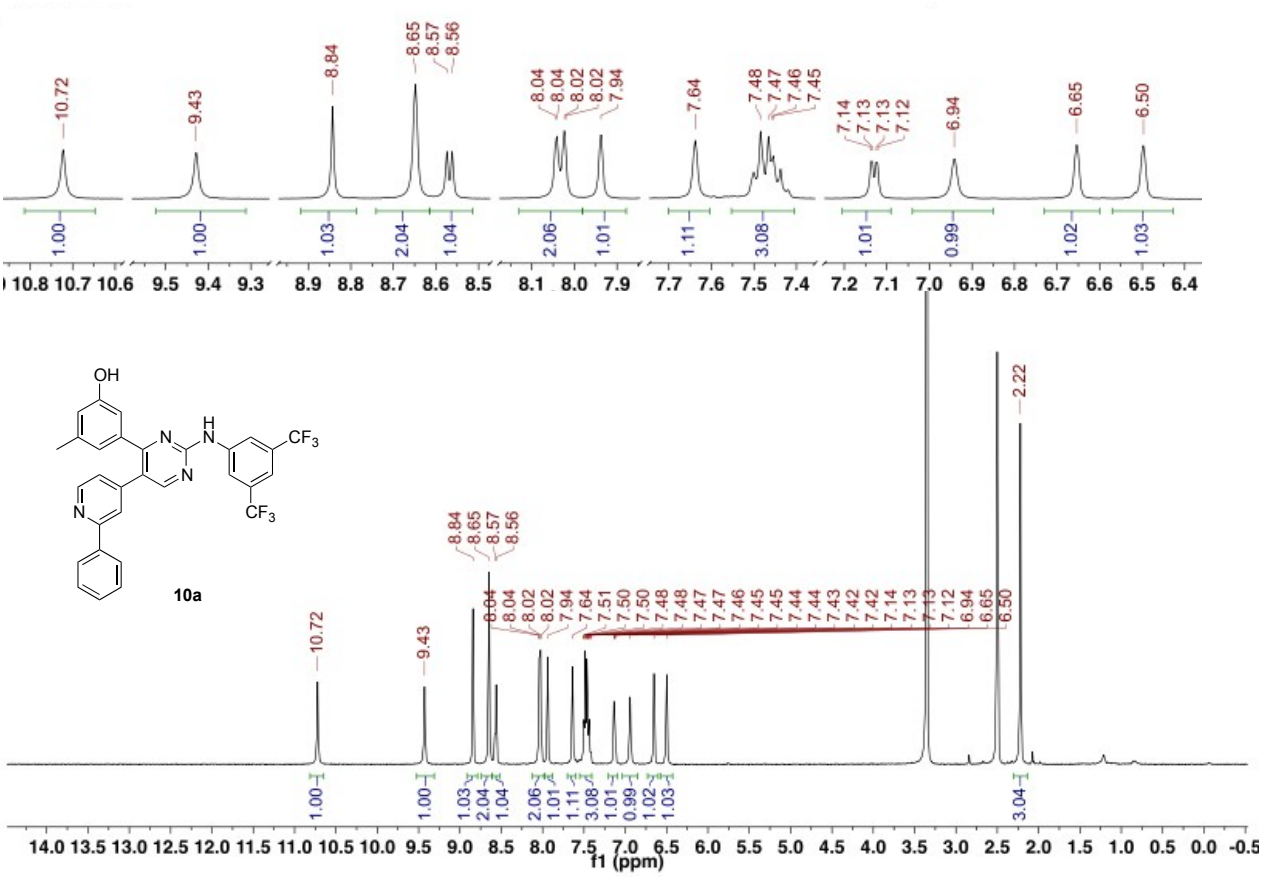
^{13}C NMR spectrum of 8j (100 MHz, DMSO)



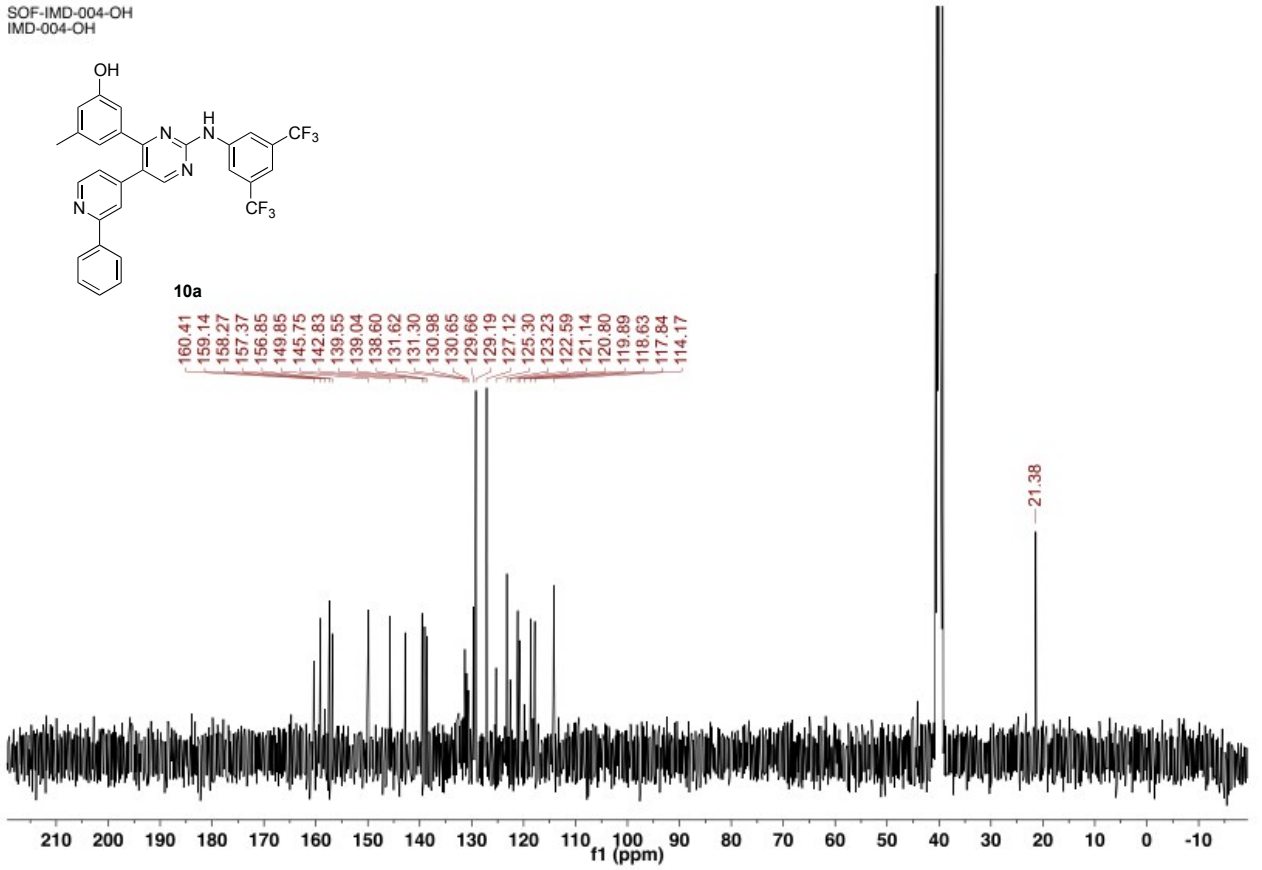
^{1}H NMR spectrum of 9 (400 MHz, DMSO)



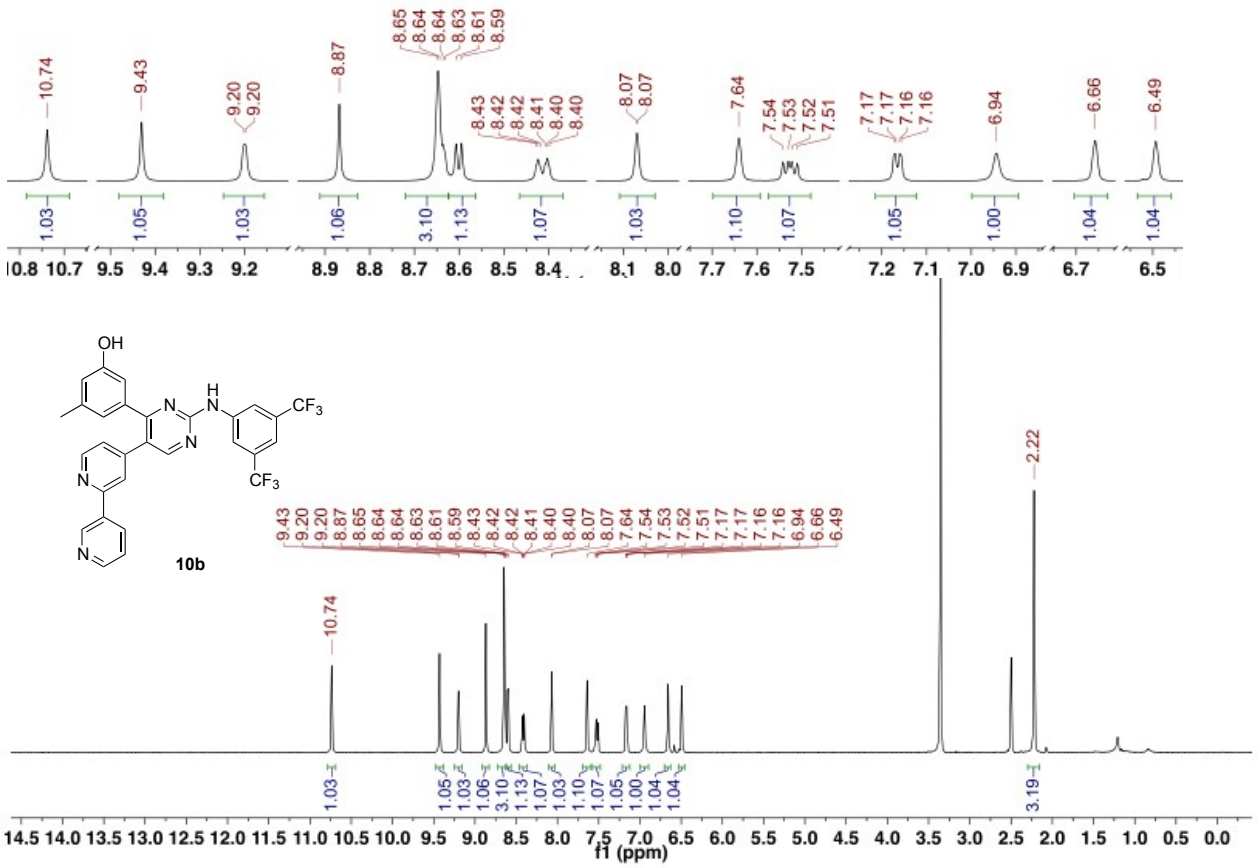
^{13}C NMR spectrum of 9 (100 MHz, DMSO)



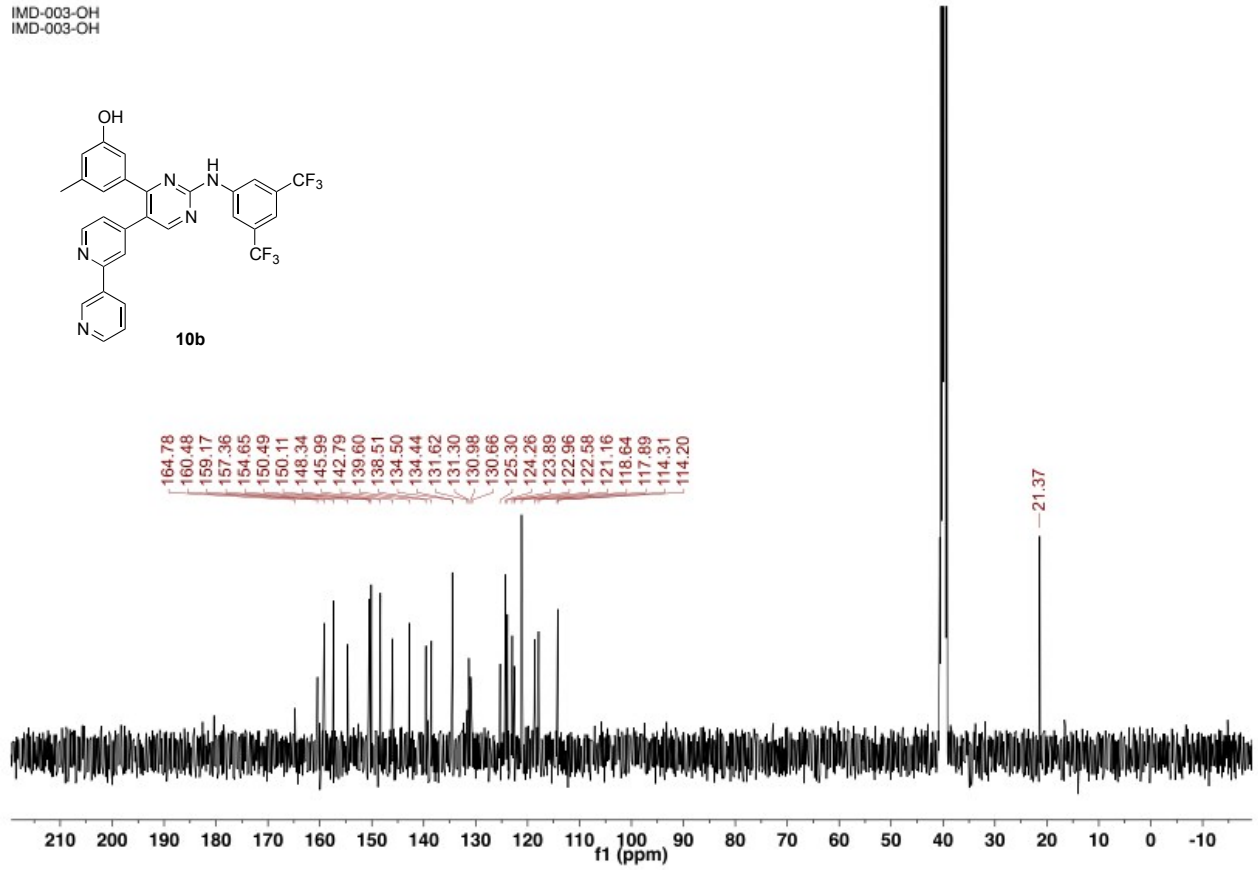
^{1}H NMR spectrum of 10a (400 MHz, DMSO)



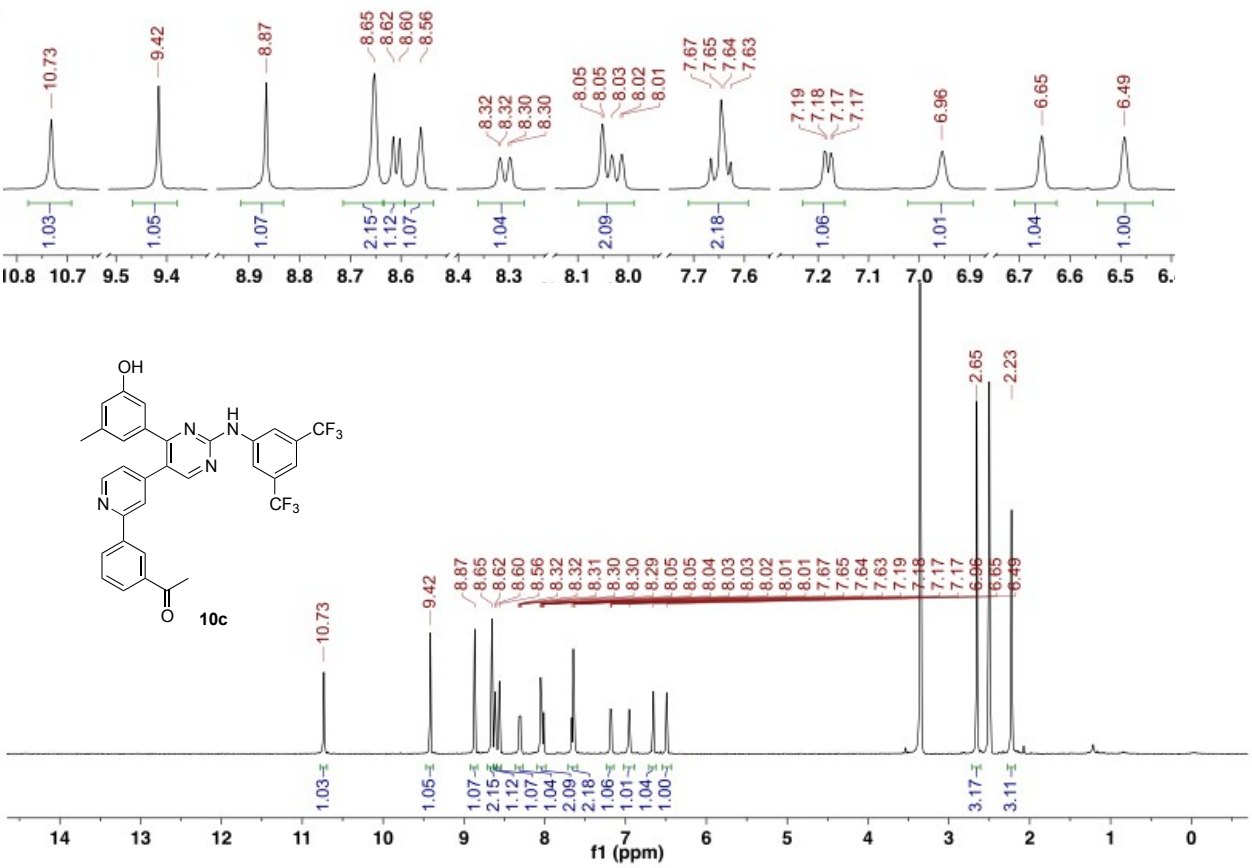
^{13}C NMR spectrum of 10a (100 MHz, DMSO)



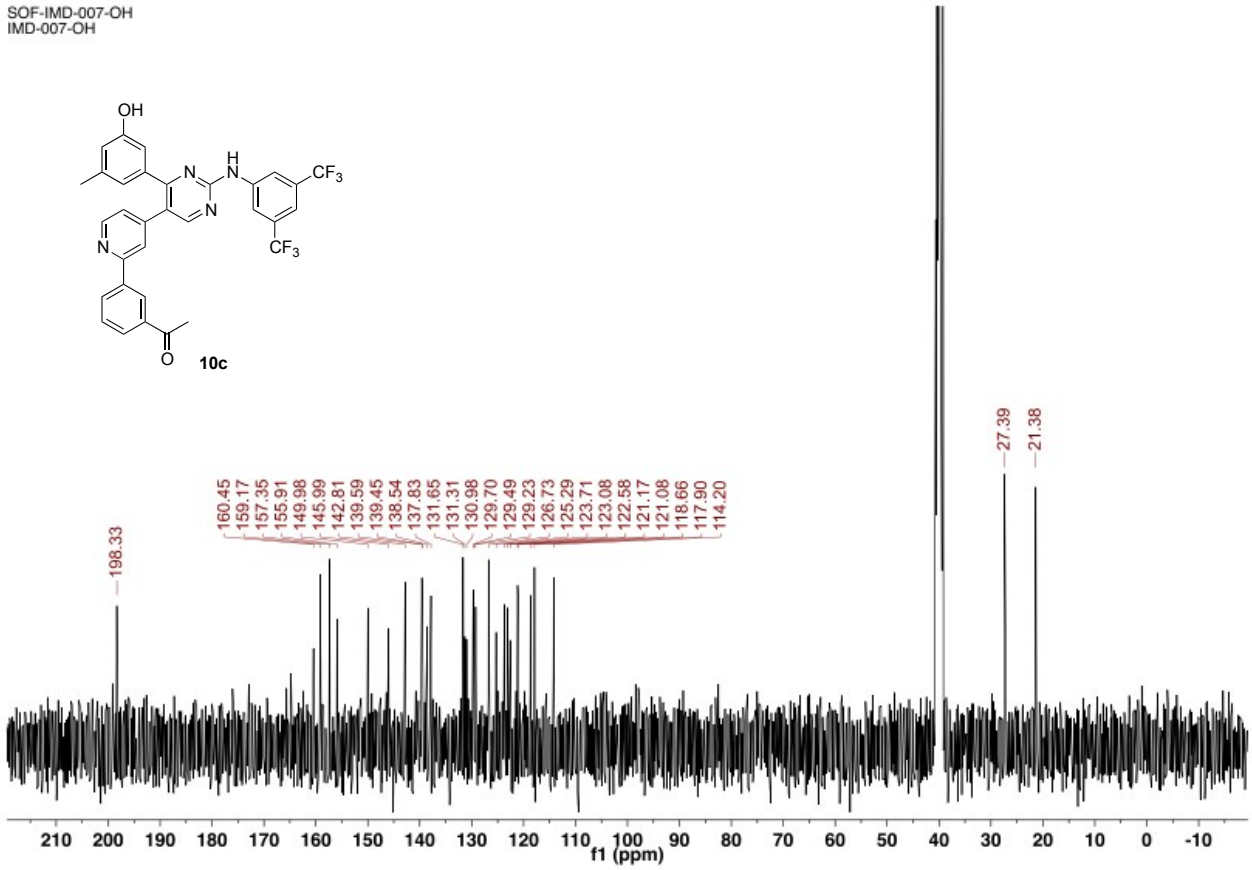
^{1}H NMR spectrum of 10b (400 MHz, DMSO)



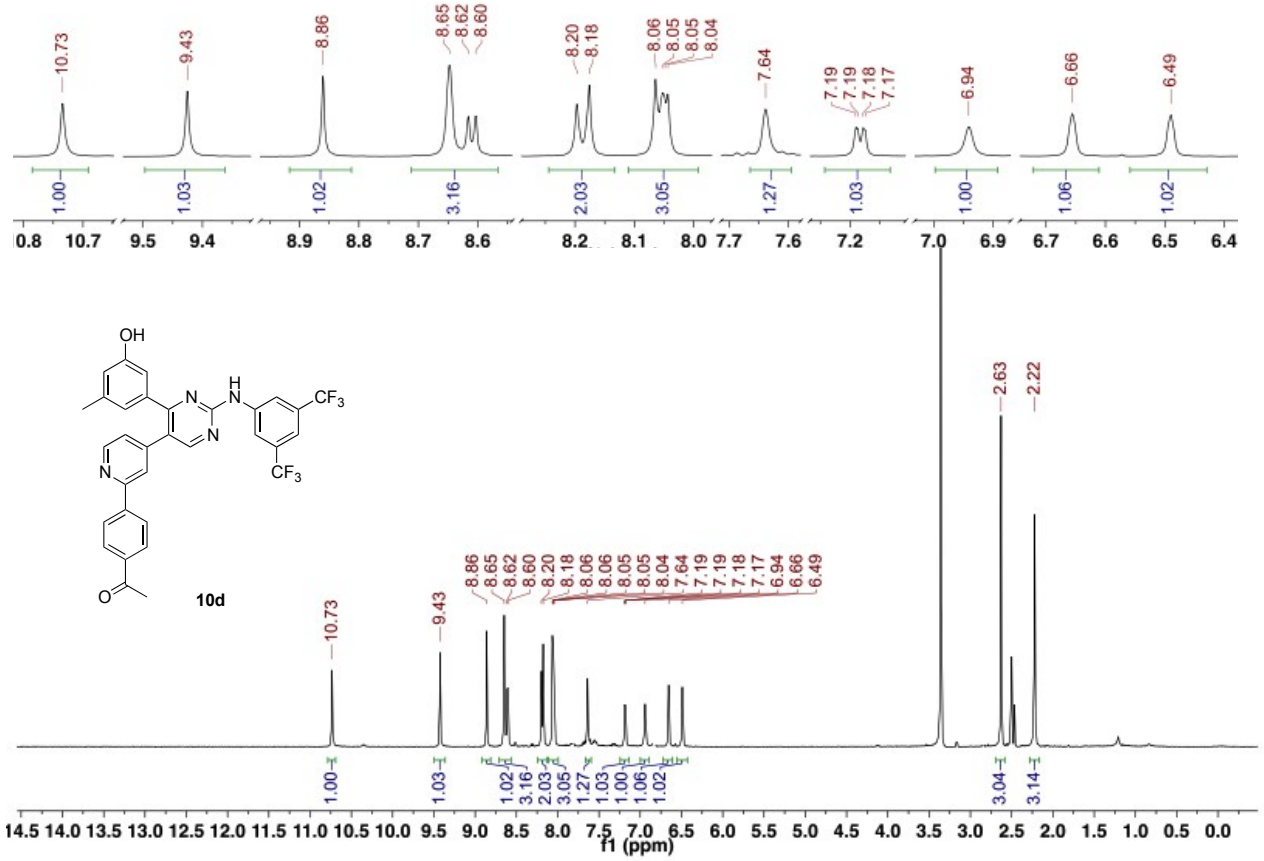
^{13}C NMR spectrum of 10b (100 MHz, DMSO)



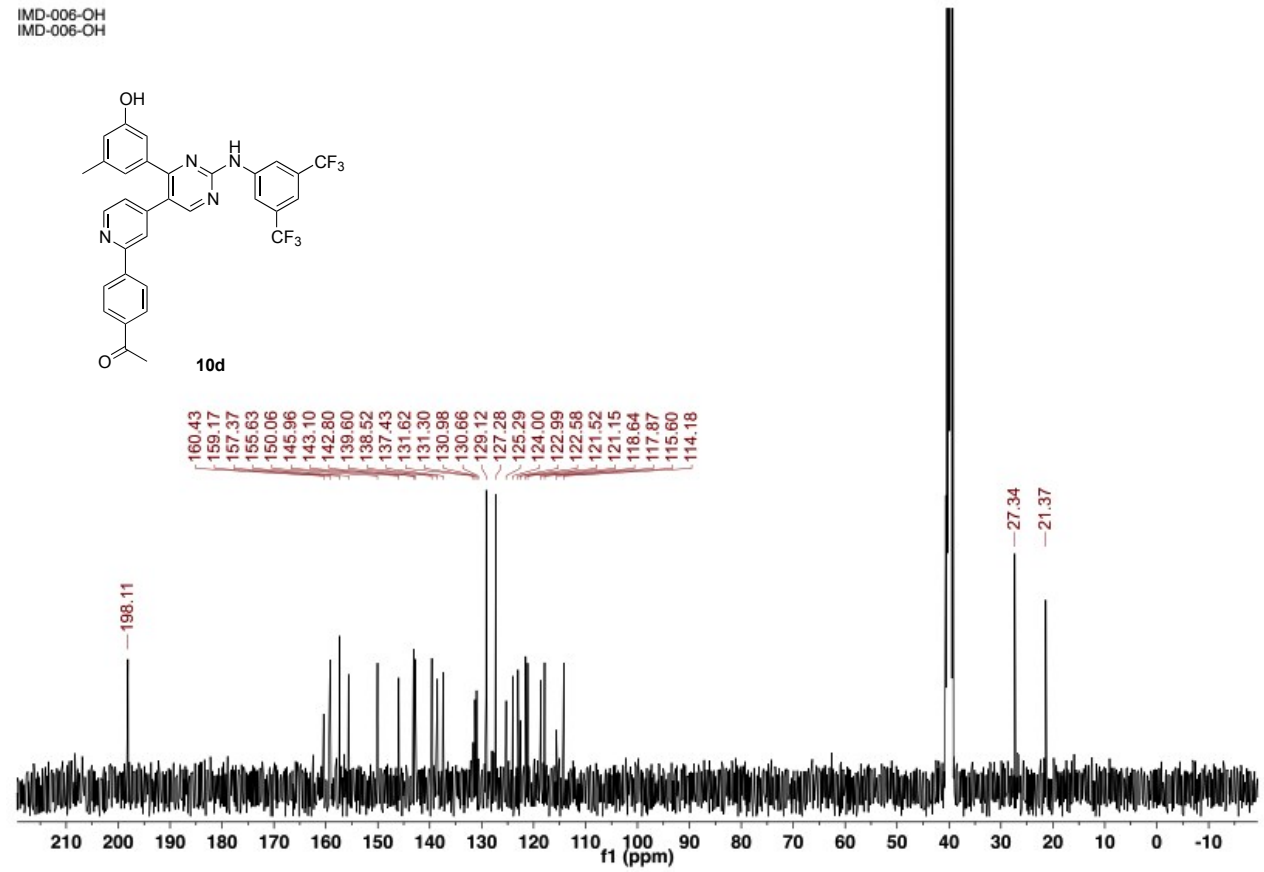
^{1}H NMR spectrum of 10c (400 MHz, DMSO)



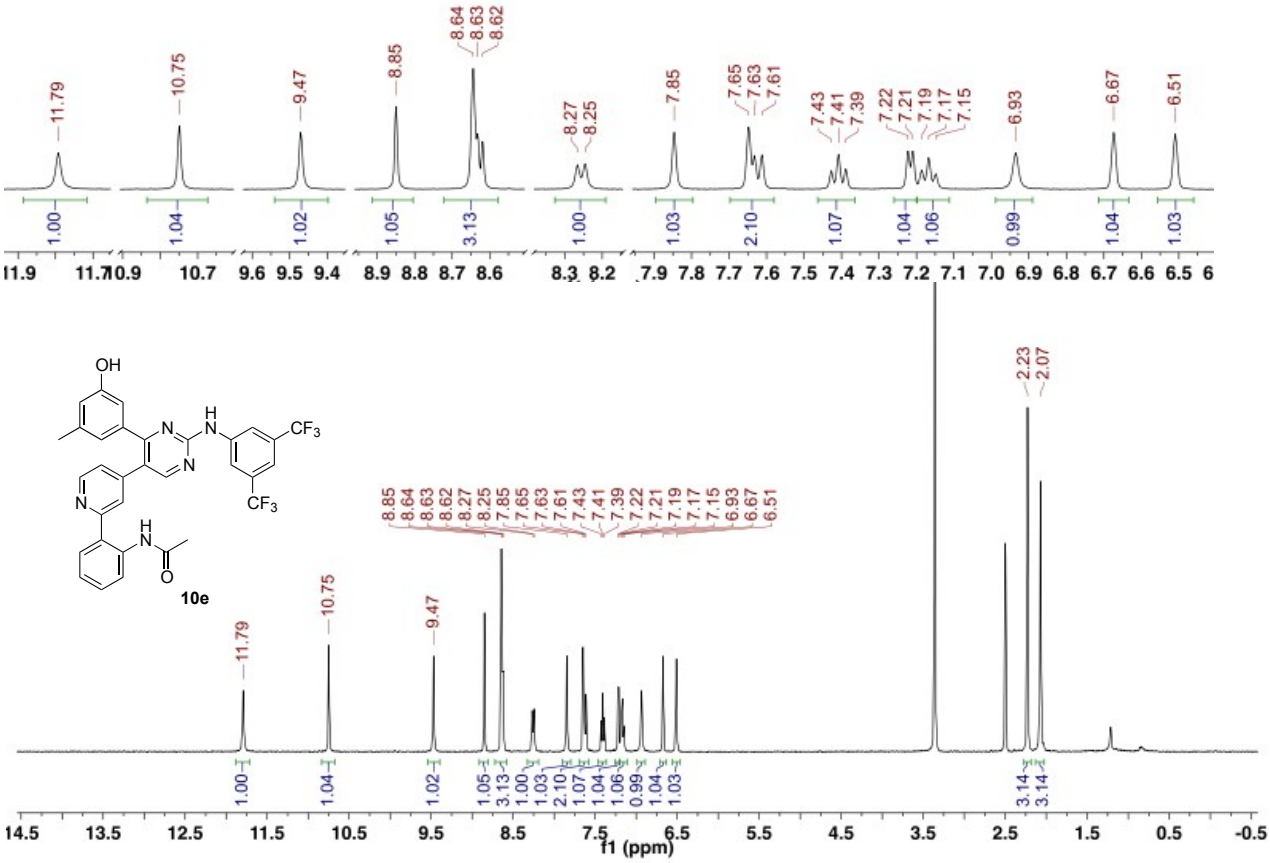
^{13}C NMR spectrum of 10c (100 MHz, DMSO)



^{1}H NMR spectrum of 10d (400 MHz, DMSO)

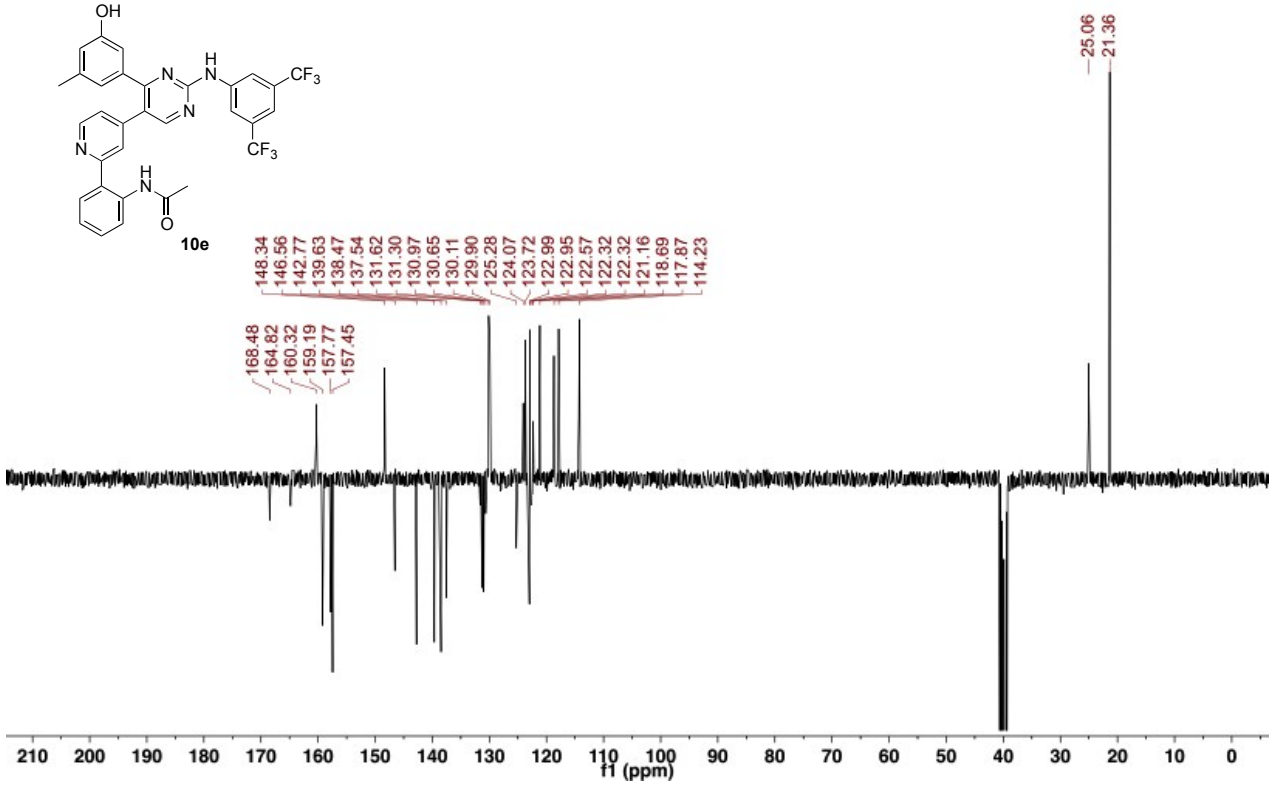


^{13}C NMR spectrum of 10d (100 MHz, DMSO)

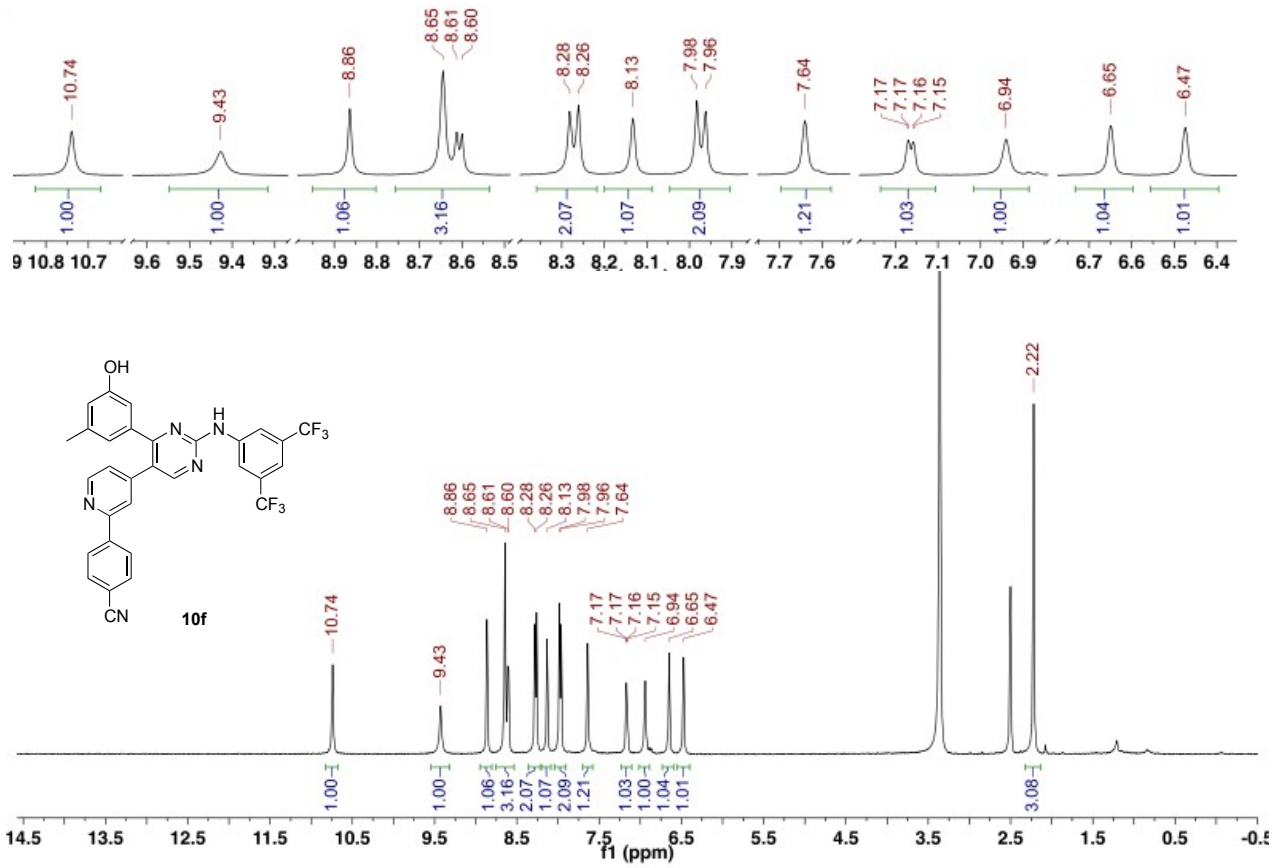


^{1}H NMR spectrum of 10e (400 MHz, DMSO)

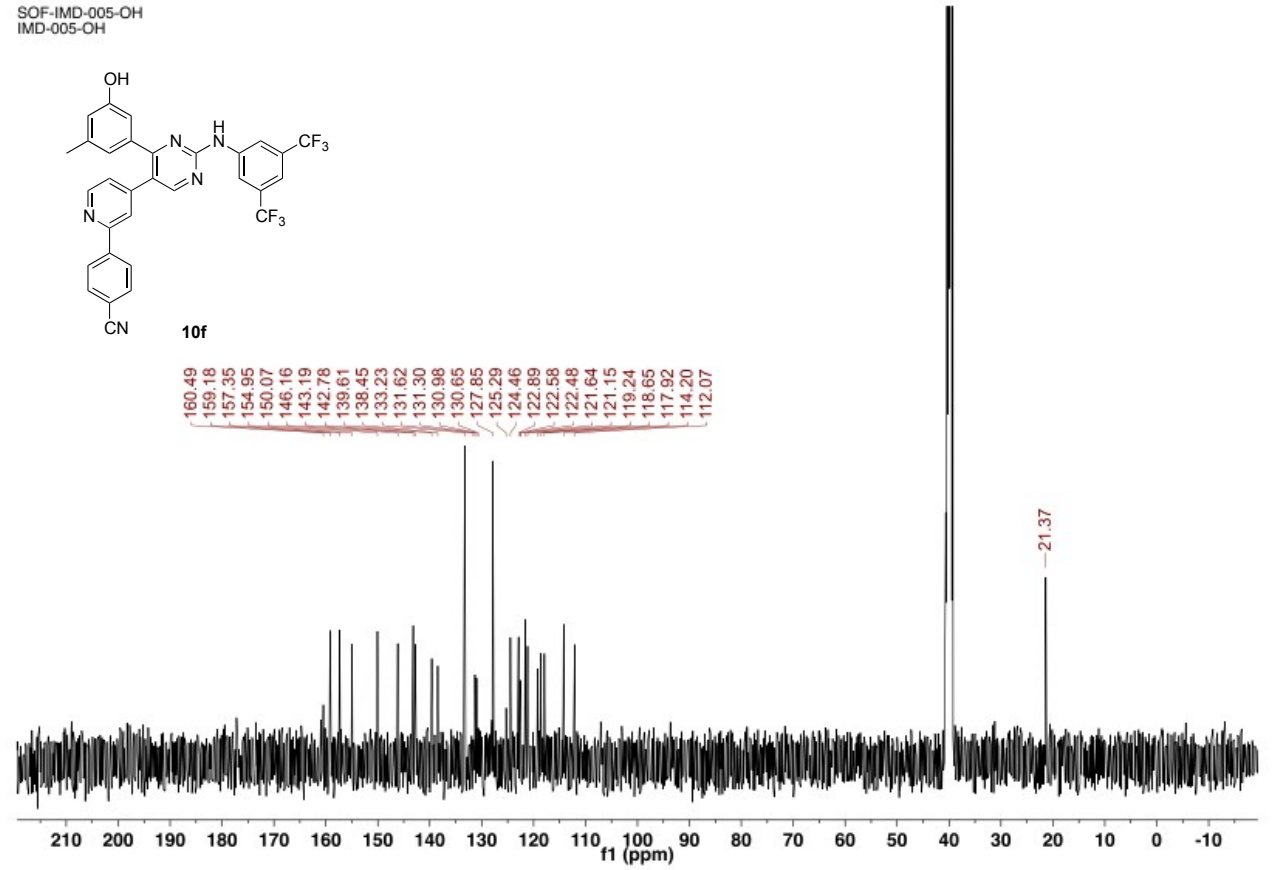
IE1041.108.fid
IE1041-18
DMSO
#106-8



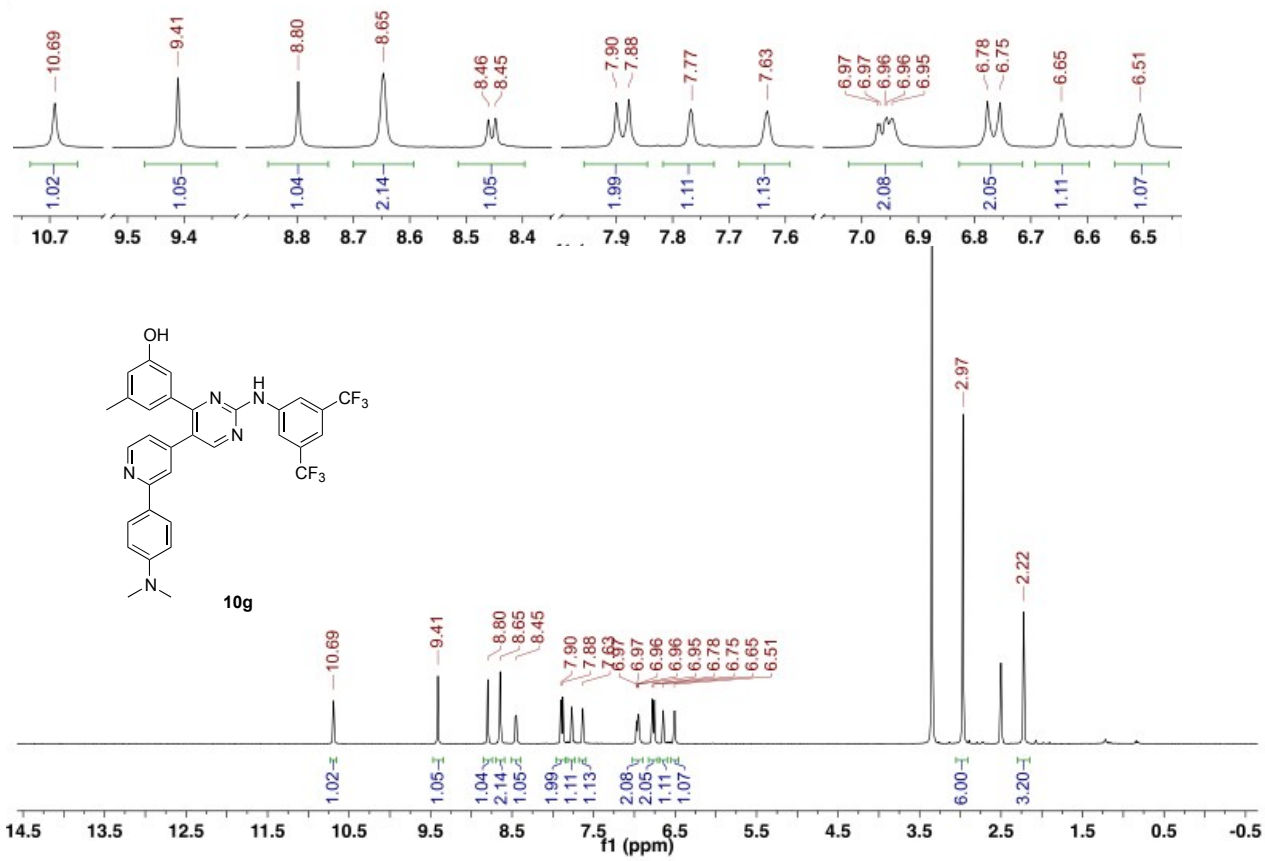
^{13}C NMR spectrum of 10e (100 MHz, DMSO)



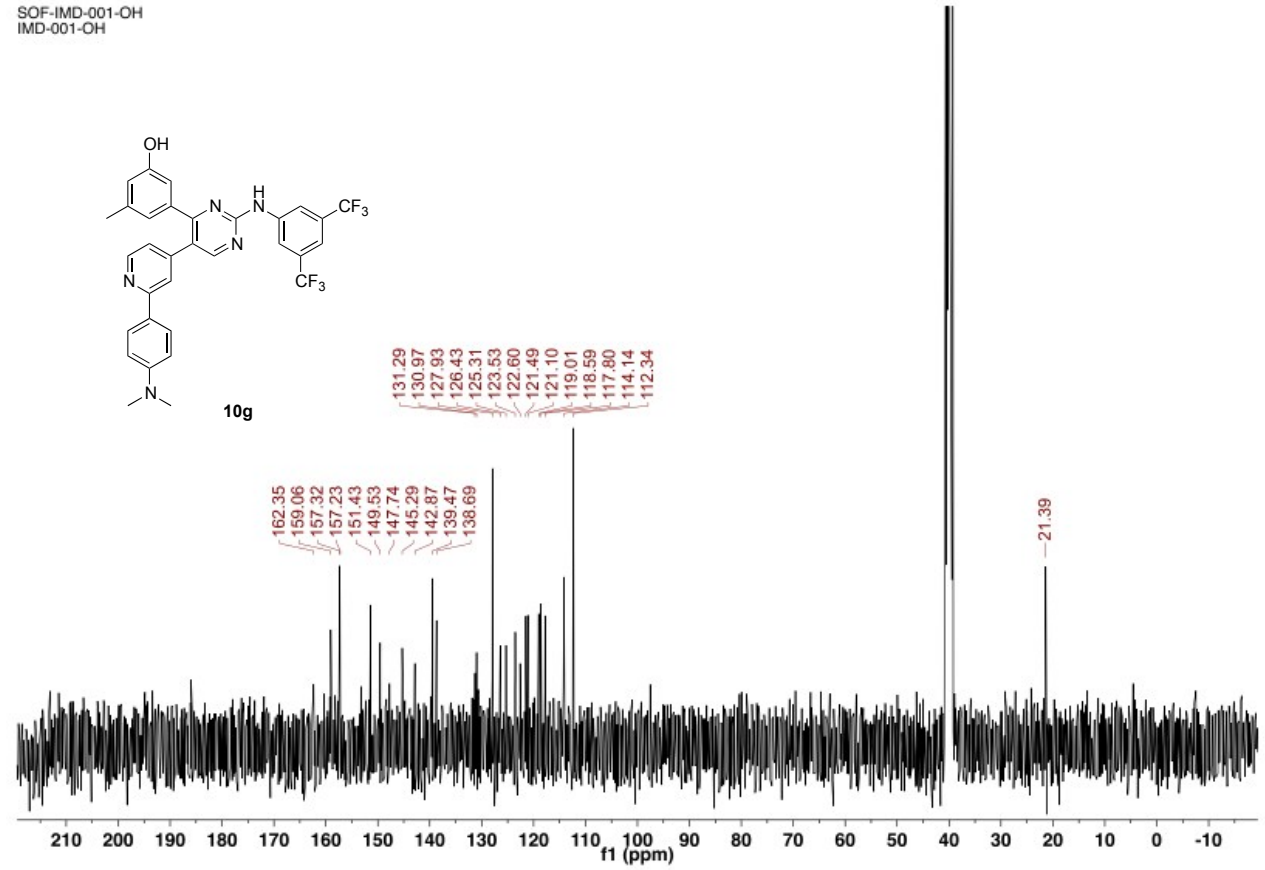
^{1}H NMR spectrum of 10f (400 MHz, DMSO)



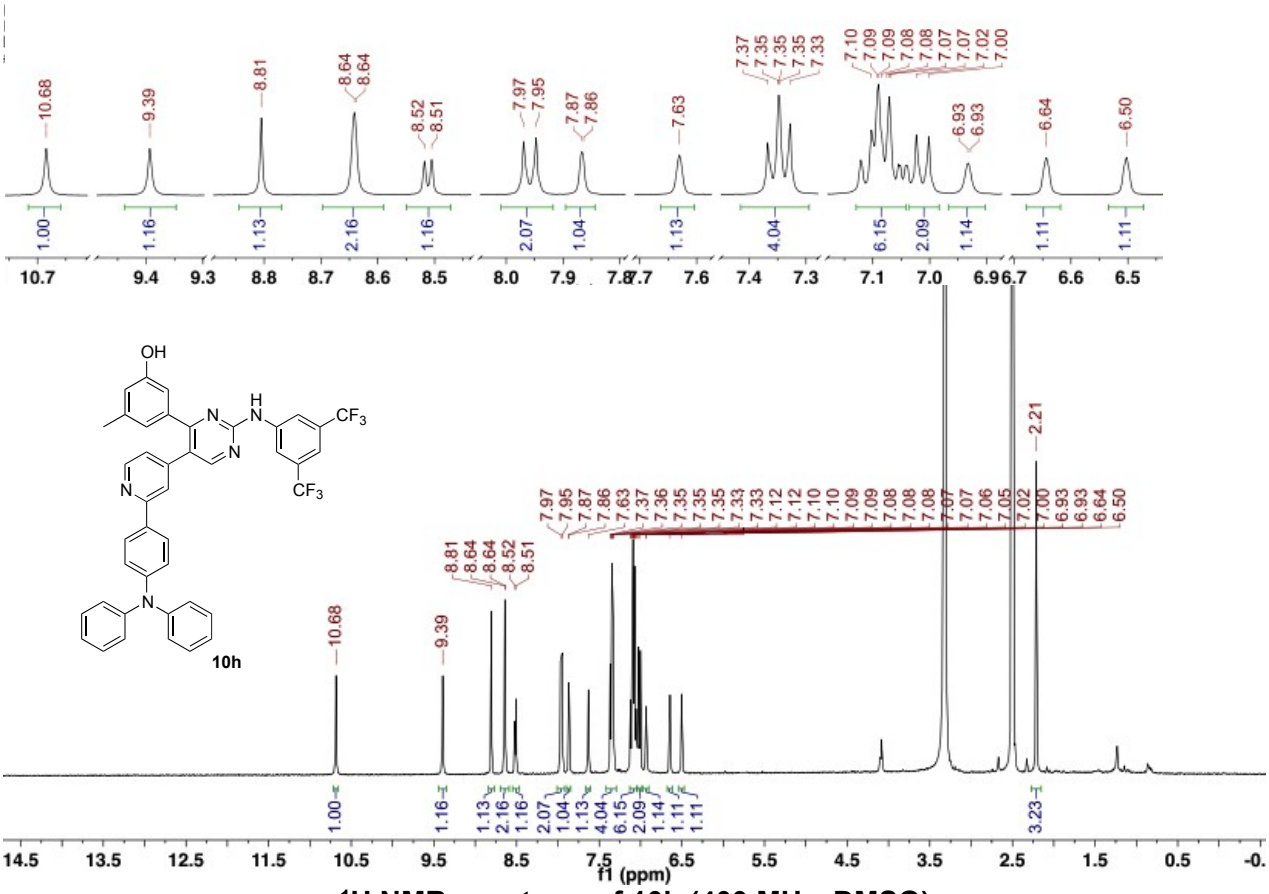
^{13}C NMR spectrum of 10f (100 MHz, DMSO)



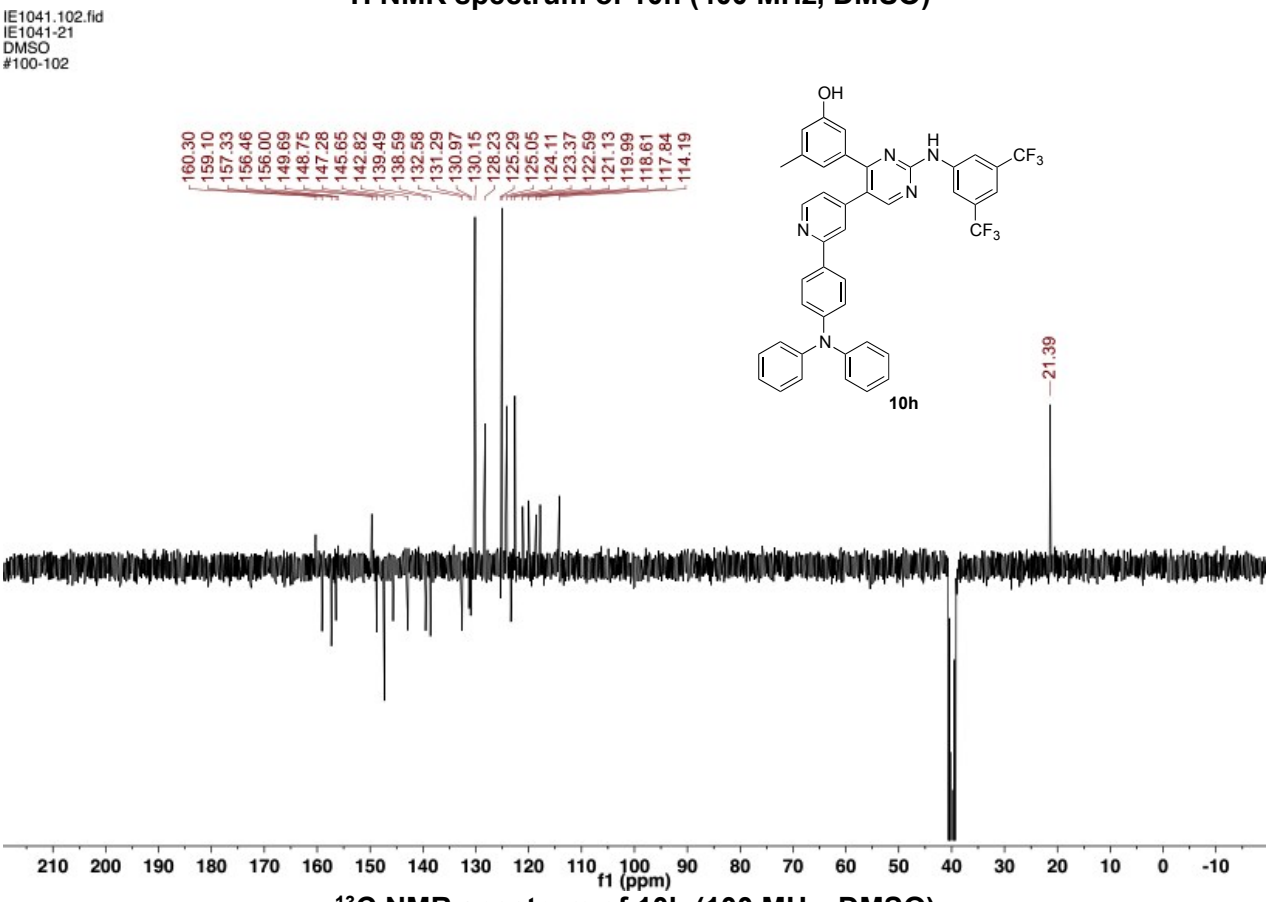
^{1}H NMR spectrum of 10g (400 MHz, DMSO)



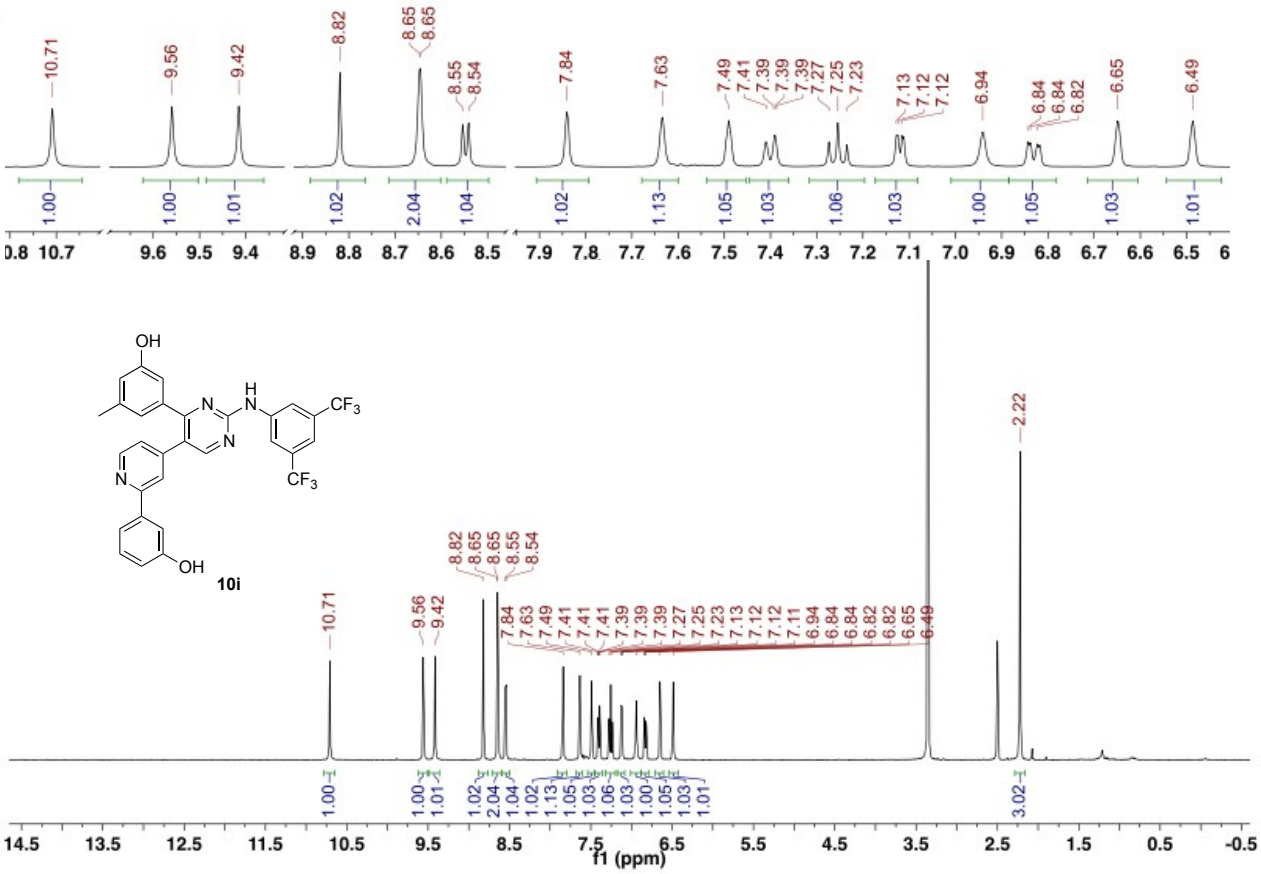
^{13}C NMR spectrum of 10g (100 MHz, DMSO)



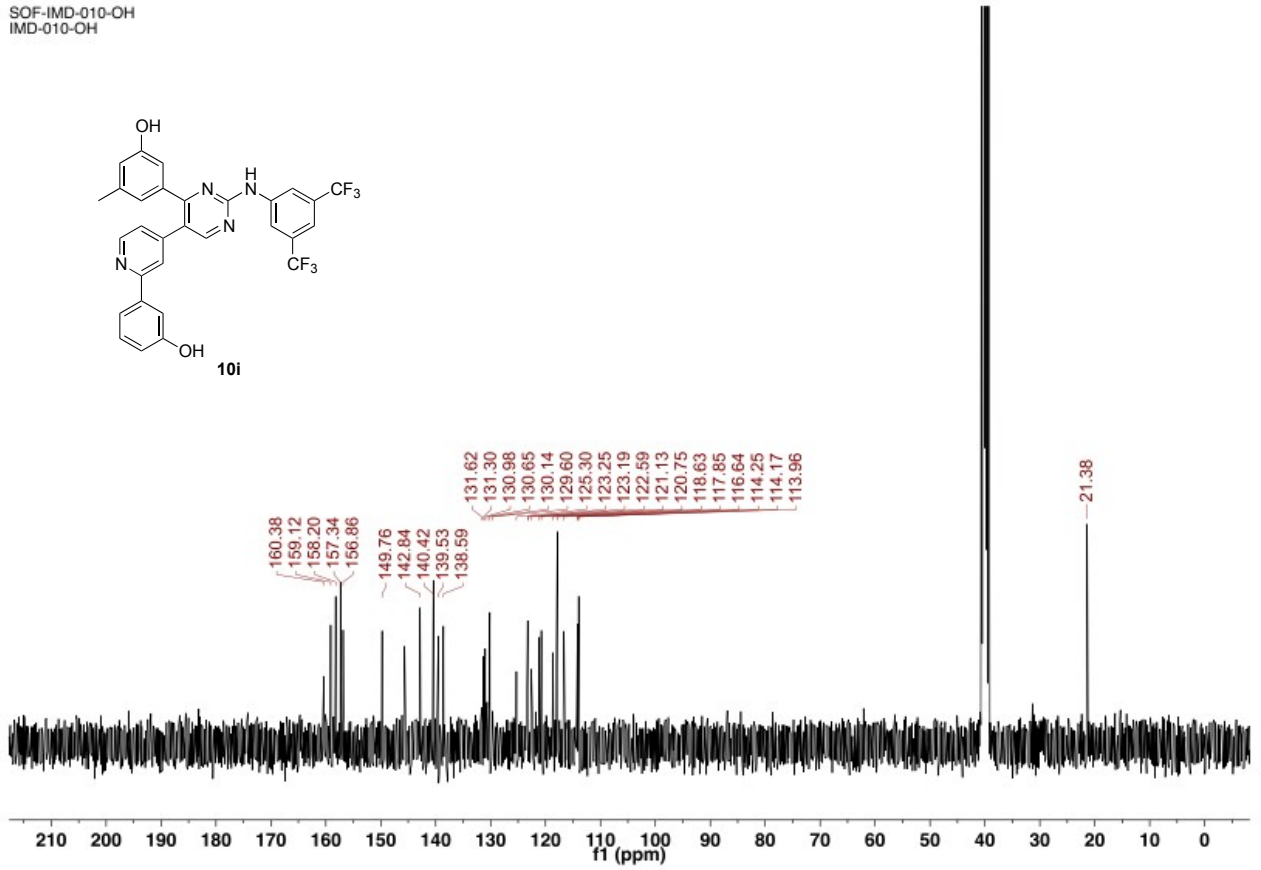
^{1}H NMR spectrum of 10h (400 MHz, DMSO)



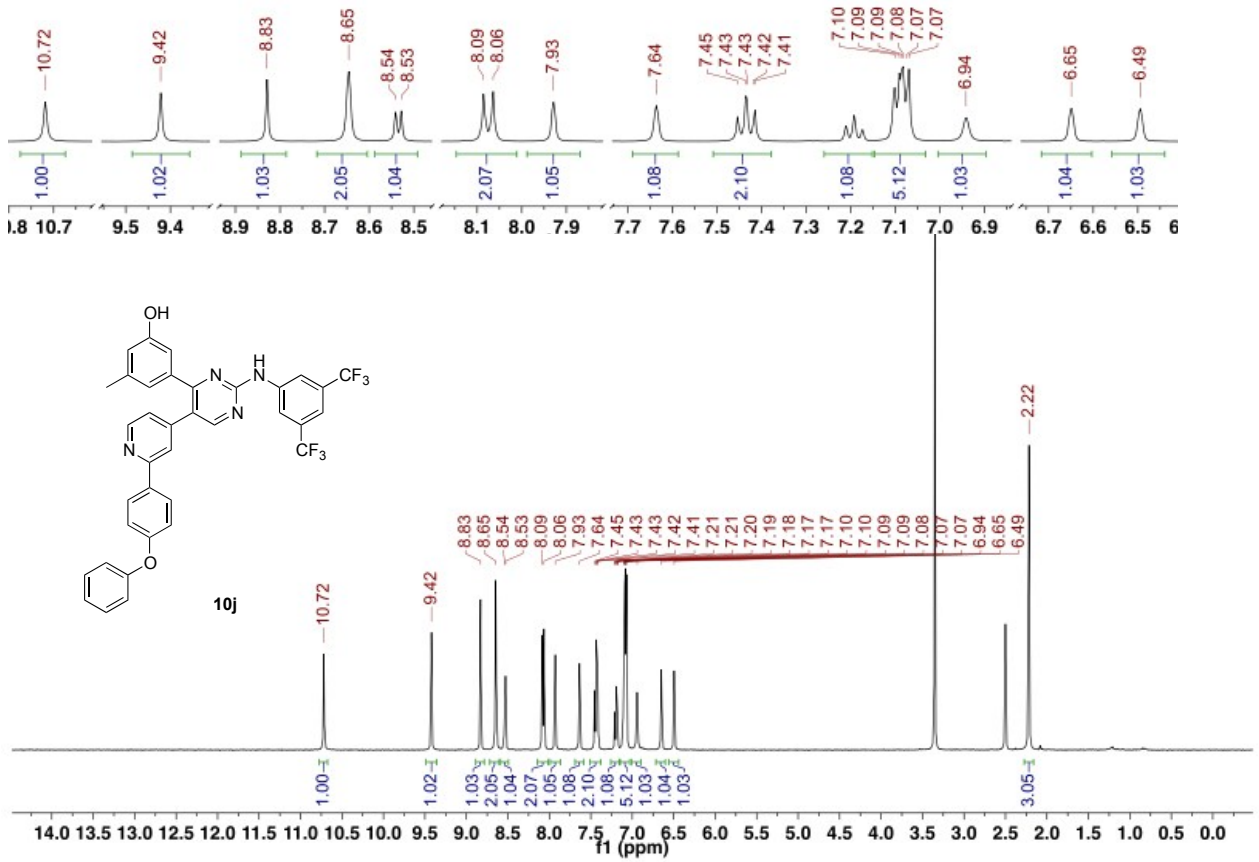
^{13}C NMR spectrum of 10h (100 MHz, DMSO)



^{1}H NMR spectrum of 10i (400 MHz, DMSO)

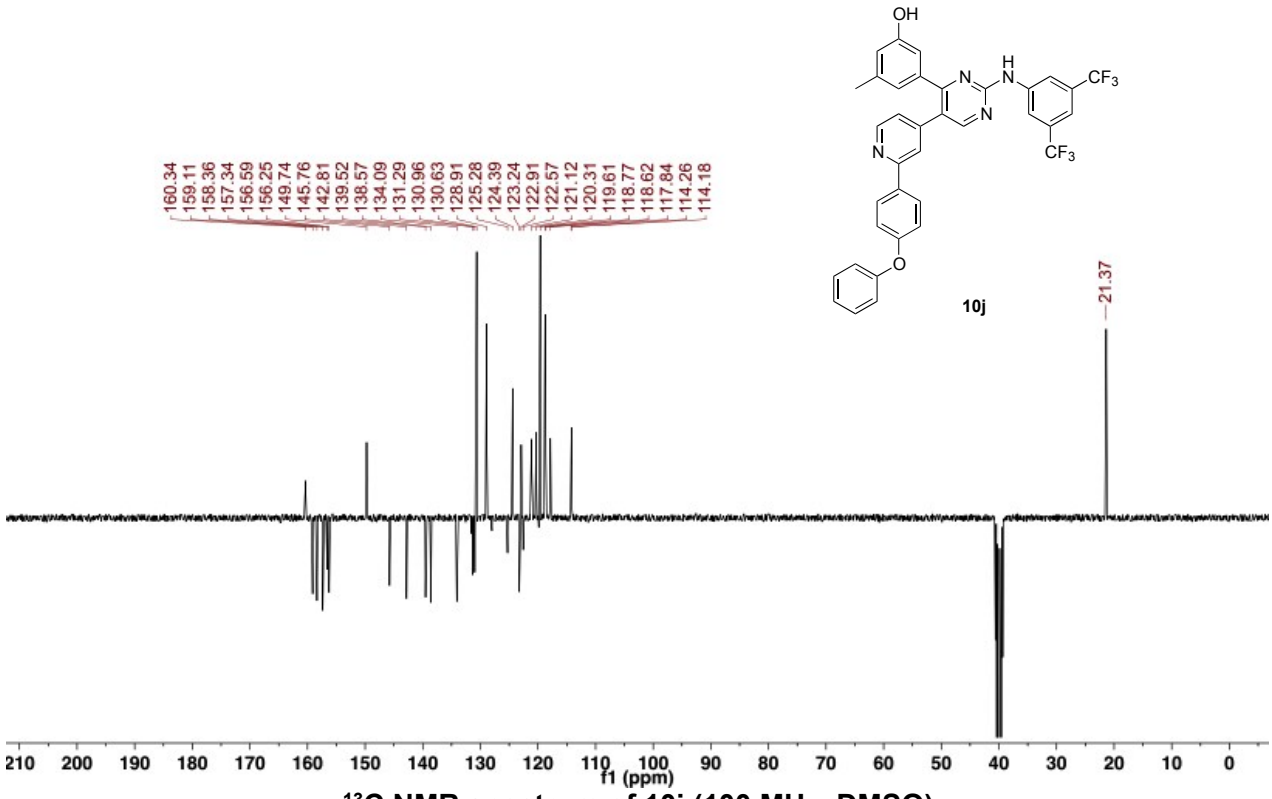


^{13}C NMR spectrum of 10i (100 MHz, DMSO)



^{1}H NMR spectrum of 10j (400 MHz, DMSO)

IE1041.114.fid
IE1041-23
DMSO
#112-4



^{13}C NMR spectrum of 10j (100 MHz, DMSO)

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