Supporting Information

The design, synthesis, and biological evaluation of 5,6,7,8-tetrahydropteridines as anti-inflammatory compounds

Rachel M. Chen,[a, b]* Stefan Emming,[b] Roseanna Cinnamon,[a†] Jacob Cameron,[a] Kate Schroder,[b] Bostjan Kobe,[a, b] Avril A. B. Robertson.[a, b]*

[a, a] *School of Chemistry and Molecular Biosciences, The University of Queensland, St Lucia, 4072, Australia*
[b, b] *Institute for Molecular Bioscience, The University of Queensland, St Lucia, 4072, Australia*
[c, c] *Australian Infectious Diseases Research Centre, The University of Queensland, St Lucia, 4072, Australia*

*Dr Rachel M. Chen uqrchen4@uq.edu.au
*Professor Avril A. B. Robertson a.robertson3@uq.edu.au

Table of Contents

MATERIALS AND METHODS

Commercially-available reagents were purchased from Combiblocks, Fluorochem, Sigma-Aldrich, and Merck and subjected to quality control by LCMS prior to use. All commercially available reagents were used without further purification unless noted otherwise. LCMS was performed with 0.05% (v/v) formic acid in water/0.05% (v/v) formic acid in acetonitrile solvent system on a Shimadzu Nexera uHPLC (Japan) equipped with a Phenomenex Luna Omega C18 column (2.1 × 50 mm, 1.6 μm) maintained at 40 °C, SPD-M20A diode array UV-Vis detector (λ = 200 and 254 nm), and Shimadzu 2020 LCMS spectrometer. HRMS was obtained by LC-MS/MS on a Shimadzu Nexera uHPLC coupled to a TripleTOF 5600 Mass Spectrometer (ABSCIEX) equipped with a duo electrospray ion source. Each sample (2 μL) was injected onto a Zorbax C18 column (2.1 mm x 100 mm, 1.8 μm; Agilent). Full scan time of flight-mass spectrometry (TOF-MS) data was acquired over a mass range 100 – 1500. RP-HPLC was performed using a Shimadzu® HPLC equipped with a Phenomenex Luna C18 100 Å column (21.2 × 150 mm, 5 μm) and purified with 0.1% TFA in H_2O/0.1% TFA in MeCN solvent system, unless otherwise specified. Fractions with >90% purity were pooled and lyophilised on a Christ® Alpha 2-4 LDplus lyophiliser. Melting ranges were measured in a glass capillary tube on a DigiMeltSRS or MPM-H2 machine. TLC was conducted using Merck aluminum-backed F254 silica plates. Flash column chromatography was performed on Merck silica gel 60 (230 – 400 mesh). NMR data was collected on a Bruker Avance 600 MHz or 900 MHz spectrometer at 298 K. ^{13}C and ^{1}H NMR chemical shifts were referenced to tetramethylsilane (TMS) where present, or residual solvent: $CDCl_3$ (^{1}H δ 7.26 ppm, ^{13}C δ 77.16), DMSO-d_6 (^{1}H δ 2.50, ^{13}C δ 39.53) or $MeOD_4$ (^{1}H δ 3.31, ^{13}C δ 49.00). NMR data was processed using MestReNova and are presented in the following format: δ (multiplicity, coupling constant, integration, assignment). Where indicated, * represents signals corresponding to the rotameric isomer.

VIRTUAL SCREENING

A 2D virtual compound library was created in ChemDraw (PerkinElmer) and converted to energy-minimised 3D structures (SYBYL) using Chem3D (MM2 energy minimisation), followed by further ligand processing (pdbqt) using Autodock Tools (Molecular Graphics Laboratory of the Scripps Research Institute). *In silico* docking of the virtual library with the X-ray crystal structure of BTK (PDB ID: 5P9J) was accomplished using AutoDock Vina (Molecular Graphics Laboratory of the Scripps Research Institute).[1] A grid box was centered on the substrate binding pocket of BTK enzyme with definitions of 40 × 40 × 40 points and 0.542 Å spacing for ligand docking experiments. The program was set to dock 20 poses for each molecule. The dockings were visualised using PyMOL v2.5.3 (Schrodinger, LLC).

BTK KINASE ASSAY

Compounds were screened for activity against BTK using the ADP Glo™ Kinase Assay with the BTK Kinase Enzyme System (Promega). Inhibitor dilutions and kinase reactions were prepared manually or using the Agilent Bravo Liquid Handling Platform. The reaction was performed at room temperature in low-volume 384-well ProxiPlates (PerkinElmer) using 5 μL, 5 μL, and 10 μL of kinase reaction, ADP Glo™ Reagent, and Kinase Detection Reagent, respectively. Each kinase reaction consisted of BTK (25 ng/mL), Poly(Glu4Tyr1) kinase substrate (0.2 mg/mL), the experimental compounds/control inhibitors/vehicle at the specified concentrations, and the reaction was initiated by addition of ATP (50 μM). The reactions were incubated for one hour before the reaction was stopped using ADP Glo™ reagent. After 1.5 hours of incubation, the Kinase Detection Reagent was added and incubated for 15 minutes. The luminescence signal was measured using the TECAN M1000 plate reader. The dose-response curves were fitted using Prism 9 (GraphPad Software). Maximal and minimal responses were calibrated to DMSO (vehicle, n = 3) and ibrutinib (full inhibition at 10 μM, n = 3), respectively.

INFLAMMASOME ACTIVATION IN THP-1 CELLS

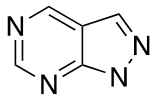
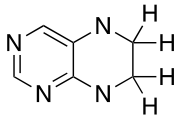
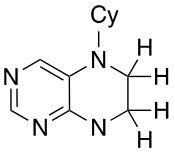
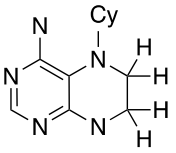
THP-1 cells were expanded in complete media (RPMI-1640, Gibco), supplemented with 10% FCS (Bovogen), 1% pyruvate (Gibco) and 1% penicillin/streptomycin (Gibco) and incubated in a humidified incubator at 37 ˚C. For differentiation, THP-1 cells were counted, supplemented with a final concentration of 30 ng/mL PMA and seeded into 96-well plates with a cell density of 100,000 cells/well. After 48 hours, the media was changed to serum-free Opti-MEM (Thermo Fisher Scientific) and cells were stimulated with LPS (100 ng/mL, Invitrogen, LPS-EK ultrapure) for three hours. Experimental compounds, control inhibitor MCC950 (a direct NLRP3 inhibitor),[2] or DMSO (vehicle) were added to the cells at the specified concentrations. THP-1 cells were incubated for one hour with the compounds and then stimulated with the NLRP3 activator, nigericin (10 μM, Sigma-Aldrich) for two hours to induce cell death. Supernatants were removed and analysed using ELISA kits according to the manufacturer's instructions (DuoSet® R&D Systems or ReadySetGo!® eBioscience). Data are expressed as the mean ± standard deviation of two independent experiments carried out in duplicate. The dose-response curves were fitted using Prism 9 (GraphPad Software). Maximal and minimal responses

were calibrated to DMSO (vehicle) and unstimulated wells, respectively. Data was analysed by a one-way ANOVA followed by a Dunnett multiple comparisons test.

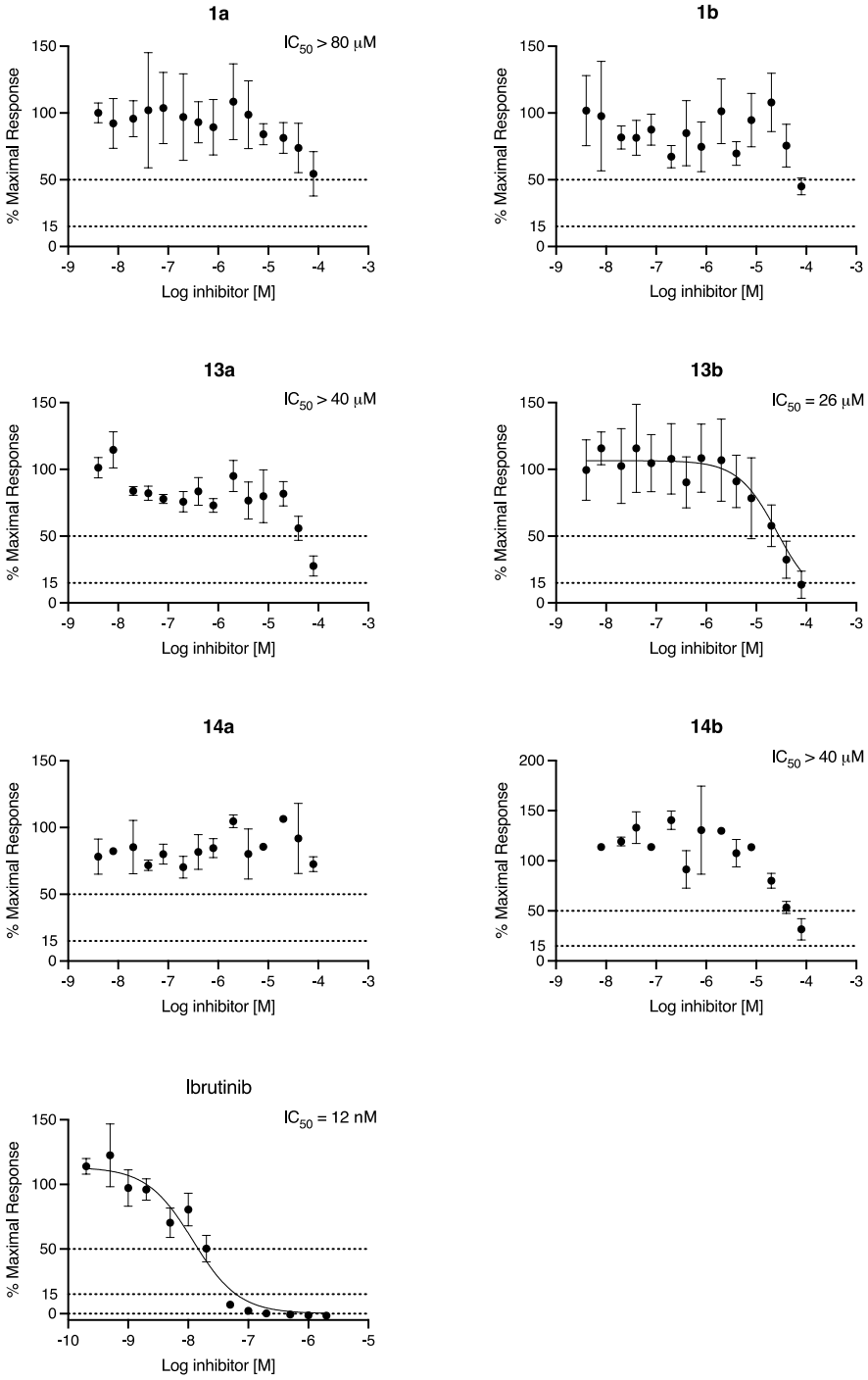
SCIFINDER^n STRUCTURE SEARCH

Compound searches were conducted by substructure search in Scifinder^n (Chemical Abstracts Services – accessed November 2023) and refined by number of components: one.

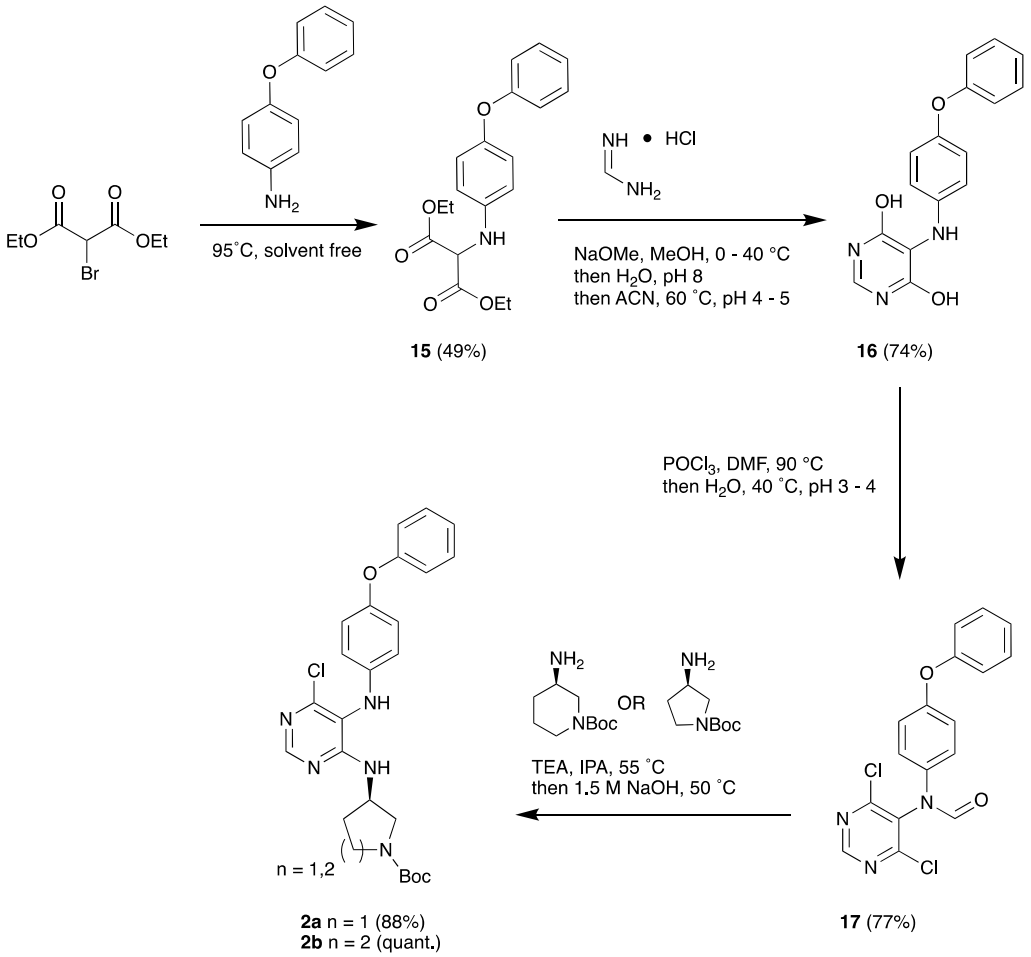
Table S1: Scifinder^n Structure Search

Search input				
Search results	224,272 substructures	935 substructures	193 substructures	0 substructures

ADP Glo™ BTK Kinase Assay



SYNTHESIS OF 2a AND 2b



Diethyl 2-((4-phenoxyphenyl)amino)malonate (**15**)[3]

4-phenoxyaniline (23.2 g, 126 mmol) and diethyl-2-bromomalonate (7.13 mL, 41.8 mmol) were stirred at room temperature under argon. After 30 mins, the temperature was increased to 95 °C for a further two hours. The tar-like solution was allowed to solidify overnight at room temperature. The black solid was triturated with Et_2O (3 x 30 mL) and the ethereal extract was washed with water (60 mL), 1 M HCl (60 mL), water (60 mL), and brine (60 mL). The organic layer was dried with anhydrous magnesium sulfate and concentrated *in vacuo*, forming brown crystals. The product was recrystallised with hexane, yielding long (2 cm) hair-like, white crystals of **15** (7.09 g, 49%), m.p. 83.0 – 84.9 ˚C. ^{1}H NMR ($CDCl_3$, 600 MHz) δ: 7.28 (m, 2H), 7.02 (tt, *J* = 7.4, 1.1 Hz, 1H), 6.91 (m, 4H), 6.66 (dd, *J* = 6.7, 2.3 Hz, 2H), 4.76 (d, *J* = 7.7 Hz, 1H), 4.72, (d, *J* = 7.8 Hz, 1H), 4.29 (m, 4H), 1.29 (t, *J* = 7.2 Hz, 6H). ^{13}C NMR ($CDCl_3$, 151 MHz) δ: 167.9, 158.8, 149.2, 142.0, 129.7, 122.4, 121.2, 117.6, 114.9, 62.6, 61.5, 14.2. HRMS (ESI-TOF) *m/z*: calcd for $C_{19}H_{22}NO_5$ $[M+H]^+$ 344.1492; found 344.1490.

5-((4-Phenoxyphenyl)amino)pyrimidine-4,6-diol (**16**)[4]

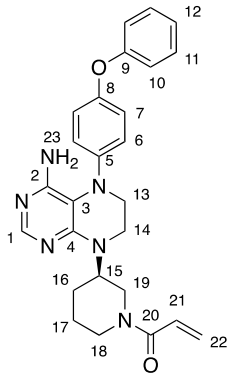
Metallic sodium (1.56 g, 67.8 mmol) was cautiously added to anhydrous MeOH (13.1 mL) under argon. The sodium methoxide solution was then added dropwise to a solution of 15 (6.48 g, 18.9 mmol) and formamidine hydrochloride (1.64 g, 20.3 mmol) in anhydrous MeOH (20 mL) at 0 ˚C under argon. After addition, the reaction was heated to 40 °C for two hours. The reaction was quenched with water (10 mL) and the pH was adjusted to 8 with 10% HCl (aq.). MeCN (9 mL) was added and the solution was heated to 60 °C. The pH was adjusted further to 4 – 5 with 10% HCl (aq.) and the reaction mixture was left to mature for one hour. The precipitate was collected by filtration and washed with chilled 90% MeOH (aq.), water, then again with 90% MeOH (aq.) to give **16** as a tan powder (4.11 g, 74%). ^{1}H NMR (DMSO-d_6, 600 MHz) δ: 11.86 (s, 2H), 7.92 (s, 1H), 7.30 (m, 2H), 7.00 (tt, *J* = 7.3, 1.1 Hz, 1H), 6.87 (dd, *J* = 8.6, 1.1 Hz, 2H), 6.81 (dd, *J* = 6.7, 2.2 Hz, 2H), 6.67 (s, 1H), 6.56 (dd, *J* = 6.7, 2.2 Hz, 2H). ^{13}C NMR (DMSO-d_6, 151 MHz) δ: 161.6, 158.9, 146.3, 144.7, 143.0, 129.7, 121.8, 120.3, 116.5, 114.9, 106.9. HRMS (ESI-TOF) *m/z*: calcd for $C_{12}H_{10}NO_3$ $[M+H]^+$ 296.1030; found 296.1025.

N-(4,6-dichloropyrimidin-5-yl)-*N*-(4-phenoxyphenyl)formamide (**17**)[4]
To an oven-dried flask was added phosphorus oxychloride (10.6 mL, 114 mmol) followed by anhydrous toluene (0.70 mL) under argon. The solution was cooled in an ice bath and anhydrous DMF (5.33 mL. 68.9 mmol) was added slowly maintaining temperature below 50 ˚C. Upon complete addition, the ice bath was removed, and 16 (6.67 g, 22.6 mmol) was added portionwise under argon. The reaction was heated to 90 ˚C, during which the suspension became fully dissolved and turned a dark red. After three hours, the reaction mixture was cooled to RT and quenched by careful addition of the reaction mixture to a vessel containing water (40 mL) with stirring. A solution of 35% potassium carbonate (aq.) was added simultaneously to the quenching solution, while maintaining the temperature below 40 ˚C and the pH at ~1. After complete addition, the pH was adjusted to 2 with further 35% potassium carbonate (aq.) solution. The slurry was let mature at RT for two hours. The precipitate was collected and washed successively with cold water and isopropyl alcohol to give **17** (6.26 g, 77%). ^{1}H NMR (DMSO-d_6, 600 MHz) δ: 9.07 (s, 1H), 8.91 (s, 1H), 7.43 (t, *J* = 7.5 Hz, 2H), 7.39 (d, *J* = 8.5 Hz, 2H), 7.19 (m, 1H, H12), 7.09 (d, *J* = 8.5 Hz, 2H), 7.05 (d, *J* = 7.5 Hz, 2H). 13C NMR (151 MHz, DMSO-d_6) δ 161.8, 160.9, 157.9, 156.3, 155.4, 133.3, 130.2, 130.0, 123.9, 123.7, 119.6, 118.8.

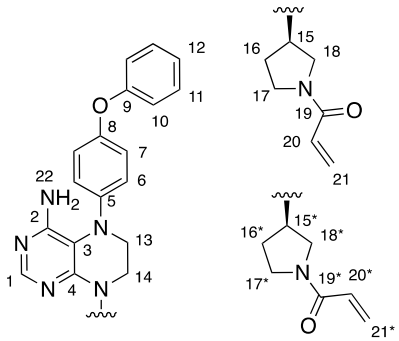
tert-Butyl (*R*)-3-((6-chloro-5-((4-phenoxyphenyl)amino)pyrimidin-4-yl)amino)piperidine-1-carboxylate (**2b**)[4]
To a suspension of **17** (4.26 g, 11.8 mmol) in isopropyl alcohol (12 mL) was added anhydrous TEA (2.50 mL, 17.7 mmol) under nitrogen. The flask was heated to 55 ˚C, and (*R*)-1-Boc-3-aminopiperidine (3.55 g, 17.7 mmol) in isopropyl alcohol (12 mL) was added slowly. After overnight stirring at 55 ˚C under nitrogen, the reaction temperature was lowered to 50 ˚C and 1.5 M NaOH (aq., 17 mL) was added and stirred for a further two hours, during which the beige suspension became clarified. The reaction was then cooled to RT and acidified to pH 2 with 1 M HCl (aq.). The aqueous layer was extracted with toluene (3 x 17 mL). The organic layers were combined, washed with an aqueous solution of 20% NaCl : 8% $NaHCO_3$ (2:1, 12 mL), dried ($MgSO_4$), and concentrated *in vacuo* to a yellow foam. The crude foam was purified using column chromatography on silica gel eluting with EtOAc/pet. spirit (5 – 30% gradient) to afford a white foam (5.90 g, quant.). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.30 (s, 1H), 7.31 (t, *J* = 7.7 Hz, 2H), 7.06 (t, *J* = 7.7 Hz, 1H), 6.99 – 6.91 (m, 4H), 6.62 (d, *J* = 8.8 Hz, 2H), 5.39 (br s, 1H), 5.18 (br s, 1H), 4.20 – 4.08 (m, 1H), 3.75 – 3.59 (m, 1H), 3.45 – 3.34 (m, 1H), 3.34 – 3.16 (m, 2H), 1.92 – 1.83 (m, 1H), 1.62 – 1.55 (m, 1H), 1.55 – 1.47 (m, 2H), 1.39 (s, 9H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 159.0, 158.2, 154.6, 155.1, 153.9, 151.3, 138.7, 129.8, 122.9, 121.0, 118.0, 117.6, 116.8, 80.0, 48.3, 46.9, 43.7, 29.8, 27.7, 22.5. HRMS (ESI) m/z $[M+H]^+$ calcd for $[C_{26}H_{31}ClN_5O_3]^+$ 496.21099; found 496.2084.

tert-Butyl (*R*)-3-((6-chloro-5-((4-phenoxyphenyl)amino)pyrimidin-4-yl)amino)pyrrolidine-1-carboxylate (**2a**)[4]
2a was prepared as described for **2b** using **17** (1.95 g, 5.41 mmol) and (*R*)-1-Boc-3-aminopyrrolidine (1.51 g, 8.12 mmol). The crude foam was purified using column chromatography on silica gel eluting with EtOAc/pet. spirit (0 – 40% gradient) to afford a white foam (2.30 g, 88%). ^{1}H NMR (600 MHz, $MeOD_4$) δ 8.22 (s, 1H), 7.31 – 7.25 (m, 2H), 7.01 (tt, *J* = 7.4, 1.2 Hz, 1H), 6.90 (d, *J* = 8.1 Hz, 2H), 6.88 – 6.84 (m, 2H), 6.61 – 6.56 (m, 2H), 4.69 – 4.62 (m, 1H), 3.70 – 3.60 (m, 1H), 3.41 – 3.33 (m, 2H), 3.29 – 3.20 (m, 1H), 2.23 – 2.15 (m, 1H), 1.99 – 1.91 (m, 1H), 1.43 (s, 9H). ^{13}C NMR (151 MHz, $MeOD_4$) δ 162.0, 161.9, 160.1, 156.4, 155.7, 155.5, 155.4, 155.3, 151.0, 150.9, 141.8, 141.7, 130.7, 123.4, 121.83, 121.77, 119.4, 118.5, 118.4, 116.5, 81.1, 52.34, 52.25, 51.9, 51.7, 45.4, 44.9, 32.0, 31.3, 28.7. HRMS (ESI) m/z $[M+H]^+$ calcd for $[C_{25}H_{29}ClN_5O_3]^+$ 482.1953; found 482.1967.

NMR ASSIGNMENT OF 7b, 10b, 1a, 1b, 13a, 13b, 14a, 14b.



1b: ^{1}H NMR (600 MHz, $MeOD_4$) δ 8.18 (s, 0.6H, H1), 8.15 (s, 0.4H, H1*), 7.40 – 7.23 (m, 2H, H11), 7.08 (t, $J_{12,11}$ = 7.3 Hz, 1H, H12), 7.03 – 6.87 (m, 6H, H6+H7+H10), 6.84 – 6.73 (m, 1H, H21), 6.22 (d, $J_{22trans,21}$ = 16.6 Hz, 1H, H22trans), 5.79 – 5.72 (m, 1H, H22cis), 4.84 (under H_2O peak, C15+C15*), 4.60 – 4.52 (m, 1H, H19a+H18a*), 4.15 – 4.08 (m, 1H, H18a+H19a*), 3.75 – 3.57 (m, 2H, H13a+H13b), 3.53 – 3.43 (m, 2H, H14a+H14b), 3.30 – 3.20 (m, 0.6H, H19b*), 3.08 (dd, J_{gem} = 13.2, $J_{18b,17}$ = 13.2 Hz, 0.6H, H18b), 2.93 (dd, J_{gem} = 11.7, $J_{19b,15}$ = 11.7 Hz, 0.6H, H19b), 2.66 (dd, J_{gem} = 13.0, $J_{18b*,17*}$ = 13.0 Hz, 0.4H, H18b*), 2.08 – 1.86 (m, 3H, H17a+H17a*+H16), 1.70 – 1.54 (m, 1H, H17b+H17b*). ^{13}C NMR (151 MHz, $MeOD_4$) δ 167.8 (C20), 159.1 (C9), 155.1 (C4), 154.3 (C8), 149.7 (C2), 145.5 (C1*), 145.4 (C1), 143.1 (C5), 130.8 (C11), 129.0 (C21), 128.7 (C22), 124.2 (C12), 123.02 (C7 or C7*), 122.97 (C7 or C7*), 121.2 (C6), 119.4 (C10), 103.5 (C3), 54.2 (C15*), 53.2 (C15), 49.0 (under $MeOD_4$, C13+C19*), 47.0 (C18), 45.3 (C19), 43.3 (C18*), 40.8 (C14*), 40.7 (C14), 28.3 (C16+C16*), 26.4 (C17), 25.5 (C17*).

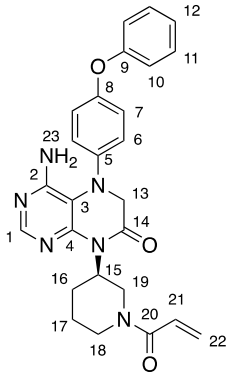


1a: ^{1}H NMR (600 MHz, $MeOD_4$) δ 8.18 (s, 0.5H, H1 or H1*), 8.18 (s, 0.5H, H1 or H1*), 7.38 – 7.26 (m, 2H, H11), 7.08 (t, $J_{12,11}$ = 7.4 Hz, 1H, H12), 6.98 – 6.95 (m, 4H, H6+H7), 6.95 – 6.92 (m, 2H, H10), 6.63 (dd, $J_{20,21trans}$ = 16.7, $J_{20,21cis}$ = 10.5 Hz, 0.5H, H20), 6.58 (dd, $J_{20*,21trans*}$ = 16.7, $J_{20*,21cis*}$ = 10.5 Hz, 0.5H, H20*), 6.29 (dd, $J_{21trans,20}$ = 16.7, J_{gem} = 1.7 Hz, 0.8H[a], H21trans), 6.28 (dd, $J_{21trans*,20*}$ = 16.7, J_{gem} = 1.7 Hz, 0.7H[a], H21trans*), 5.77 (dd, $J_{21cis,20}$ = 10.5, J_{gem} = 1.7 Hz, 0.5H, H21cis), 5.74 (dd, $J_{21cis*,20*}$ = 10.5, J_{gem} = 1.7 Hz, 0.5H, H21cis*), 5.71 – 5.65 (m, 0.5H, H15*), 5.65 – 5.58 (m, 0.5H, H15), 3.93 (dd, J_{gem} = 10.7, $J_{18a*,15*}$ = 7.9 Hz, 0.5H. H18a*), 3.91 – 3.86 (m, 0.5H, H17a), 3.85 – 3.77 (m, 1H, H17a*+H18a), 3.73 (dt br, 1H, H13a), 3.70 – 3.66 (m, 0.5H, H17b), 3.66 – 3.59 (m, 2H, H13b+H18b*), 3.54 – 3.47 (m, 1H, H17b*+H18b), 3.47 – 3.39 (m, 2H, H14), 2.36 – 2.24 (m, 1H, H16a+H16b), 2.24 – 2.15 (m, 1H, H16a*+H16b*). ^{13}C NMR (151 MHz, $MeOD_4$) δ 166.9 (C19 or C19*), 166.8 (C19 or C19*), 159.1 (C9), 155.3 (C4), 154.3 (C8), 149.9 (C2 or C2*), 149.8 (C2 or C2*), 145.5 (C1 or C1*), 145.4 (C1 or C1*) 143.07 (C5 or C5*), 143.05 (C5 or C5*), 130.8 (C11), 129.7 (C20*), 129.2 (C20), 128.72 (C21 or C21*), 128.68 (C21 or C21*), 124.2 (C12), 123.01 (C7 or C7*), 122.99 (C7 or C7*), 121.2 (C6), 119.4 (C10), 103.8 (C3), 55.8 (C15*), 54.4 (C15), 49.0 (under $MeOD_4$, C13) 48.5 (C18*), 47.9 (C18), 46.4 (C17), 45.4 (C17*), 40.7 (C14 or C14*), 40.6 (C14 or C14*), 29.3 (C16), 27.4 (C16*).

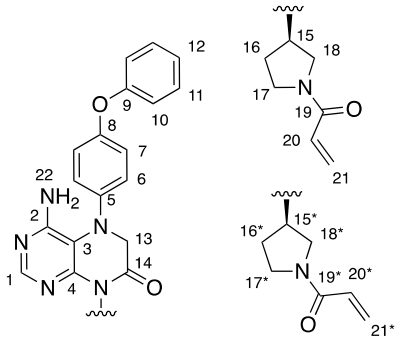
[a]Integration of these signals is inaccurate due to overlap of multiplets.

The NMR spectra of **1a** and **1b** showed that the compounds existed in two rotameric forms (0.6 : 0.4 ratio), arising from restricted rotation about the acrylamide bond. This was illustrated by duplicate signals in both the ^{13}C and ^{1}H NMR spectra, especially for the signals associated with the piperidine and acrylamide moieties. A similar observation was made for acrylamide-containing compounds **13a**, **13b**, **14a**, and **14b**. Despite using variable temperature NMR (35 ˚C and 50 ˚C) and changing the NMR solvent to DMSO-d_6, the signals corresponding to the rotamers did not coalesce. Therefore, each rotameric species were assigned* using a

combination of COSY, ROESY, HSQC, and HMBC NMR spectroscopy (refer to supplementary section: COPIES OF 1H, 13C, AND 2D NMR SPECTRA).

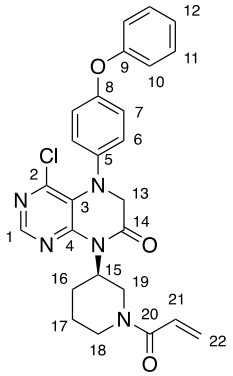


13b: ^{1}H NMR (600 MHz, $MeOD_4$) δ 8.22 (s, 0.4H, H1), 8.21 (s, 0.6H, H1*), 7.33 (t, J = 7.8 Hz, 2H, H11), 7.11 – 7.06 (m, 1H, H12), 6.99 – 6.94 (m, 4H, H7+H10), 6.91 (d, $J_{6,7}$ = 8.8 Hz, 2H, H6), 6.80 (dd, $J_{21*,22trans*}$ = 16.7, $J_{21*,22cis*}$ = 10.7 Hz, 0.6H, H21*), 6.76 (dd, $J_{21,22trans}$ = 16.7, $J_{21,22cis}$ = 10.7 Hz, 0.4H, H21), 6.21 (d, $J_{22trans}$ = 16.7 Hz, 1H, H22trans), 5.79 – 5.74 (m, 0.6H, H22cis*), 5.74 – 5.71 (m, 0.4H, H22cis), 4.86 – 4.75 (m, 1H, H15+H15*), 4.63 – 4.58 (m, 0.6H, H18a*), 4.52 – 4.45 (m, 0.5H, H19a), 4.36 (d, J_{gem} = 16.5 Hz, 1H, H13a), 4.33 – 4.26 (m, 1H, H13b), 4.19 (dd, $J_{19a*,15*}$ = 12.2 Hz, J_{gem} = 12.2 Hz, 0.5H, H19a*), 4.14 (br d, 0.4H, H18a), 4.04 (br d, 0.4H, H19b*), 3.80 (dd, $J_{19b,15}$ = 11.8 Hz, J_{gem} = 11.8 Hz, 0.5H, H19b), 3.16 – 3.08 (m, 0.5H, H18b), 2.79 – 2.64 (m, 1.5H, H16a+H16a*+H18b*), 1.94 – 1.86 (m, 1H, H17a+H17a*), 1.84 (br d, 0.4H, H16b), 1.77 (br d, 0.5H, H16b*), 1.69 – 1.51 (m, 1H, H17b+H17b*). ^{13}C NMR (151 MHz, $MeOD_4$) δ 170.1 (C14*), 169.7 (C14), 167.59 (C20*), 167.57 (C20), 159.8 (C2), 159.2 (C9), 155.1 (C1), 154.1 (C8), 153.6 (C4), 142.0 (C5), 130.8 (C11), 129.14 (C21*), 129.07 (C21), 128.54 (C22), 128.46 (C22*), 124.1 (C12), 121.3 (C6 or C7), 121.2 (C6 or C7), 119.4 (C10), 109.6 (C3), 57.0 (C13), 56.9 (C13*), 53.2 (C15*), 52.3 (C15), 49.0 (under $MeOD_4$, C19*), 47.3 (C18), 45.4 (C19), 43.7 (C18*), 28.2 (C16), 28.1 (C16*), 27.3 (C17), 26.2 (C17*).

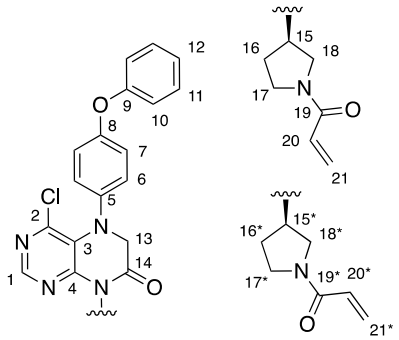


13a: ^{1}H NMR (600 MHz, MeOD4) δ 8.19 (s, 0.5H, H1 or H1*), 8.18 (s, 0.5H, H1 or H1*), 7.33 – 7.28 (m, 2H, H11), 7.06 (t, $J_{12,11}$ = 7.4 Hz, 1H, H12), 6.93 (br d, 4H, H7+H10), 6.90 – 6.86 (m, 2H, H6), 6.64 (dd, $J_{20,21trans}$ = 16.8, $J_{20,21cis}$ = 10.5 Hz, 0.5H, H20), 6.53 (dd, $J_{20*,21trans*}$ = 16.8, $J_{20*,21cis*}$ = 10.5 Hz, 0.5H, H20*), 6.27 (dd, $J_{21trans,20}$ = 16.8, J_{gem} = 1.9 Hz, 0.6H[a], H21trans), 6.26 (dd, $J_{21trans*,20*}$ = 16.8, J_{gem} = 1.9 Hz, 0.6H[a], H21trans*), 5.78 – 5.62 (m, 2H, H21cis+H21cis*+H15+H15*), 4.36 – 4.28 (m, 2H, H13), 4.12 (dd, J = 10.2, 7.8 Hz, 0.5H, H18a*), 4.01 (td, J_{gem} = 9.6, $J_{17a,16a}$ = 9.6, $J_{17a,16b}$ = 4.3 Hz, 0.5H, H17a), 3.95 – 3.85 (m, 1.6H, H17a*+H18a+H18b*), 3.79 (dd, J_{gem} = 12.4, $J_{18b,15}$ = 9.2 Hz, 0.5H, H18b), 3.72 – 3.64 (m, 0.5H, H17b), 3.50 (dt, J_{gem} = 12.3, $J_{17b*,16a*}$ = 8.3, $J_{17b*,16b*}$ = 8.3 Hz, 0.5H, H17b*), 2.70 (dq, J_{gem} = 12.6, $J_{16a,15}$ = 8.4, $J_{16a,17a}$ = 8.4, $J_{16a,17b}$ = 8.4 Hz, 0.5H, H16a), 2.63 (dq, J_{gem} = 12.4, $J_{16a*,15}$ = 9.0, $J_{16a*,17a*}$ = 9.0, $J_{16a*,17b*}$ = 9.0 Hz, 0.5H, H16a*), 2.27 (dtd, J_{gem} = 12.6, $J_{16b,15}$ = 8.3, $J_{16b,17b}$ = 8.3, $J_{16b,17a}$ = 4.2 Hz, 0.5H, H16b), 2.15 (dtd, J_{gem} = 12.4, $J_{16b*,15}$ = 8.1, $J_{16b*,17a*}$ = 3.6 Hz, 0.5H, H16b*). ^{13}C NMR (151 MHz, MeOD) δ 170.0 (C14), 169.9 (C14*), 166.63 (C19), 166.59 (C19*), 159.8 (C2), 159.2 (C9), 155.1 (C1), 154.08 (C8), 154.06 (C8*), 153.54 (C4), 153.47 (C4*), 142.02 (C5), 141.97 (C5*), 130.8 (C11), 129.9 (C20*), 129.5 (C20), 128.33 (C21), 128.31 (C21*), 124.1 (C12), 121.23 (C6+C7), 121.19 (C6*+C7*), 119.4 (C10), 109.7 (C3), 109.6 (C3*), 56.9 (C13), 56.8 (C13*), 53.0 (C15*), 51.7 (C15), 49.0 (under $MeOD_4$, C18*), 49.0 (under $MeOD_4$, C18), 47.3 (C17), 46.1 (C17*), 29.6 (C16), 27.6 (C16*).

[a]Integration of these signals is inaccurate due to overlap of multiplets.



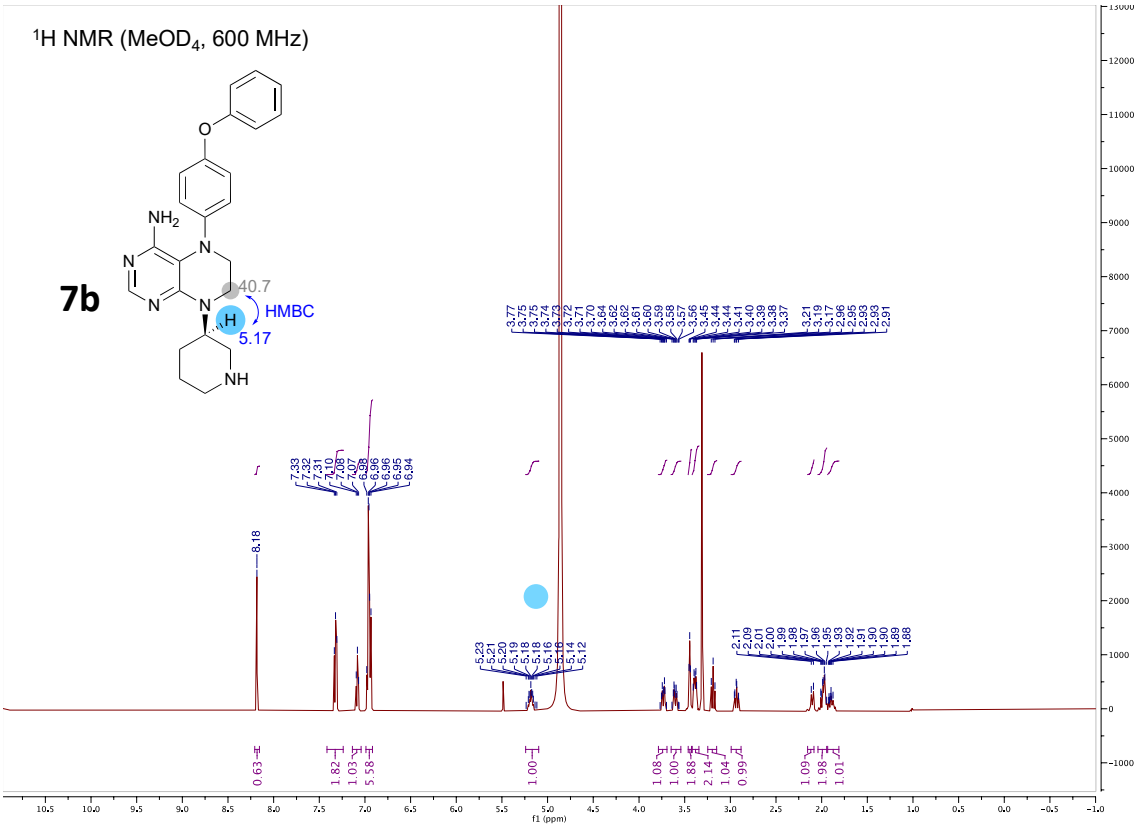
14b: ^{1}H NMR (600 MHz, $MeOD_4$) δ 8.53 (s, 1H, H1), 7.36 – 7.30 (m, 2H, H11), 7.12 – 7.06 (m, 1H, H12), 6.99 – 6.92 (m, 6H, H6+H7+H10), 6.85 – 6.71 (m, 1H, H21+H21*), 6.22 (dd, $J_{22trans}$ = 16.8, J_{gem} = 1.9 Hz, 1H, H22trans), 5.76 (dd, $J_{22cis,21}$ = 10.6, J_{gem} = 1.9 Hz, 0.6H, H22cis), 5.74 – 5.70 (m, 0.4H, H22cis*), 5.01 – 4.92 (m, 1H, H15), 4.62 (br d, 0.5H, H18a*), 4.55 (br d, 0.5H, H19a), 4.48 – 4.38 (m, 2H, H13), 4.23 – 4.4 (m, 0.3H, H19a*), 4.15 – 4.06 (m, 1H, H19b*+H18a), 3.81 (t, J_{gem} = 11.9, $J_{19b,15}$ = 11.9 Hz, 0.6H, H19b), 3.18 – 3.11 (m, 0.6H, H18b), 2.82 – 2.65 (m, 1.4H, H16a+H16a*+H18b*), 1.97 – 1.82 (m, 2H, H16b+H16b*+H17a+H17a*), 1.70 – 1.55 (m, 1H, H17b+H17b*). ^{13}C NMR (151 MHz, MeOD) δ 169.4 (C14), 167.7 (C20), 158.9 (C9), 155.6 (C4), 154.9 (C8), 153.4 (C2), 152.8 (C1), 152.7 (C1*), 142.7 (C5), 130.9 (C11), 129.0 (C21), 128.6 (C22), 125.6 (C3), 124.3 (C12), 123.2 (C6 or C7), 123.1 (C6* or C7*), 120.9 (C6 or C7), 119.6 (C10), 56.5 (C13), 56.4 (C13*), 53.0 (C15), 49.0 (under $MeOD_4$, C19*), 47.4 (C18*), 45.1 (C19), 43.7 (C18), 27.9 (C16 or C16*), 27.8 (C16 or C16*), 27.3 (C17 or C17*), 26.3 (C17 or C17*).

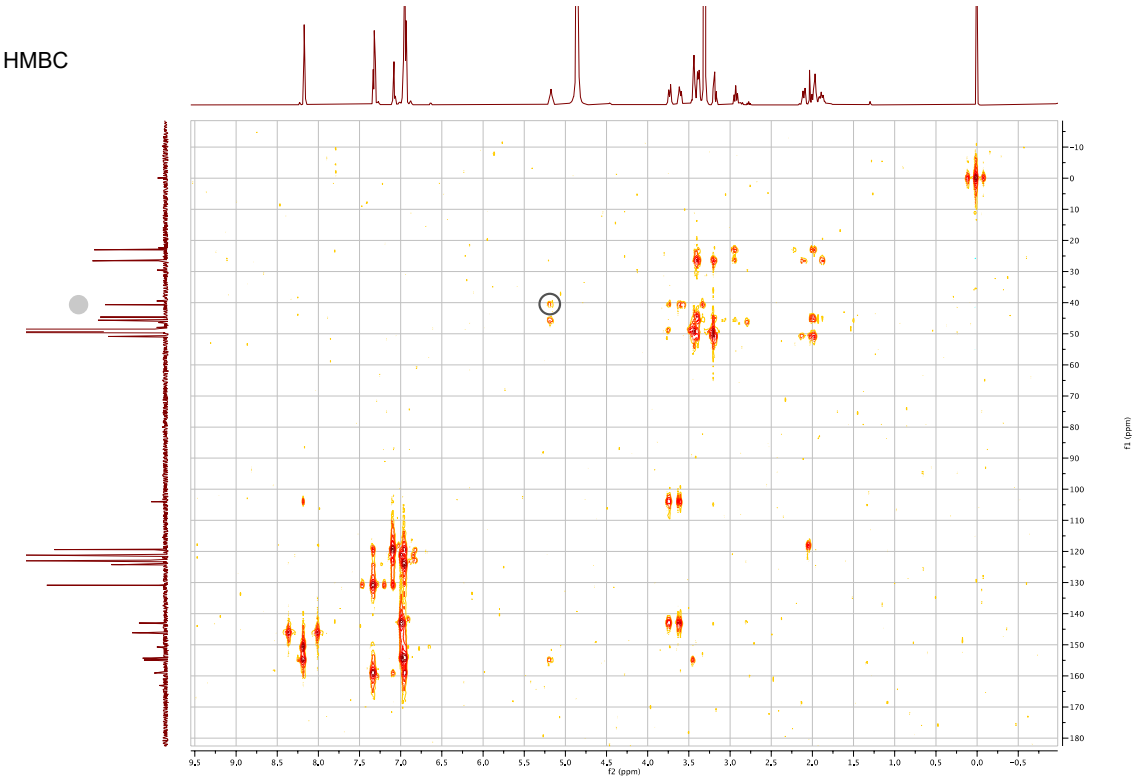


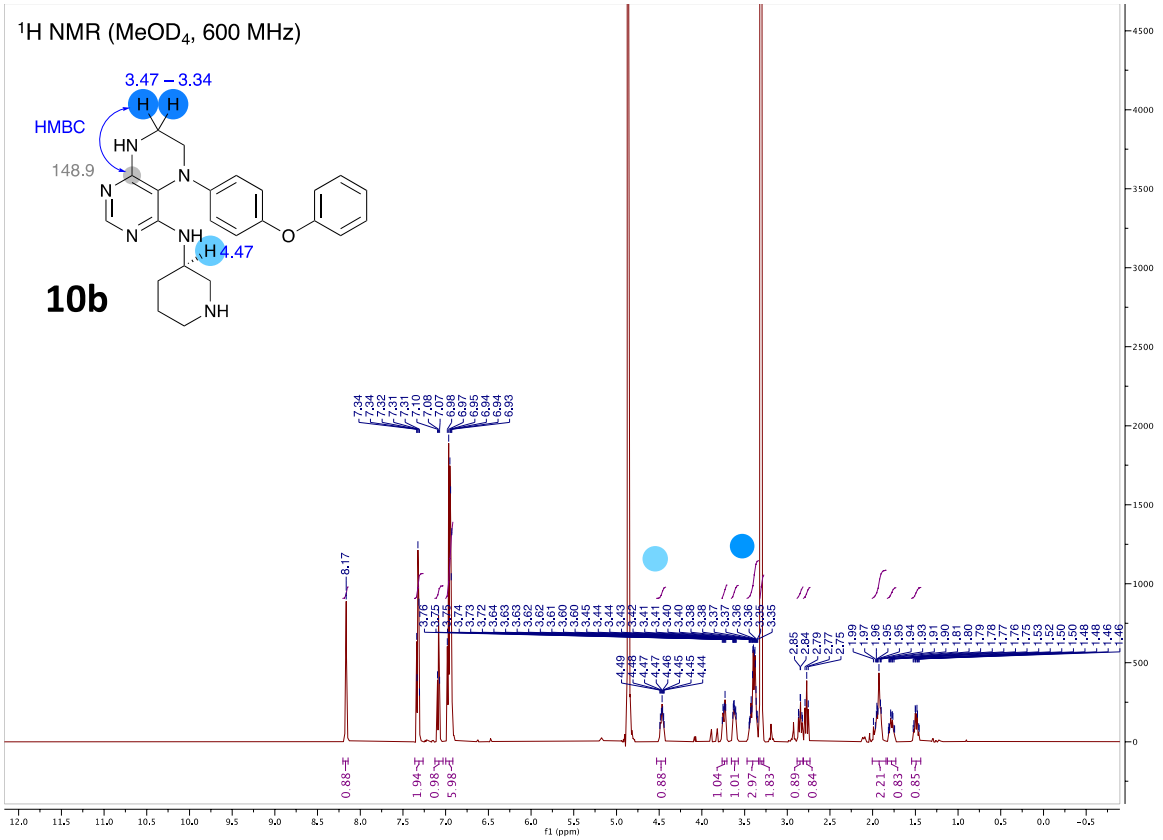
14a: ^{1}H NMR (600 MHz, $MeOD_4$) δ 8.52 (s, 0.5H, H1 or H1*), 8.51 (s, 0.5H, H1 or H1*), 7.39 – 7.29 (m, 2H, H11), 7.09 (t, $J_{12,11}$ = 7.4 Hz, 1H, H12), 6.99 – 6.92 (m, 6H, H10+H6+H7), 6.68 (dd, $J_{20,21trans}$ = 16.8, $J_{20,21cis}$ = 10.4 Hz, 0.5H, H20), 6.56 (dd, $J_{20*,21trans*}$ = 16.8, $J_{20*,21cis*}$ = 10.4 Hz, 0.5H, H20*), 6.29 (dd, $J_{21trans,20}$ = 16.8, J_{gem} = 2.0 Hz, 0.8H[a], H21trans), 6.28 (dd, $J_{21trans*,20*}$ = 16.8, J_{gem} = 2.0 Hz, 0.8H[a], H21trans*), 5.93 – 5.85 (m, 0.5H, H15*), 5.85 – 5.80 (m, 0.5H, H15), 5.77 (dd, $J_{21cis,20}$ = 10.4, J_{gem} = 2.0 Hz, 0.5H, H21cis), 5.73 (dd, $J_{21cis*,20*}$ = 10.4, J_{gem} = 2.0 Hz, 0.5H, H21cis*), 4.45 (ABq, 2H, H13), 4.16 (dd, J = 10.5, 7.5 Hz, 0.5H, H18a*), 4.06 (td, J_{gem} = 9.6, $J_{17a,16a}$ = 9.6, $J_{17a,16b}$ = 4.5 Hz, 0.5H, H17a), 4.02 – 3.93 (m, 1.5H, H17a*+H18a+H18b*), 3.86 (dd, J_{gem} = 12.7, $J_{18b,15}$ = 9.2 Hz, 0.5H, H18b), 3.79 – 3.70 (m, 0.5H, 17b), 3.56 (ddd, J_{gem} = 12.3, $J_{17b*,16a*}$ = 8.7, $J_{17b*,16b*}$ = 8.2 Hz, 0.5H, H17b*), 2.74 (dq, J_{gem} = 12.8, $J_{16a,15}$ = 8.1, $J_{16a,17a}$ = 8.1, $J_{16a,17b}$ = 8.1 Hz, 0.5H, H16a), 2.67 (dq, J_{gem} = 12.4, $J_{16a*,15*}$ = 8.7, $J_{16a*,17a*}$ = 8.7, $J_{16a*,17b*}$ = 8.7 Hz, 0.5H, H16a*), 2.37 (dddd, J_{gem} = 12.8, $J_{16b,15}$ = 8.4, $J_{16b,17b}$ = 8.4, $J_{16b,17a}$ = 4.5 Hz, 0.5H, H16b), 2.26 (dddd, J_{gem} = 12.4, $J_{16b*,15*}$ = 8.7, $J_{16b*,17b*}$ = 8.2, $J_{16b*,17a*}$ = 3.9 Hz, 0.5H, H16b*). ^{13}C NMR (151 MHz, $MeOD_4$) δ 169.61 (C14 or C14*), 169.58 (C14 or C14*), 166.6 (C19 and C19*), 158.9 (C9), 155.5 (C4 or C4*), 155.4 (C4 or C4*), 154.9 (C8), 153.1 (C2), 152.6 (C1), 142.62 (C5 or C5*), 142.60 (C5 or C5*), 130.9 (C11), 129.9 (C20*), 129.5 (C20), 128.4 (C21 and C21*), 125.7 (C3), 124.3 (C12), 123.1 (C6, C6*, C7, or C7*), 123.1 (C6, C6*, C7, or C7*), 120.9 (C6 or C7), 119.6 (C10), 56.4 (C13), 56.3 (C13*), 53.5 (C15*), 52.3 (C15), 49.5 (under $MeOD_4$, C18*), 48.5 (C18), 47.3 (C17), 46.1 (C17*), 29.4 (C16), 27.5 (C16*).

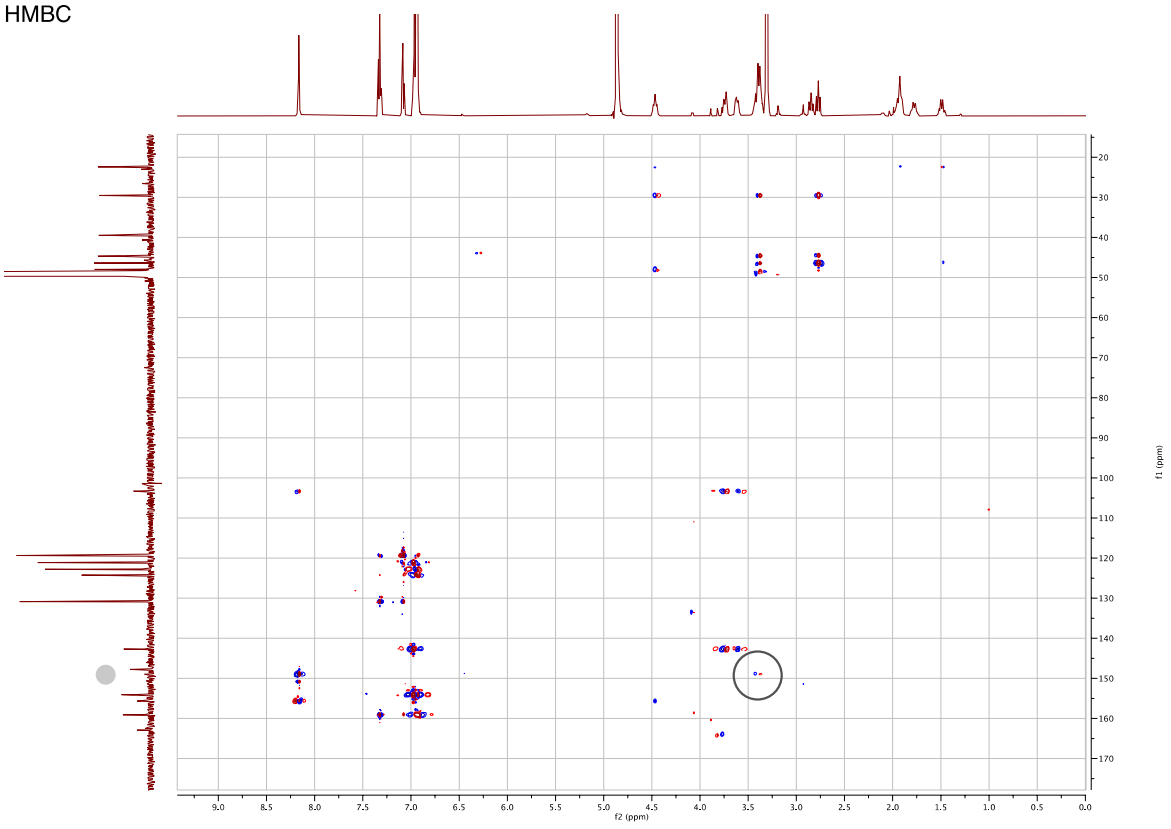
[a]Integration of these signals is inaccurate due to overlap of multiplets.

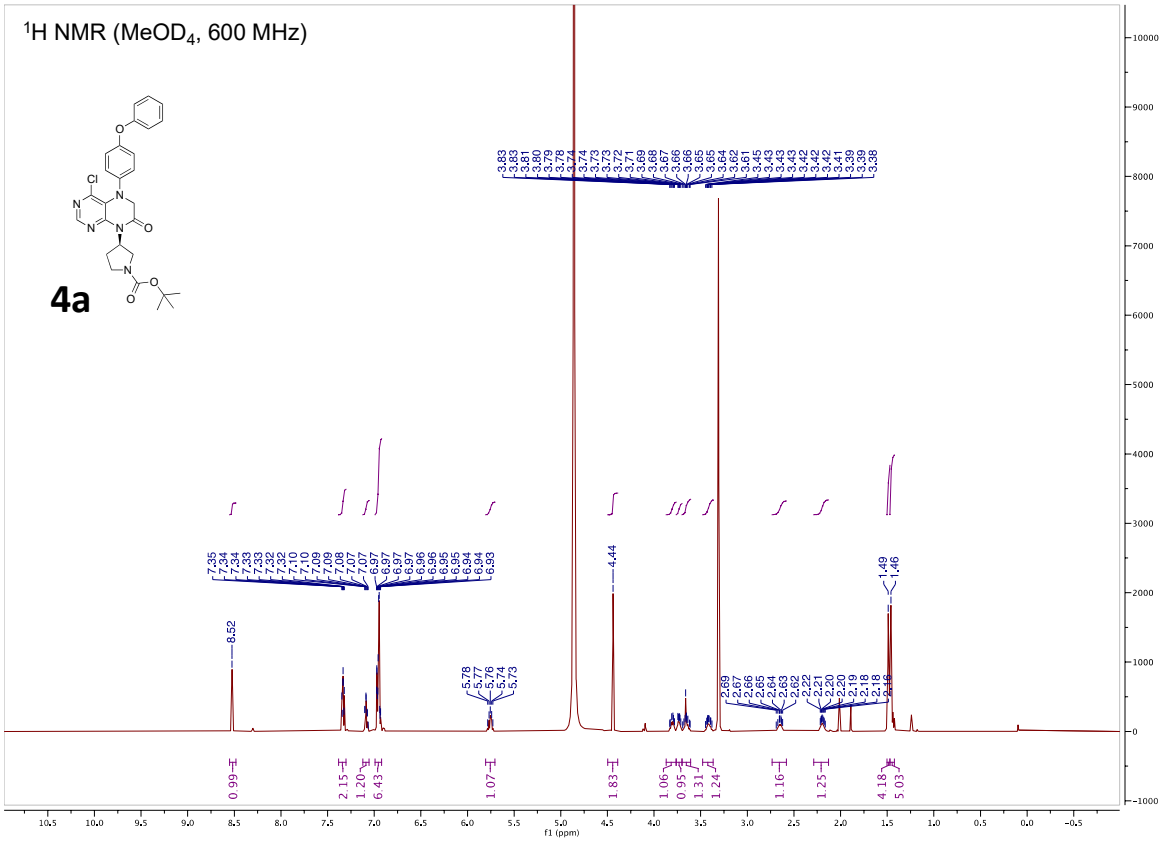
COPIES OF ^{1}H, ^{13}C, AND 2D NMR SPECTRA

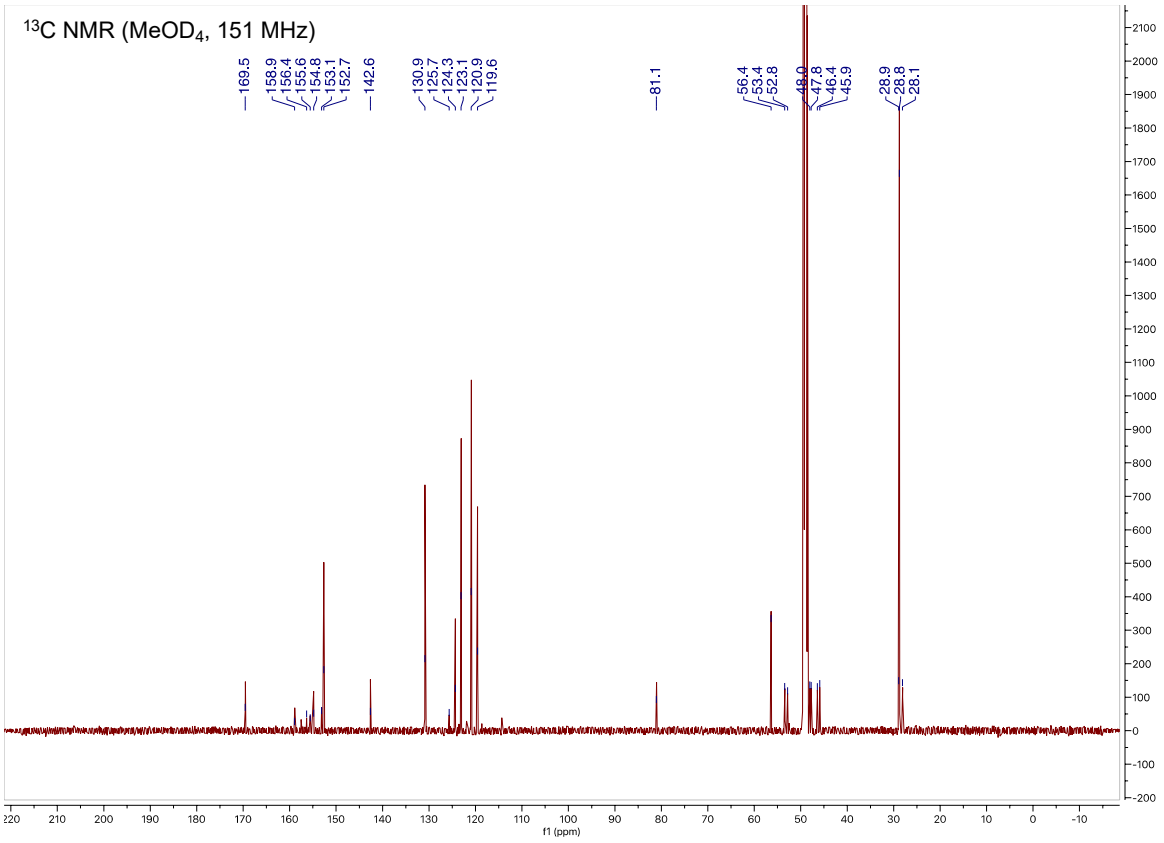


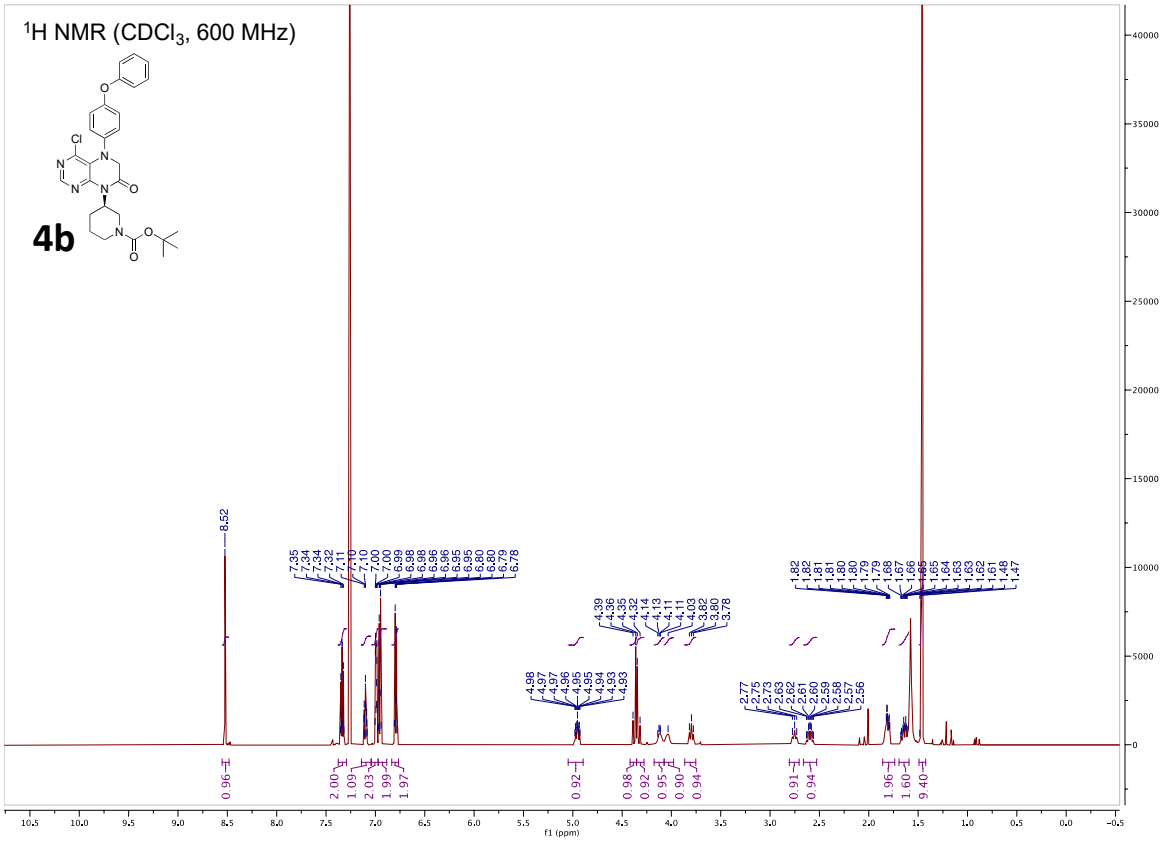


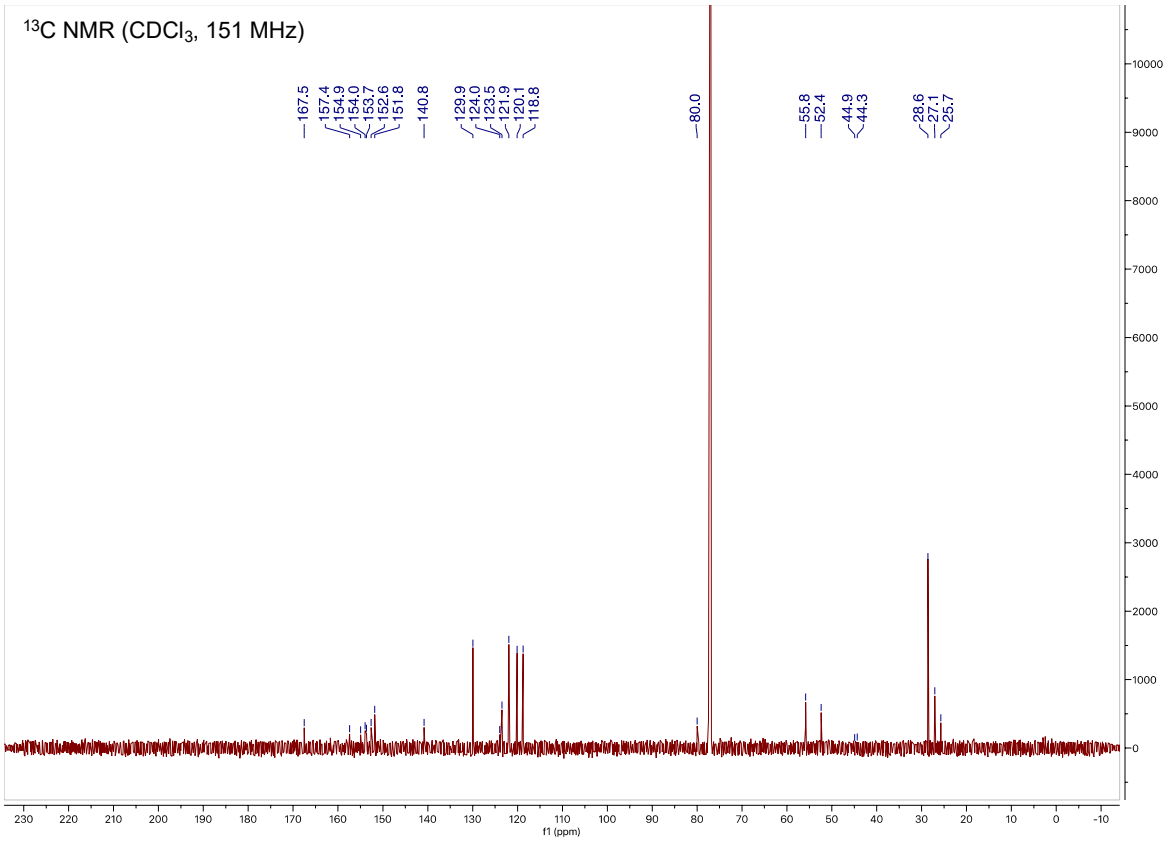

^{1}H NMR (MeOD$_4$, 600 MHz)
3.47 – 3.34
HMBC
148.9
4.47
10b
f1 (ppm)

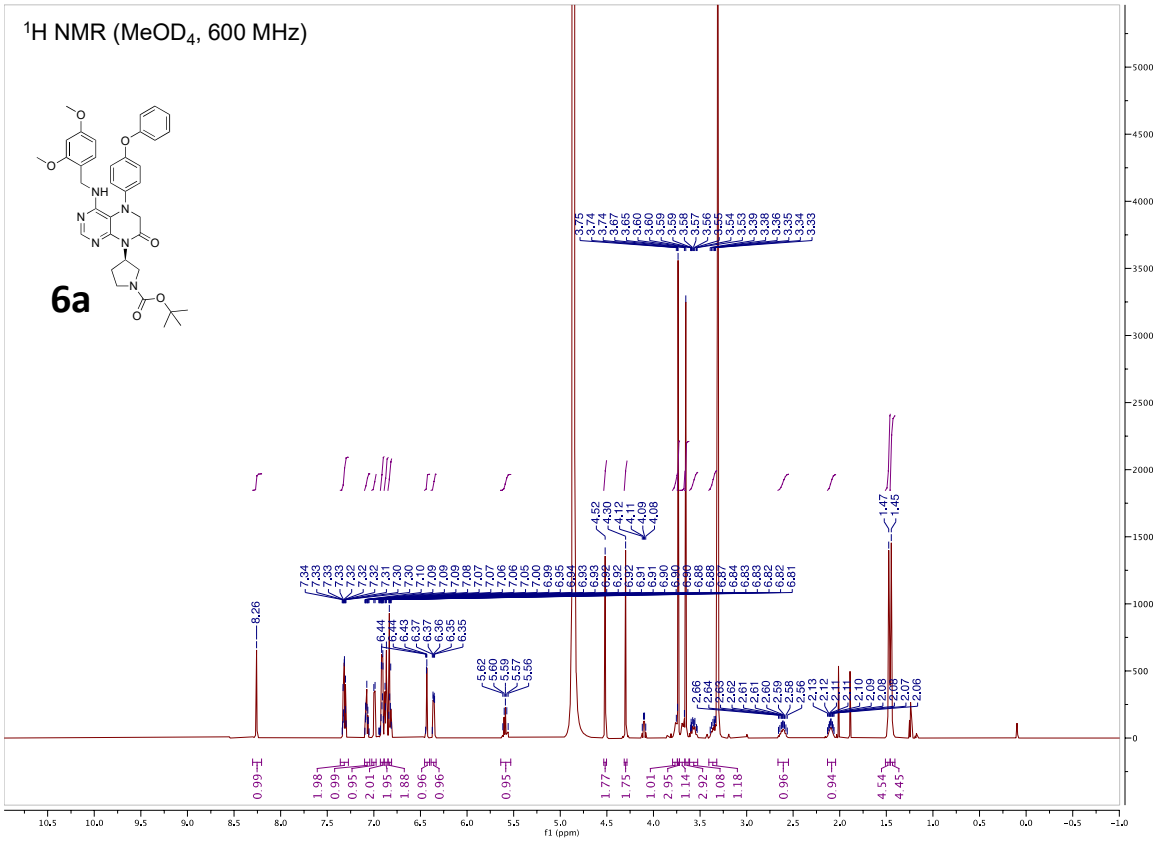

HMBC
f2 (ppm)
f1 (ppm)

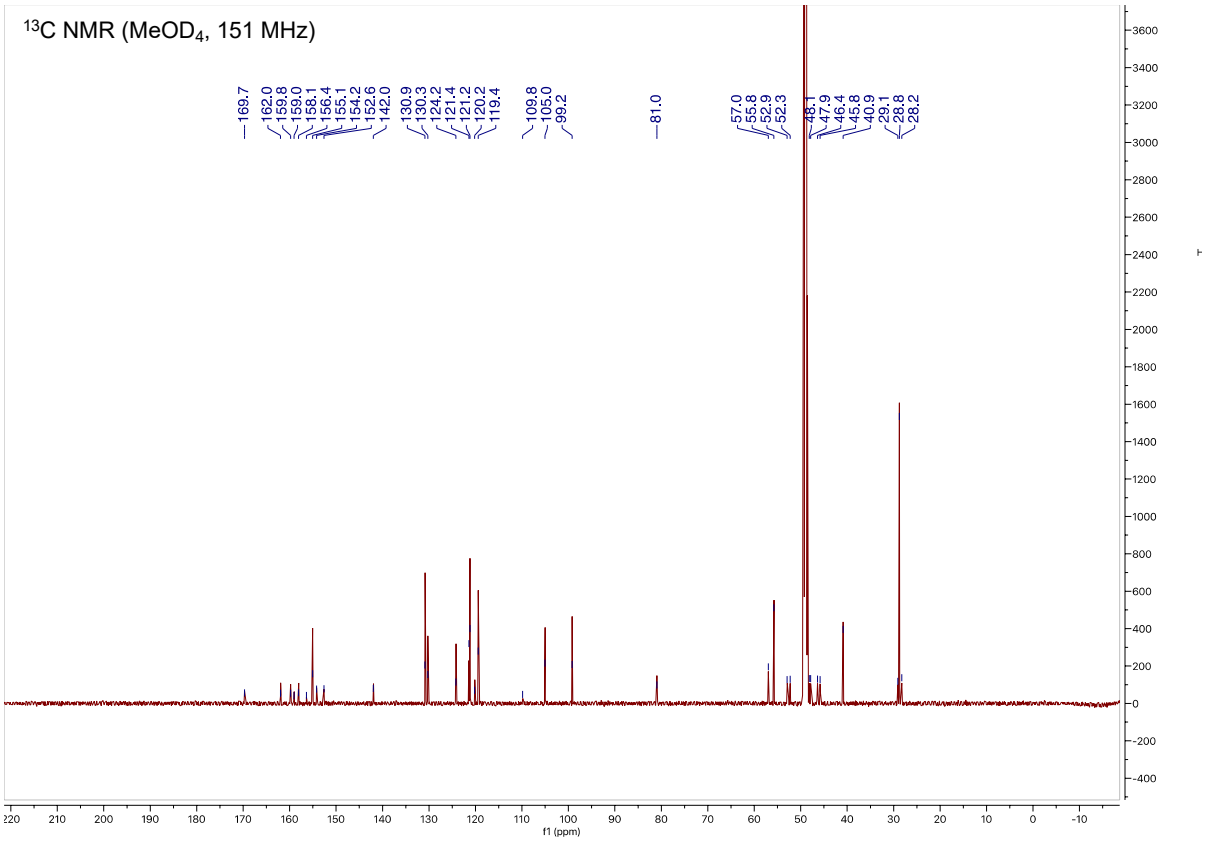

^{1}H NMR (MeOD$_4$, 600 MHz)
4a
f1 (ppm)

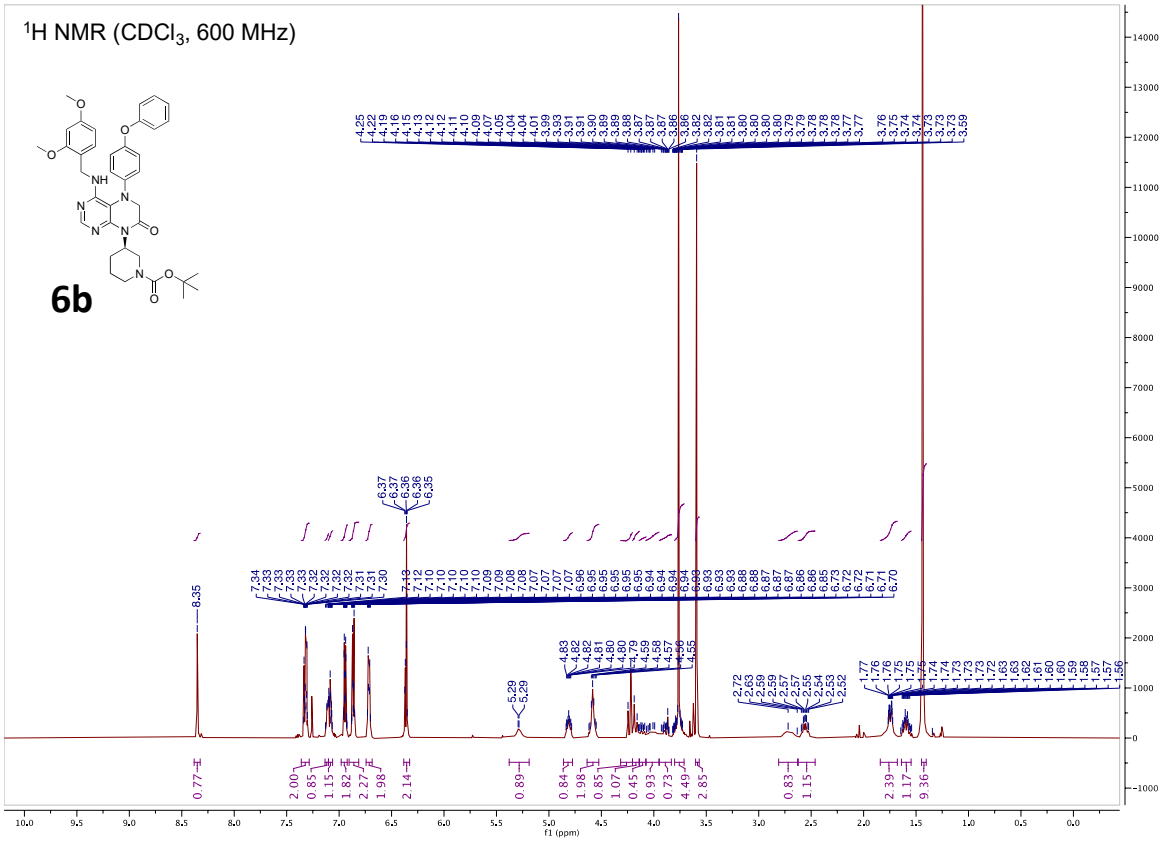

^{13}C NMR (MeOD$_4$, 151 MHz)
f1 (ppm)

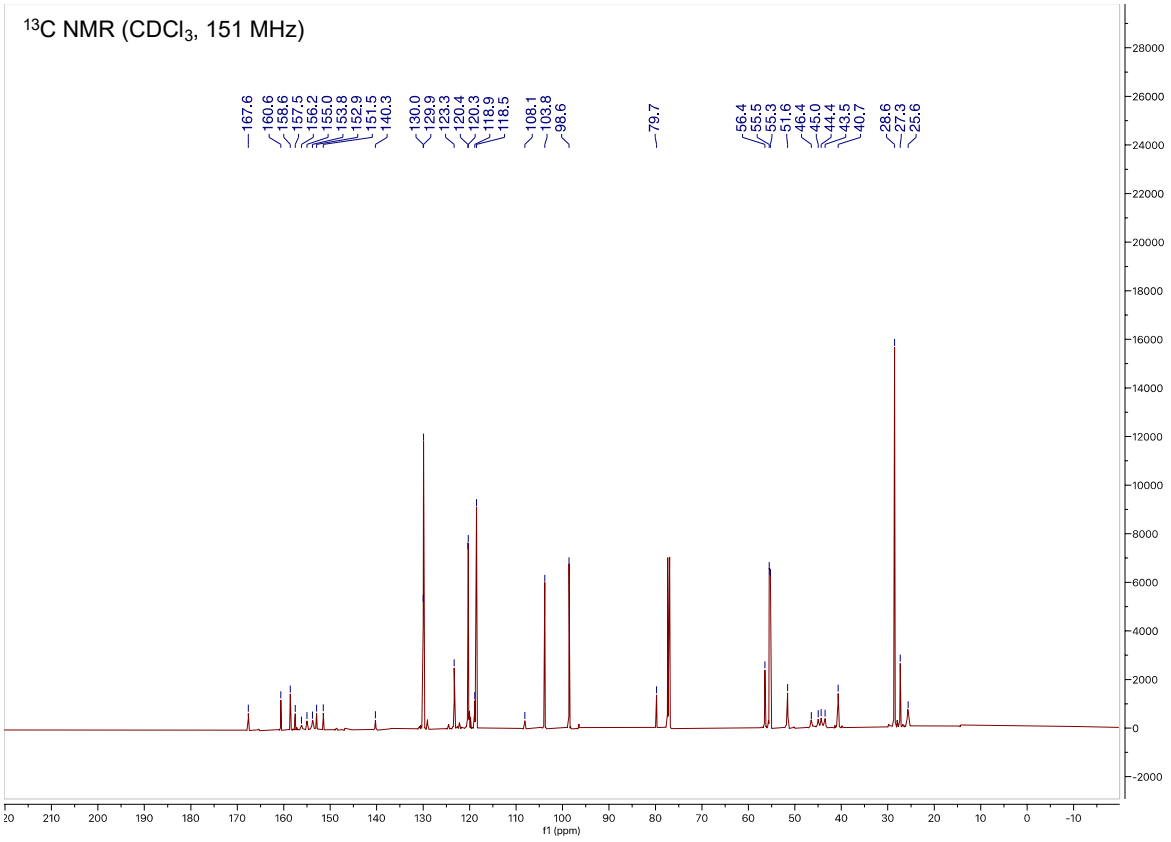

^{1}H NMR ($CDCl_3$, 600 MHz)
4b
8.52
7.35
7.34
7.34
7.32
7.11
7.10
7.10
7.00
7.00
6.99
6.98
6.98
6.96
6.96
6.95
6.80
6.80
6.79
6.78
4.98
4.97
4.97
4.96
4.95
4.95
4.94
4.94
4.93
4.93
4.39
4.38
4.36
4.35
4.32
4.14
4.13
4.11
4.11
4.03
3.82
3.80
3.78
2.77
2.75
2.73
2.63
2.62
2.61
2.60
2.59
2.58
2.57
2.56
1.82
1.82
1.81
1.81
1.80
1.80
1.79
1.79
1.68
1.67
1.66
1.65
1.64
1.63
1.62
1.61
1.48
1.47
0.96
2.00
1.09
2.03
1.99
1.97
0.92
0.98
0.92
0.95
0.90
0.94
0.91
0.94
1.96
1.60
9.40
f1 (ppm)

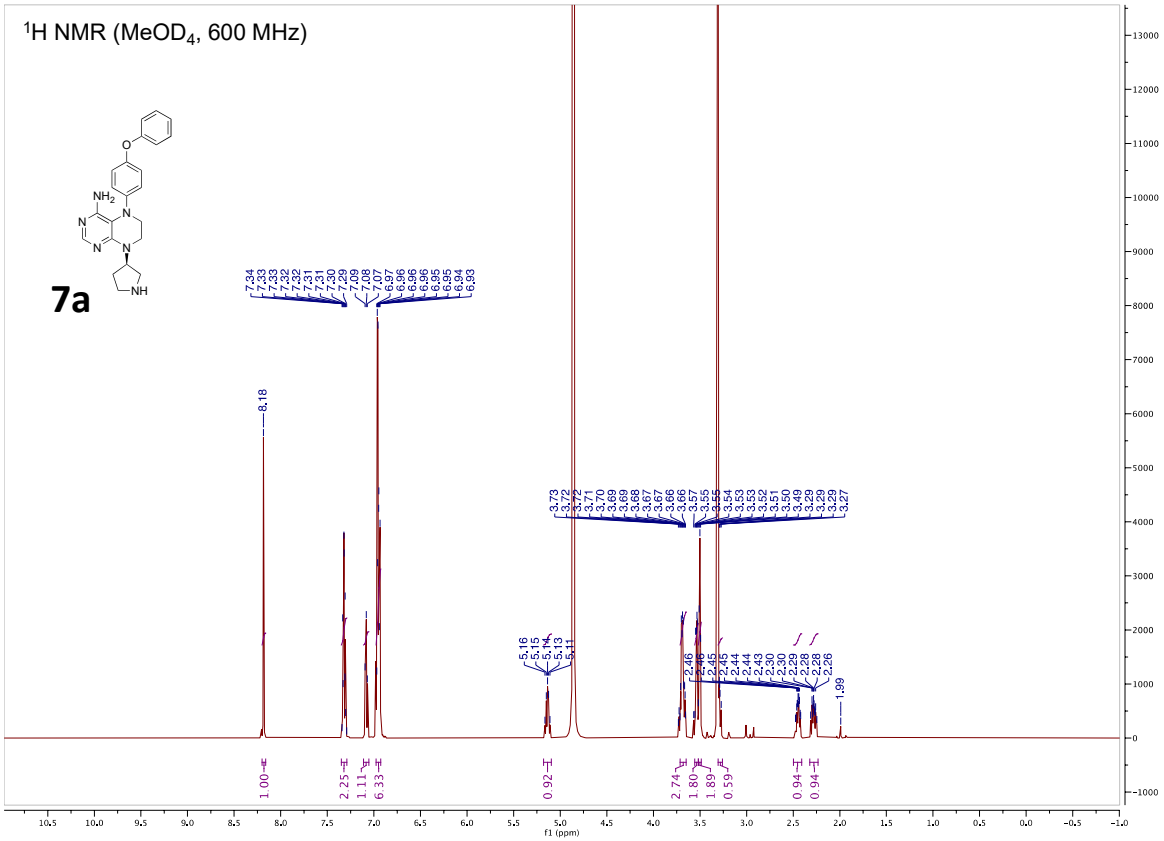

^{13}C NMR ($CDCl_3$, 151 MHz)
167.5
157.4
154.9
154.0
153.7
152.6
151.8
140.8
129.9
124.0
123.5
121.9
120.1
118.8
80.0
55.8
52.4
44.9
44.3
28.6
27.1
25.7
f1 (ppm)

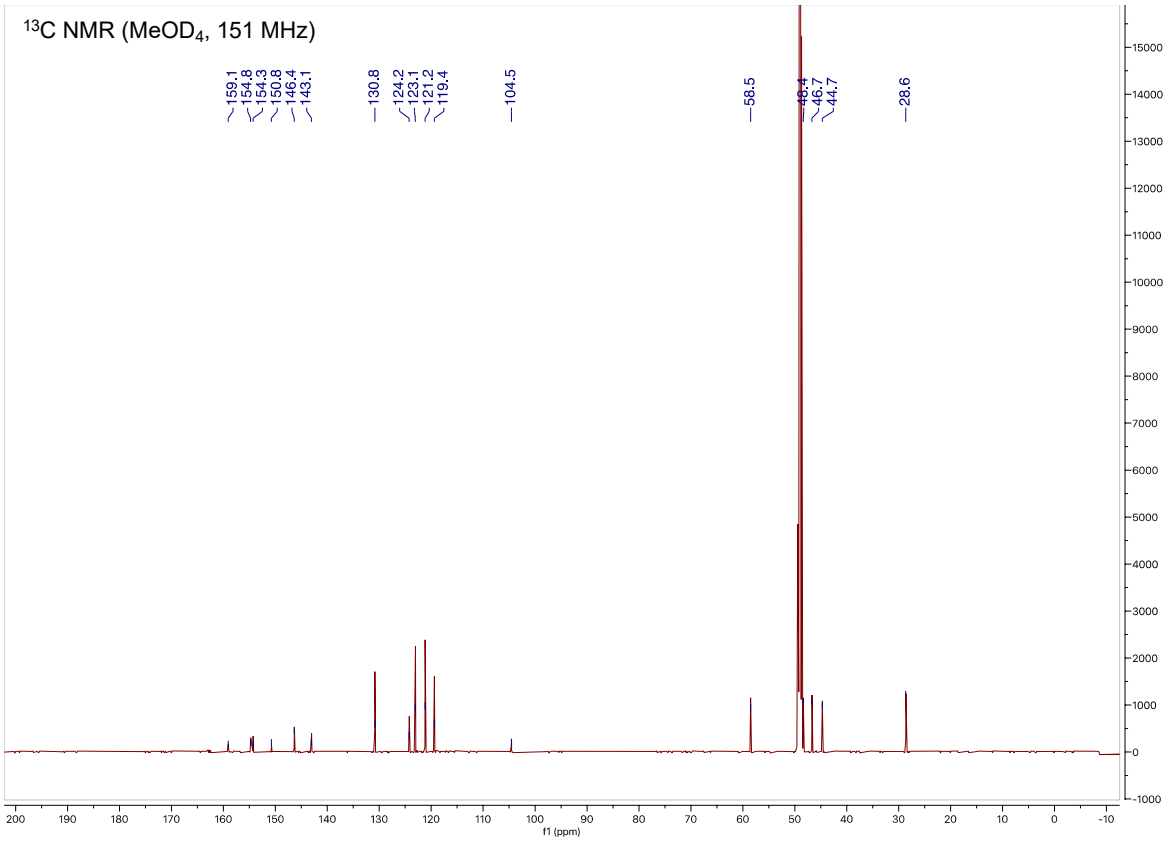

^{1}H NMR (MeOD$_4$, 600 MHz)
6a
f1 (ppm)

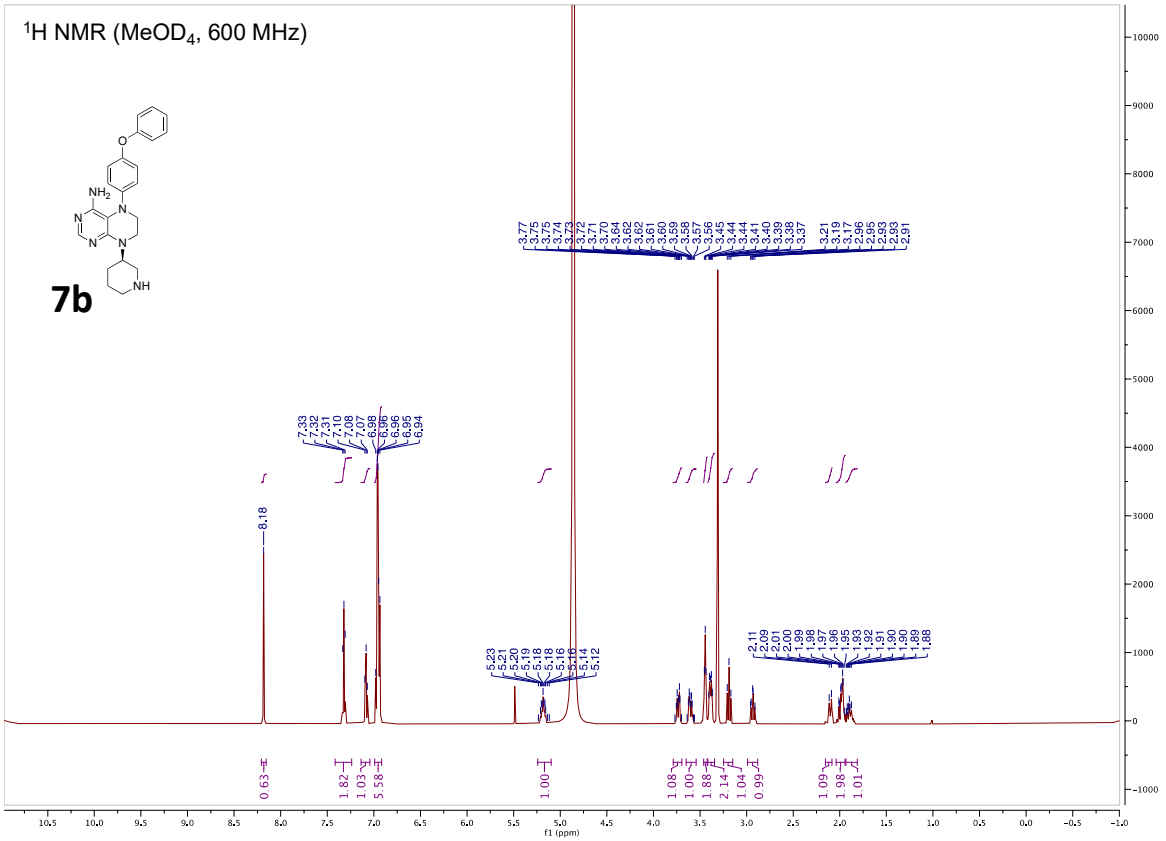

^{13}C NMR (MeOD$_4$, 151 MHz)
f1 (ppm)

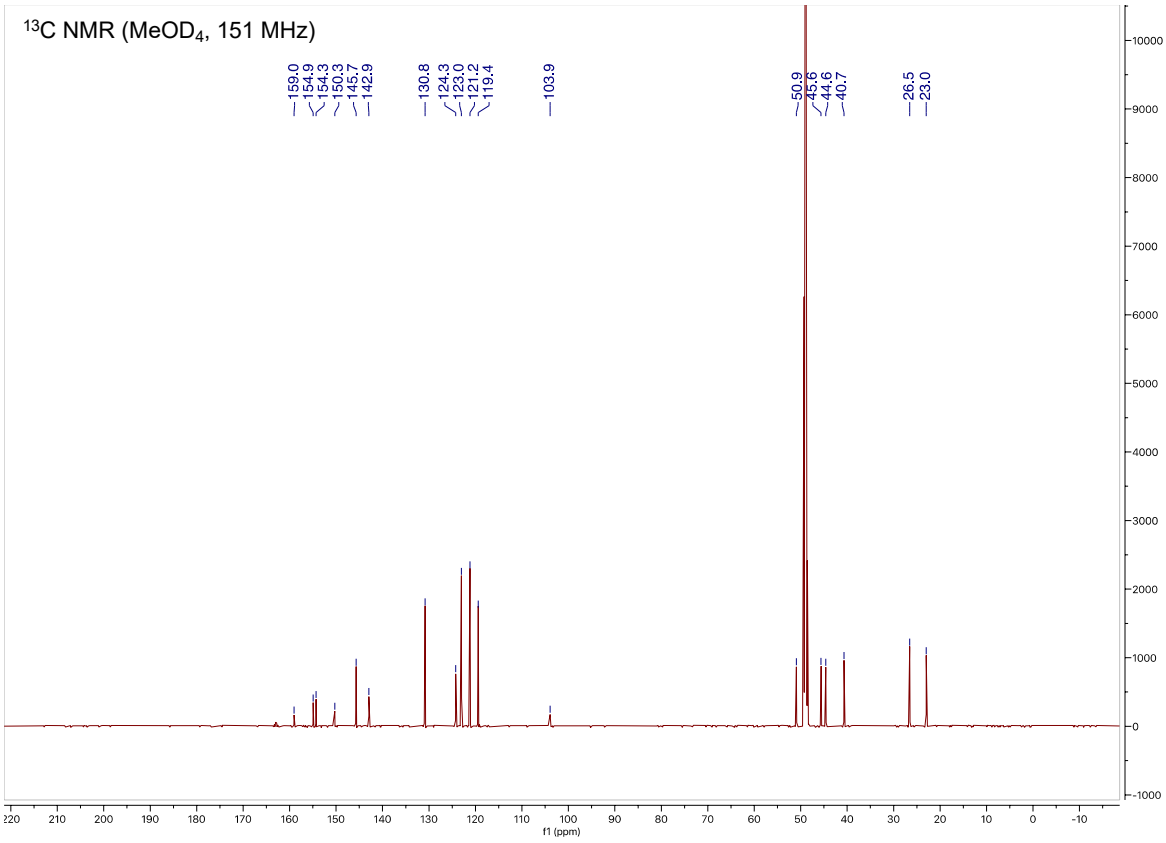

^{1}H NMR ($CDCl_3$, 600 MHz)
6b
f1 (ppm)

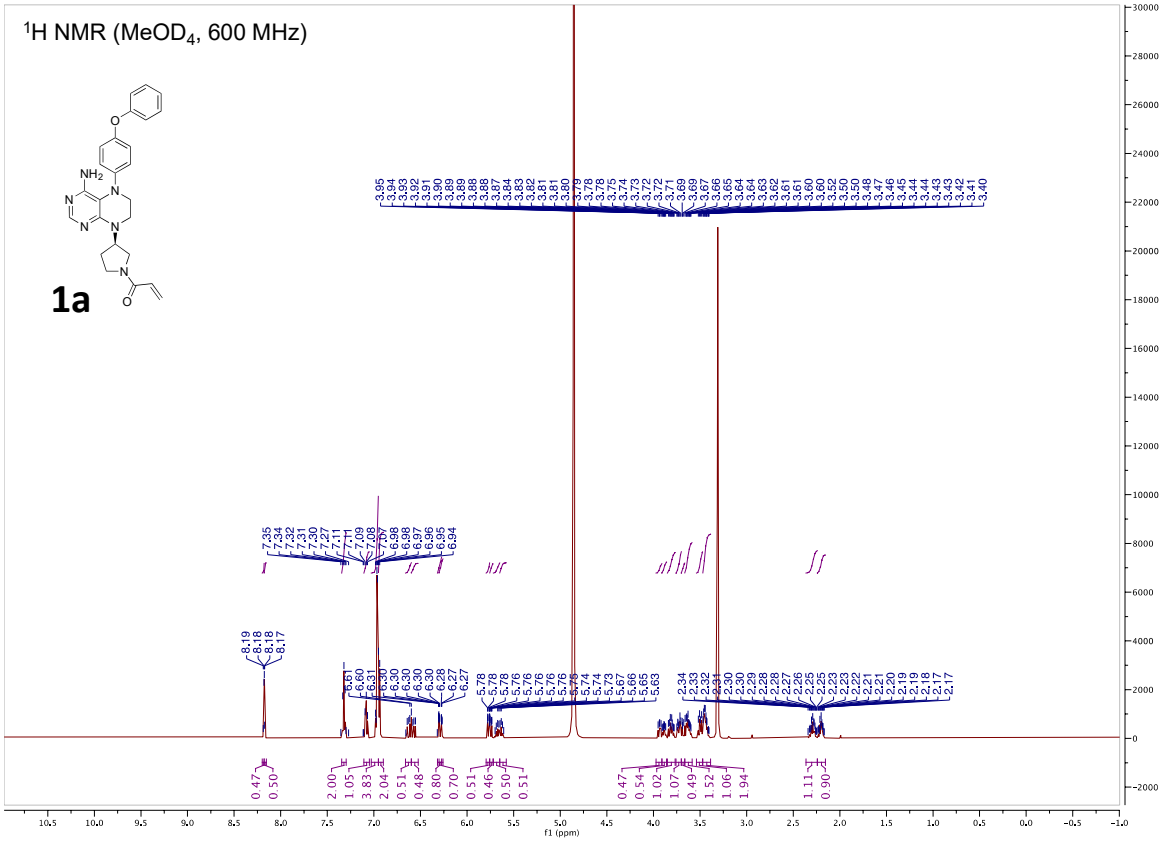

^{13}C NMR ($CDCl_3$, 151 MHz)
f1 (ppm)

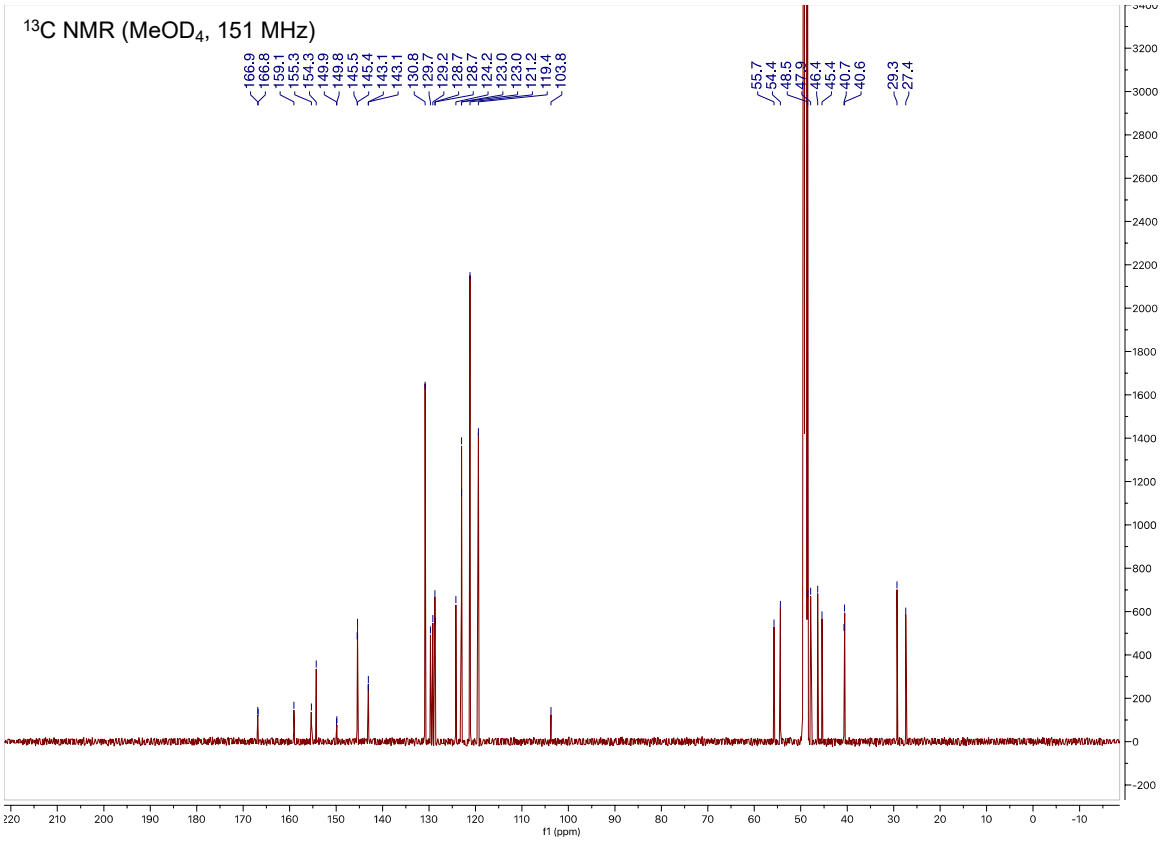

^{1}H NMR (MeOD$_4$, 600 MHz)
7a
8.18
7.34
7.33
7.33
7.32
7.32
7.31
7.31
7.30
7.29
7.09
7.08
7.07
6.97
6.96
6.96
6.96
6.95
6.95
6.94
6.93
5.16
5.15
5.14
5.13
5.11
3.73
3.72
3.72
3.71
3.70
3.69
3.69
3.68
3.67
3.67
3.66
3.66
3.57
3.55
3.55
3.54
3.53
3.53
3.52
3.51
3.50
3.49
3.29
3.29
3.29
3.27
2.46
2.46
2.45
2.45
2.44
2.44
2.43
2.30
2.30
2.28
2.28
2.28
2.26
1.99
1.00
2.25
1.11
6.33
0.92
2.74
1.80
1.89
0.59
0.94
0.94
f1 (ppm)

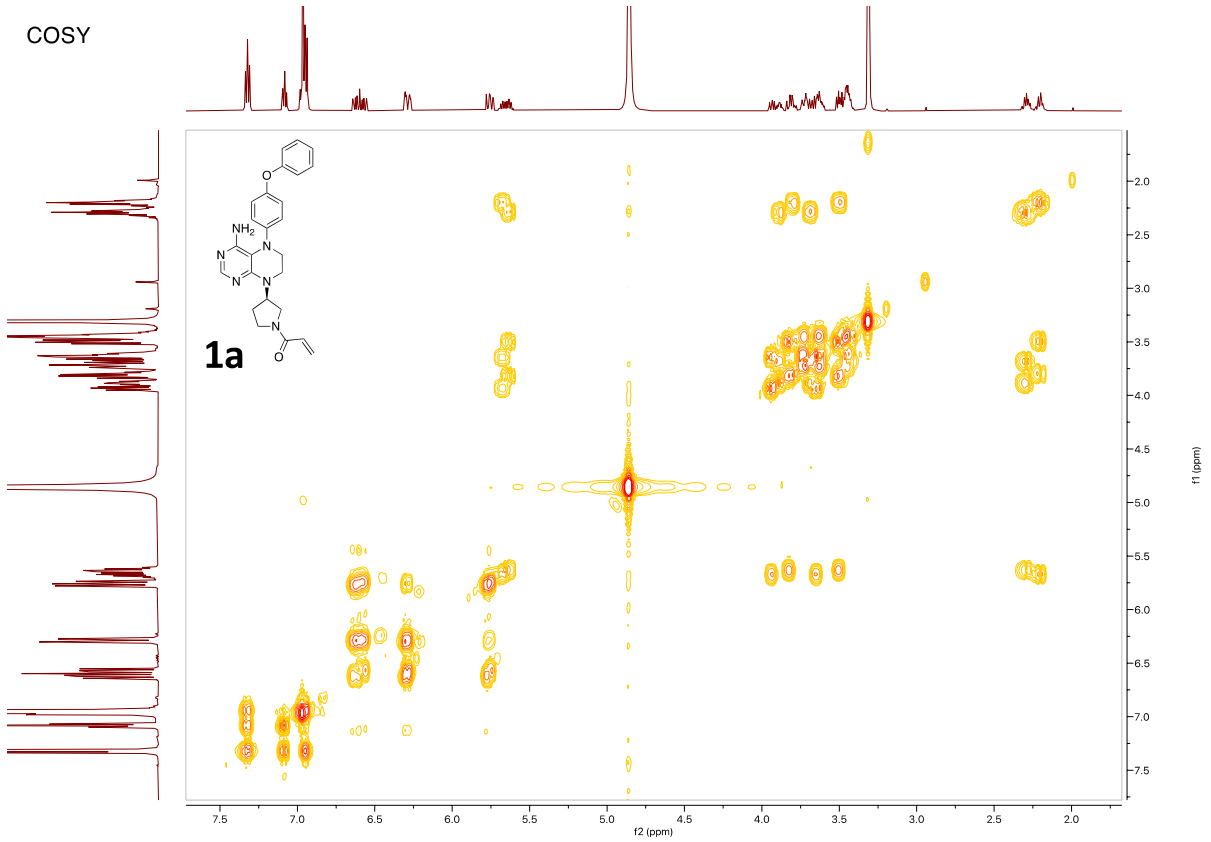

^{13}C NMR (MeOD$_4$, 151 MHz)
159.1
154.8
154.3
150.8
146.4
143.1
130.8
124.2
123.1
121.2
119.4
104.5
58.5
46.4
46.7
44.7
28.6
f1 (ppm)

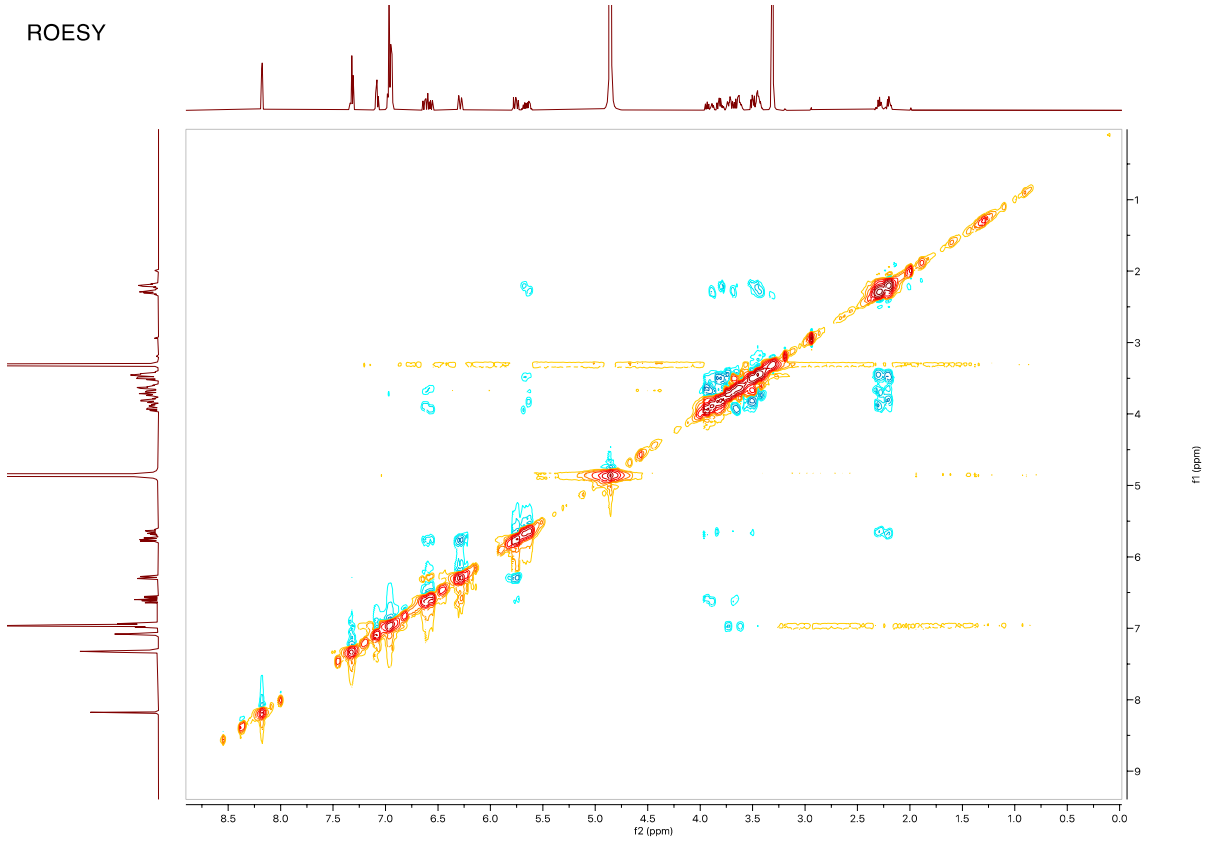

^{1}H NMR (MeOD$_4$, 600 MHz)
7b
NH$_2$
O
N
NH
8.18
7.33
7.32
7.31
7.10
7.08
7.07
6.98
6.96
6.96
6.95
6.94
5.23
5.21
5.20
5.19
5.18
5.18
5.16
5.16
5.14
5.12
3.77
3.75
3.75
3.74
3.73
3.72
3.71
3.70
3.64
3.62
3.62
3.61
3.60
3.59
3.58
3.57
3.56
3.45
3.44
3.44
3.41
3.40
3.39
3.38
3.37
3.21
3.19
3.17
2.96
2.95
2.93
2.93
2.91
2.11
2.09
2.01
2.00
1.99
1.98
1.97
1.96
1.95
1.93
1.92
1.91
1.90
1.90
1.89
1.88
0.63
1.82
1.03
5.58
1.00
1.08
1.00
1.88
2.14
1.04
0.99
1.09
1.98
1.01
f1 (ppm)

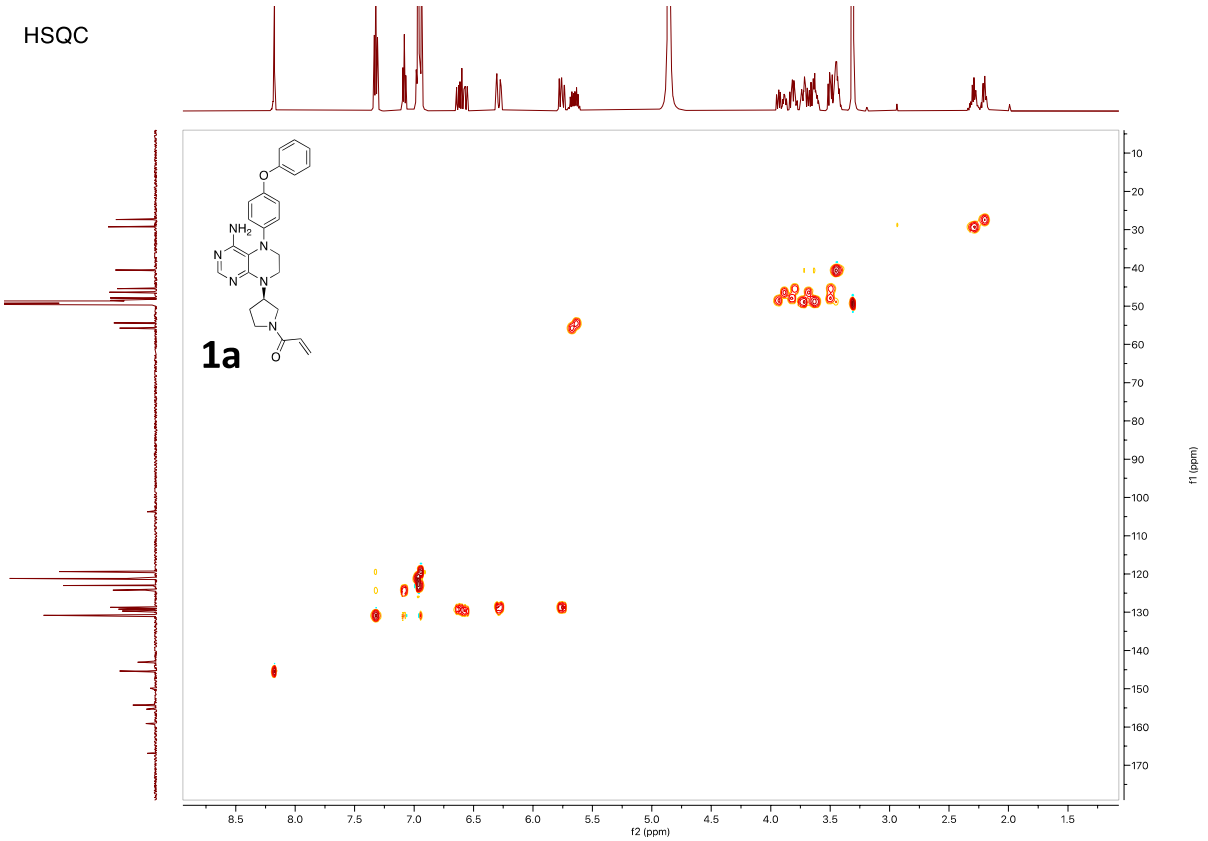

^{13}C NMR (MeOD$_4$, 151 MHz)
159.0
154.9
154.3
150.3
145.7
142.9
130.8
124.3
123.0
121.2
119.4
103.9
50.9
45.6
44.6
40.7
26.5
23.0
f1 (ppm)

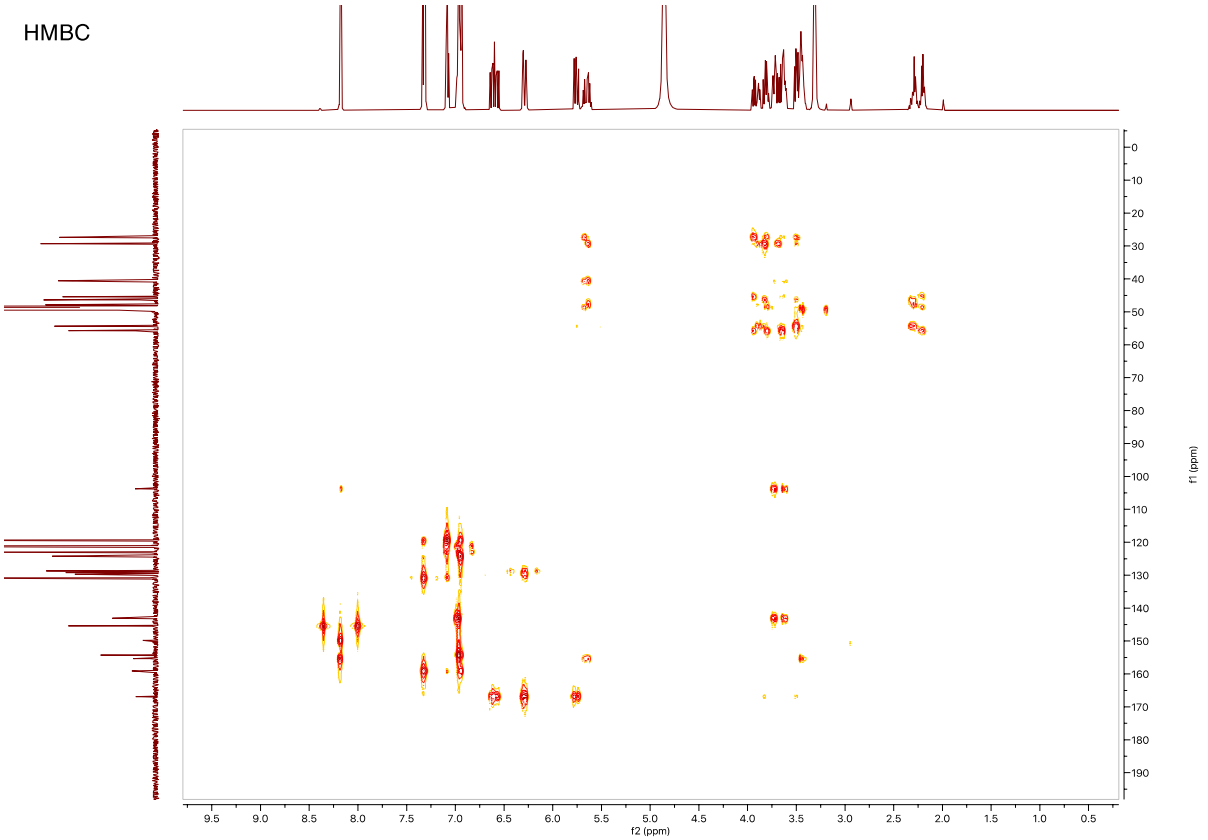

^{1}H NMR (MeOD$_4$, 600 MHz)
1a
f1 (ppm)

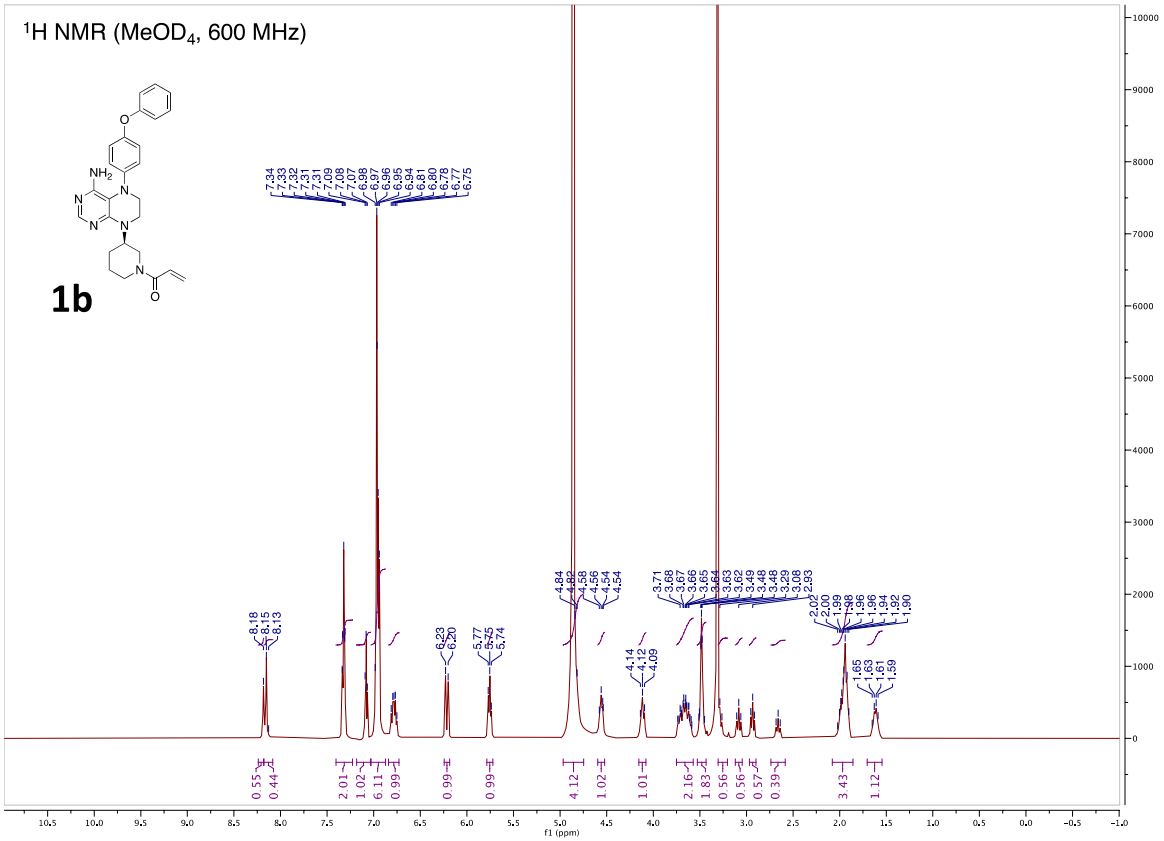

^{13}C NMR (MeOD$_4$, 151 MHz)
f1 (ppm)

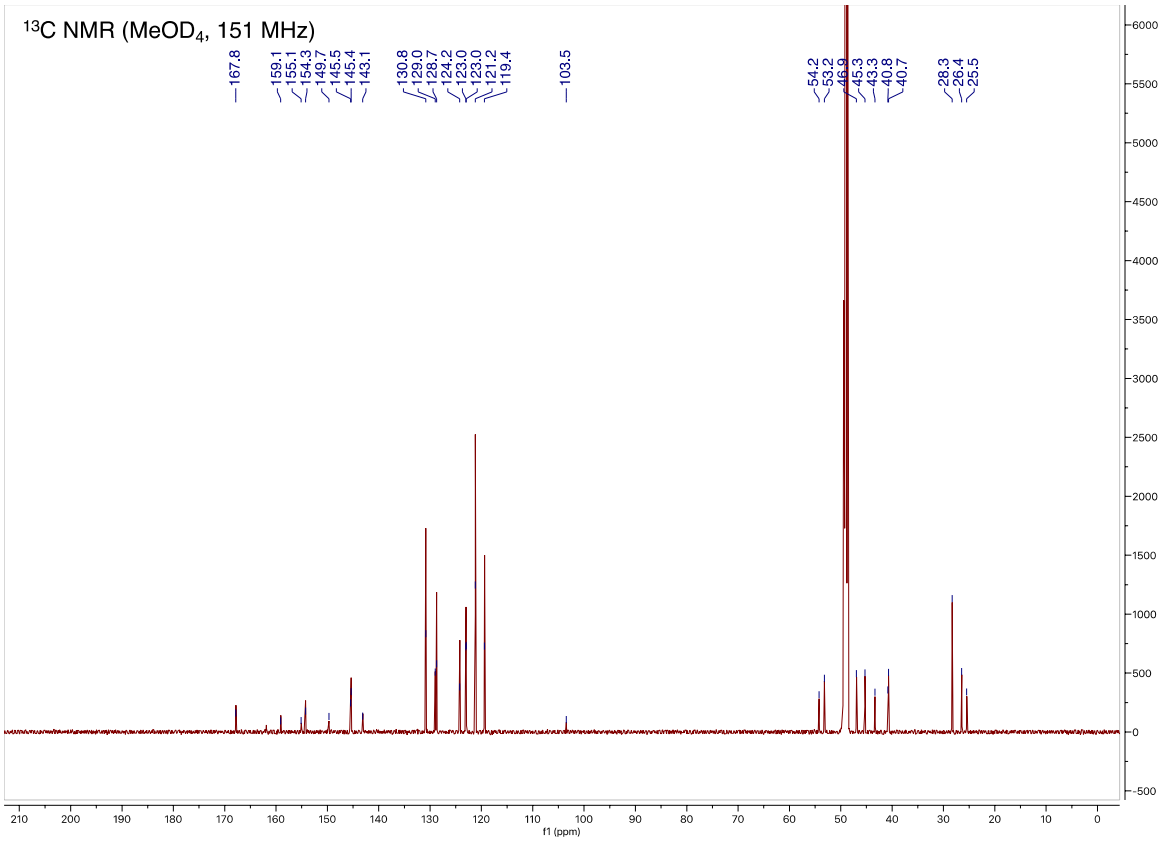

COSY
1a
f2 (ppm)
f1 (ppm)

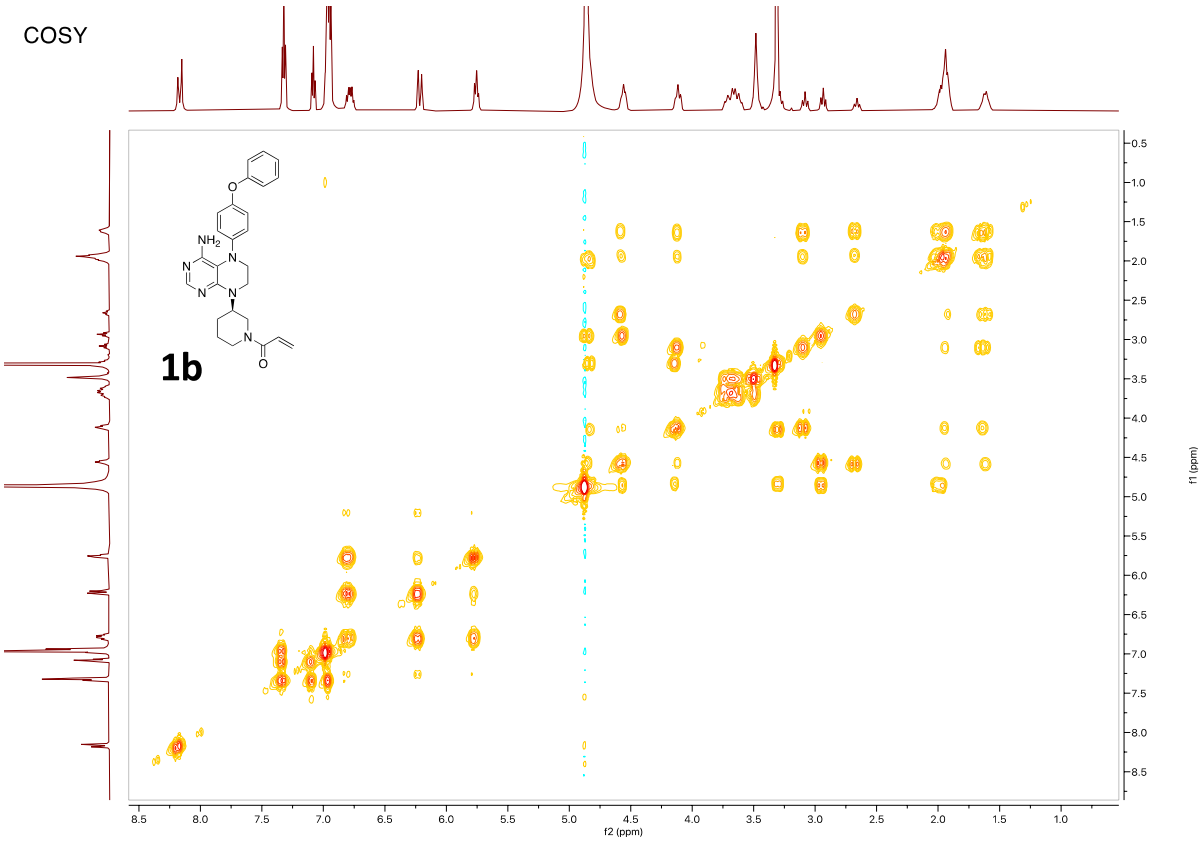

ROESY
f2 (ppm)
f1 (ppm)

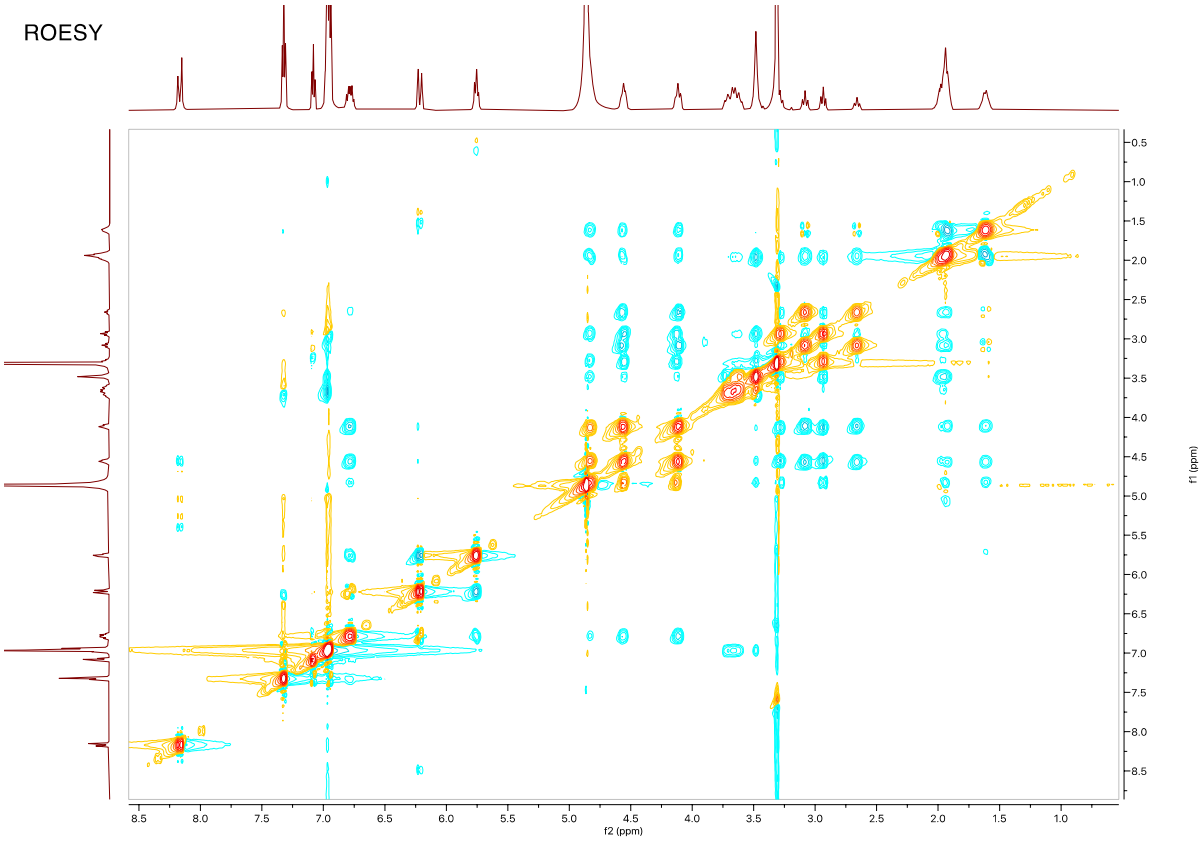

HSQC
1a
f2 (ppm)
f1 (ppm)

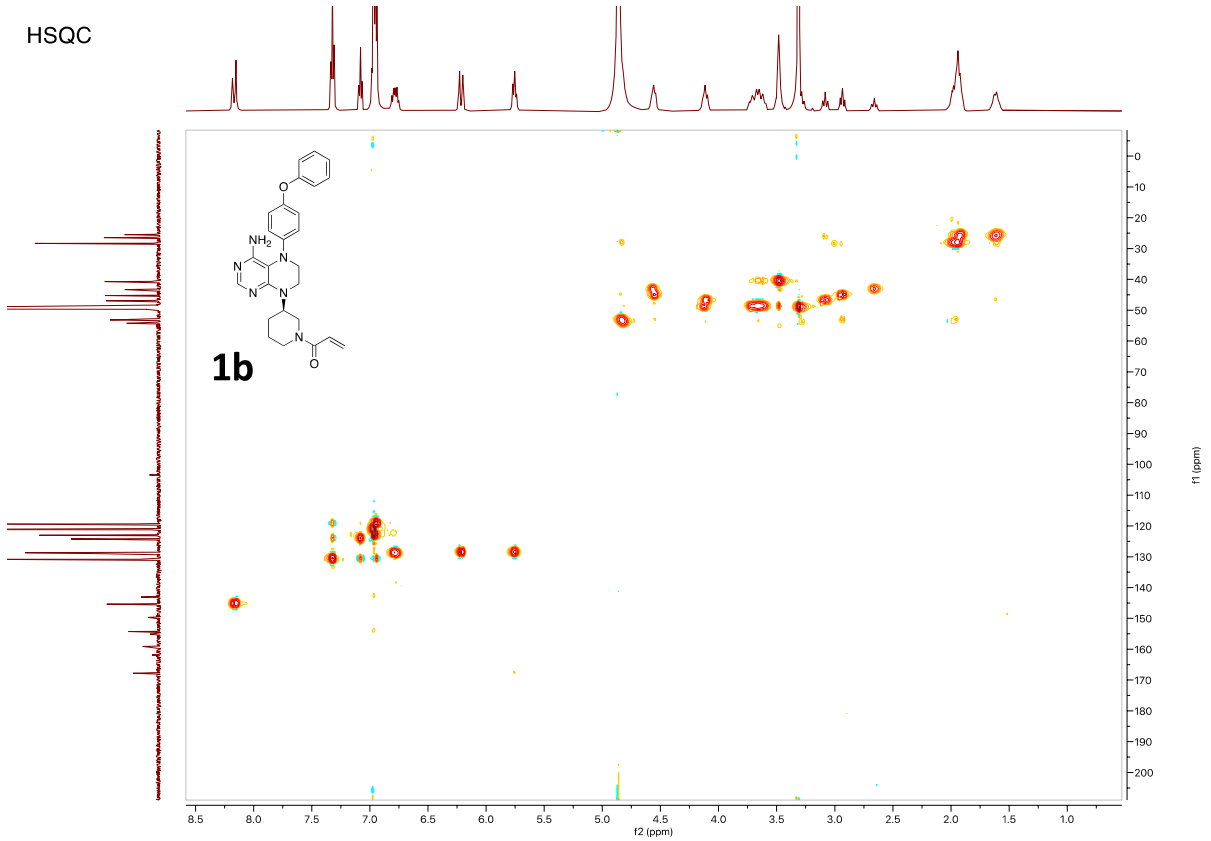

HMBC
f2 (ppm)
f1 (ppm)

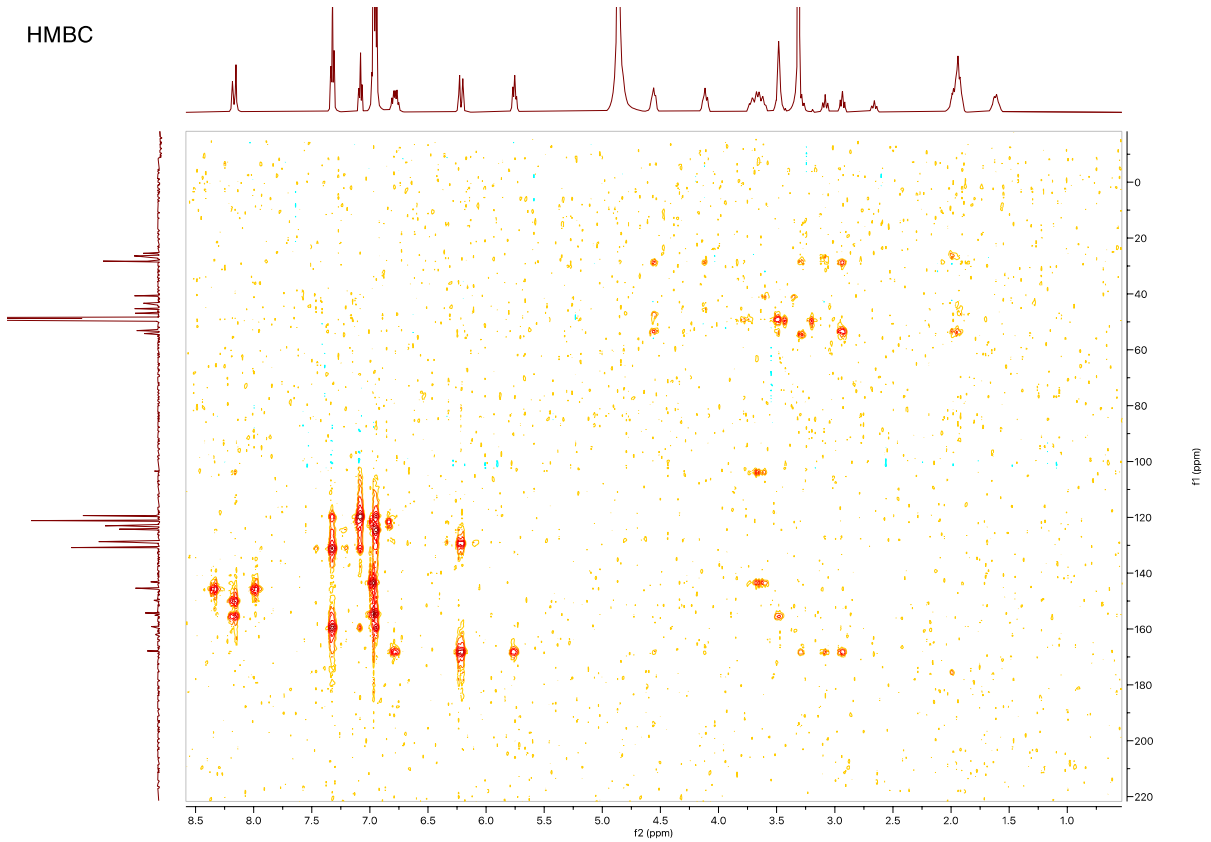

^{1}H NMR (MeOD$_4$, 600 MHz)
1b
f1 (ppm)

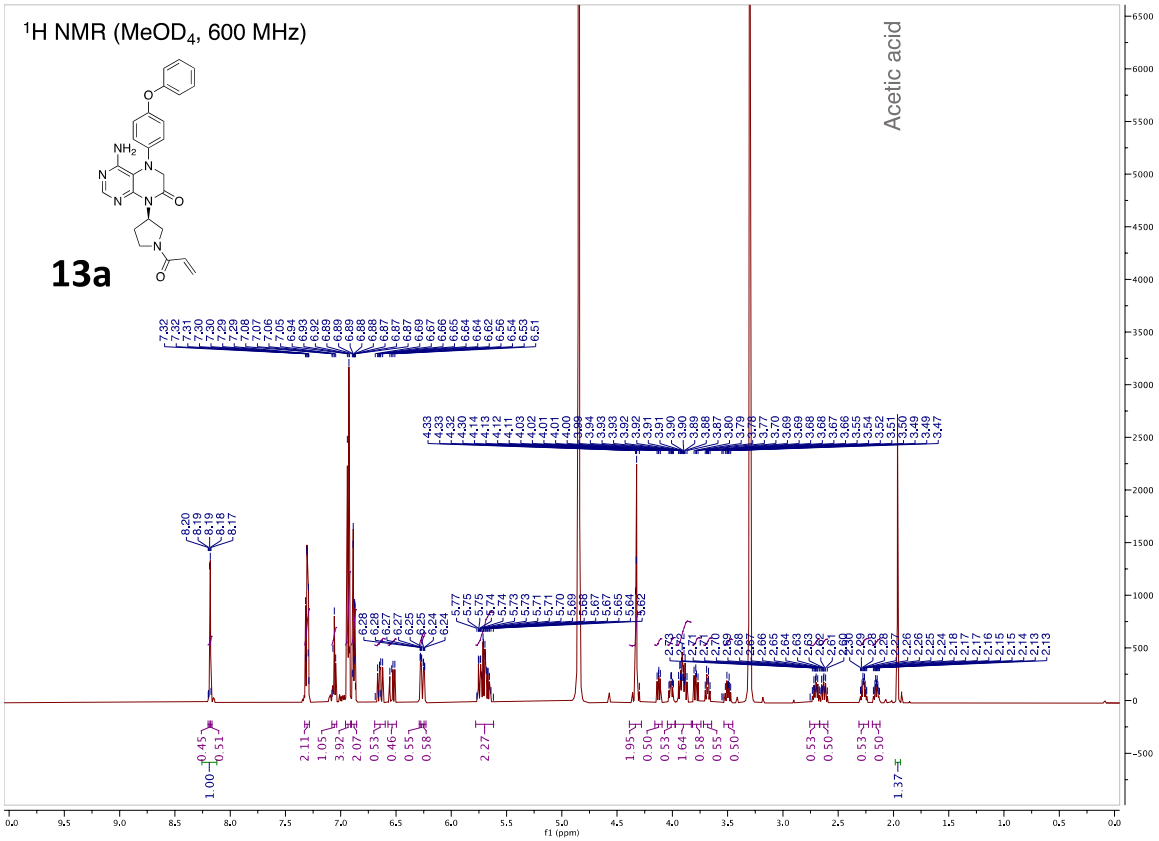

^{13}C NMR (MeOD$_4$, 151 MHz)
167.8
159.1
155.1
154.3
149.7
145.5
145.4
143.1
130.8
129.0
128.7
124.2
123.0
123.0
121.2
119.4
103.5
54.2
53.2
46.9
45.3
43.3
40.8
40.7
28.3
26.4
25.5
f1 (ppm)

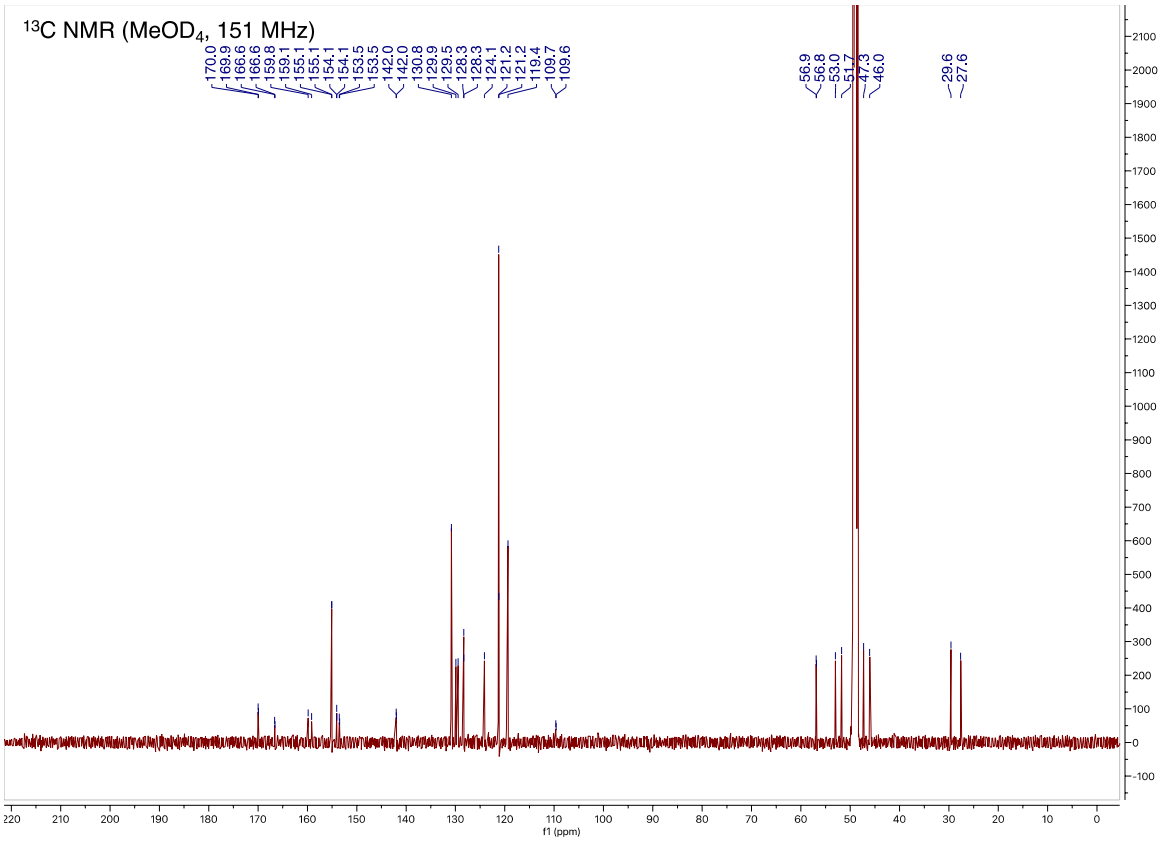

COSY
1b
f2 (ppm)
f1 (ppm)

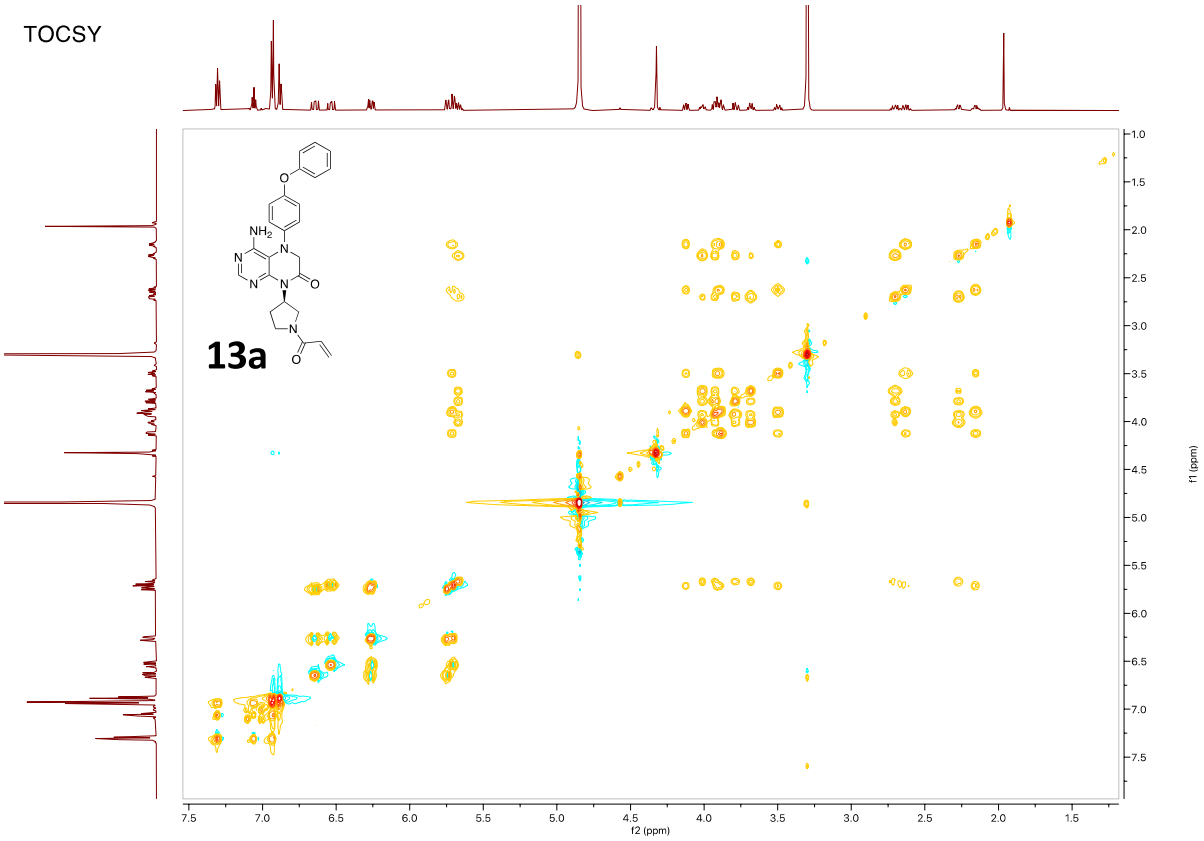

ROESY
f2 (ppm)
f1 (ppm)

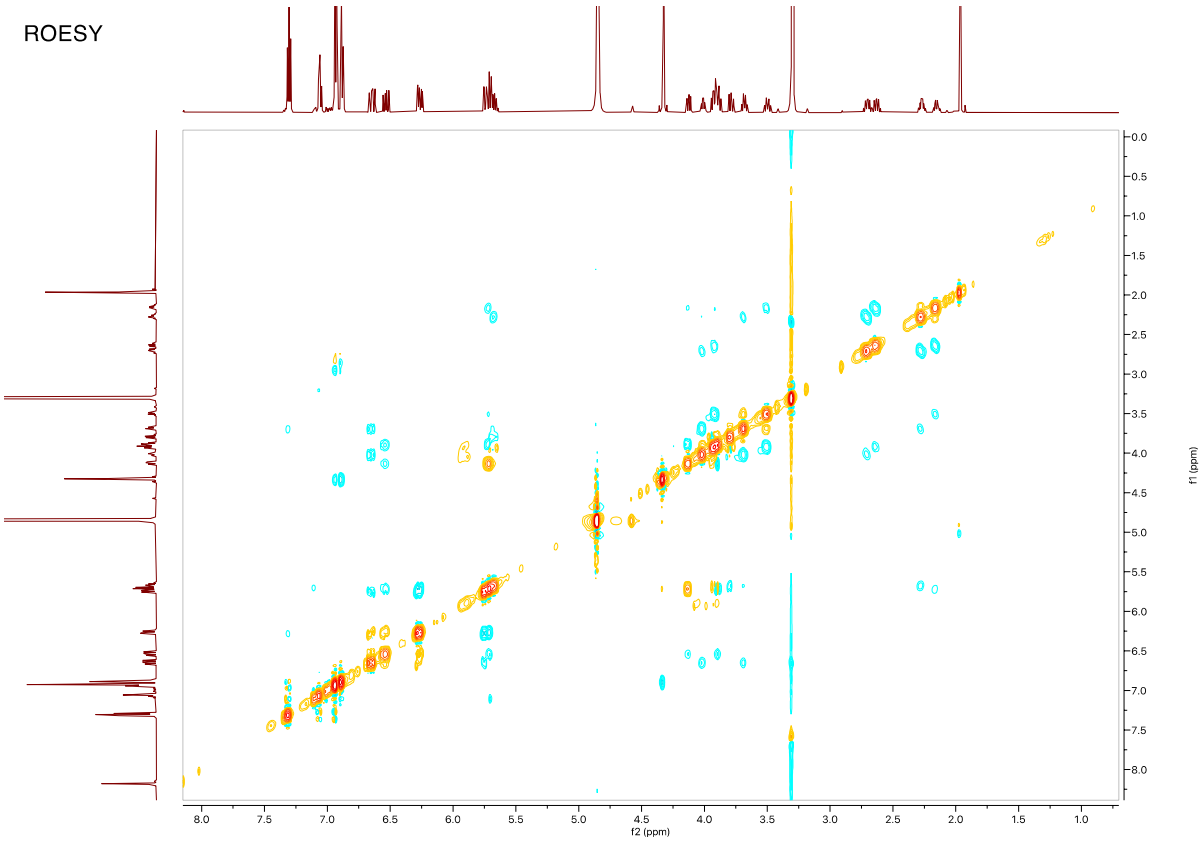

HSQC
1b
f2 (ppm)
f1 (ppm)


HMBC
f2 (ppm)
f1 (ppm)

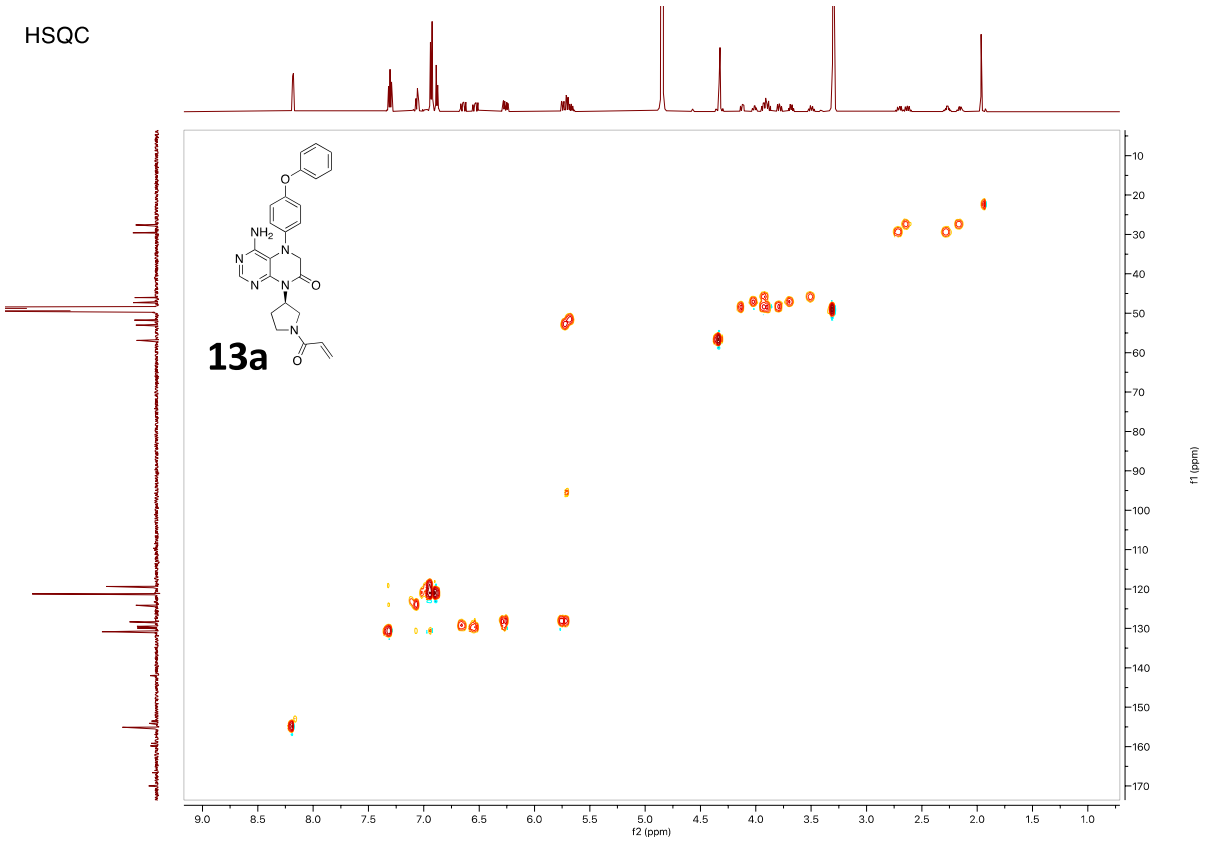

^{1}H NMR (MeOD$_4$, 600 MHz)
Acetic acid
13a
f1 (ppm)


^{13}C NMR (MeOD$_4$, 151 MHz)
f1 (ppm)

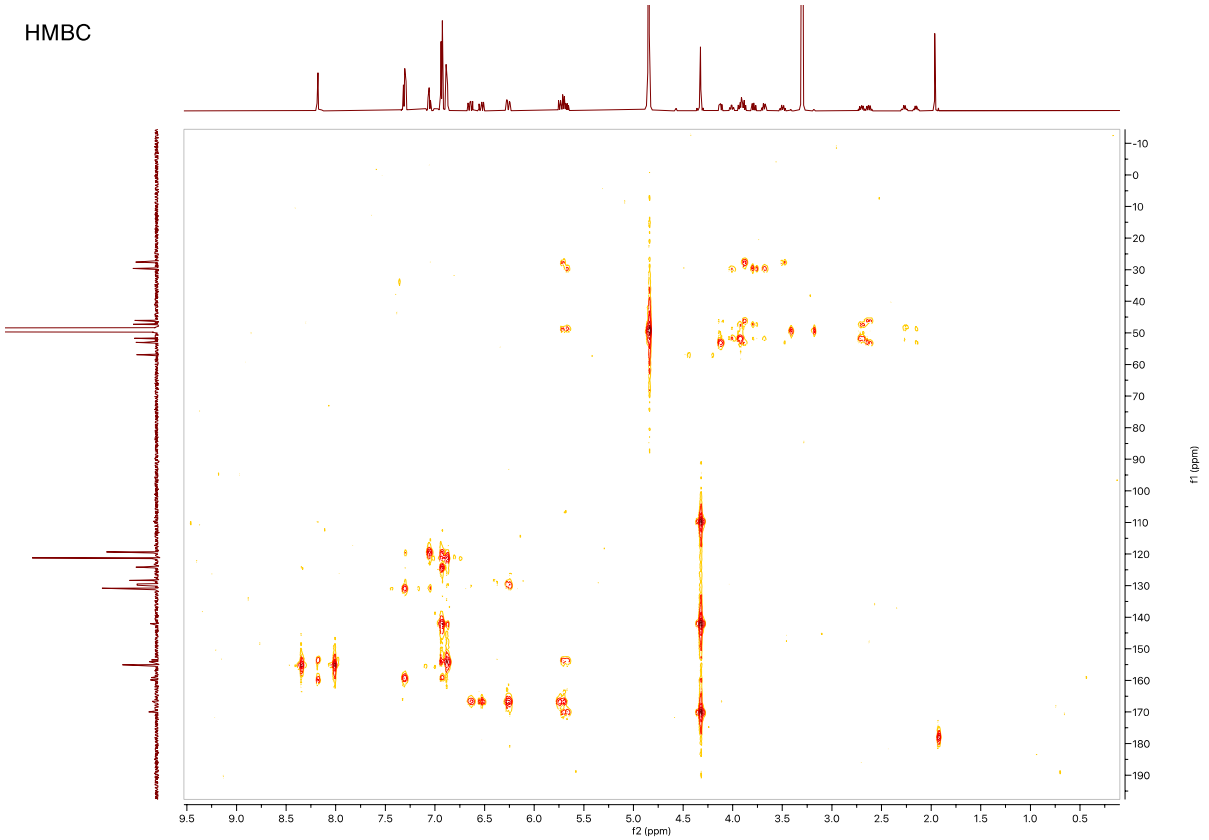

TOCSY
13a
f2 (ppm)
f1 (ppm)

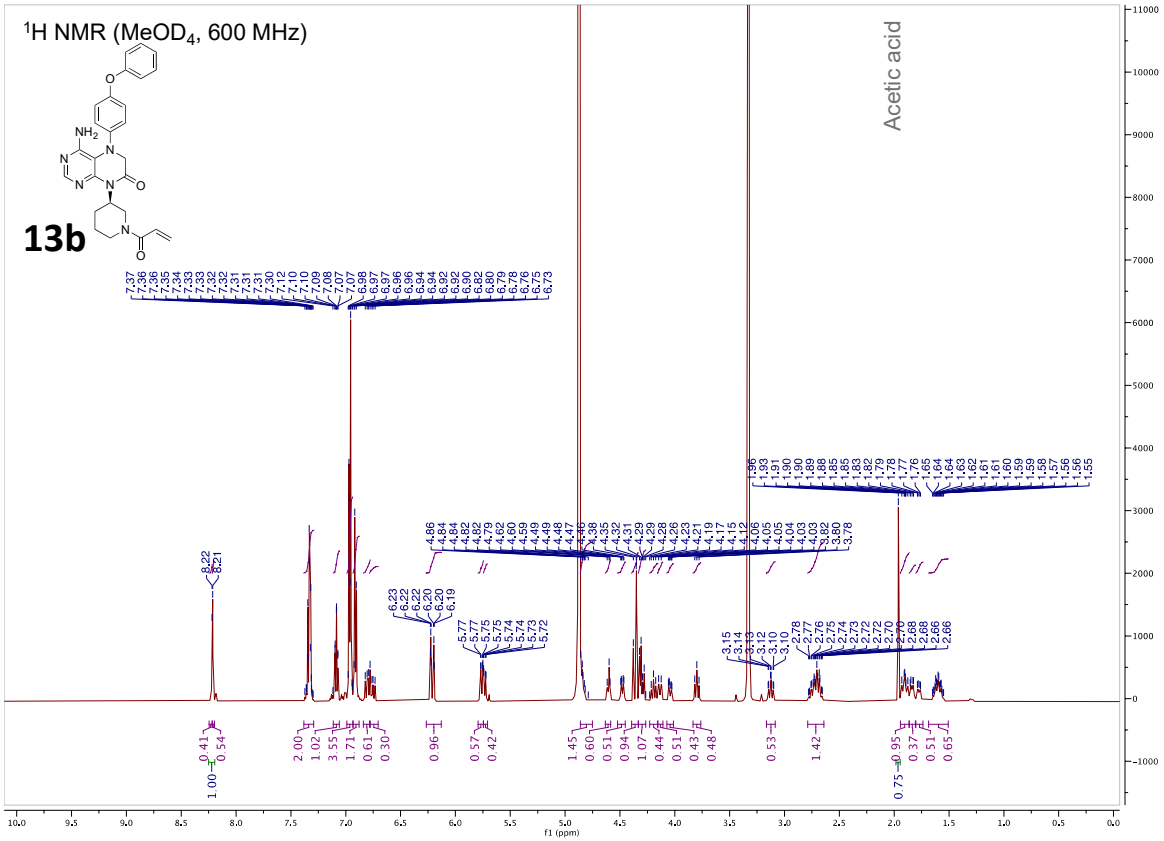

ROESY
f2 (ppm)
f1 (ppm)

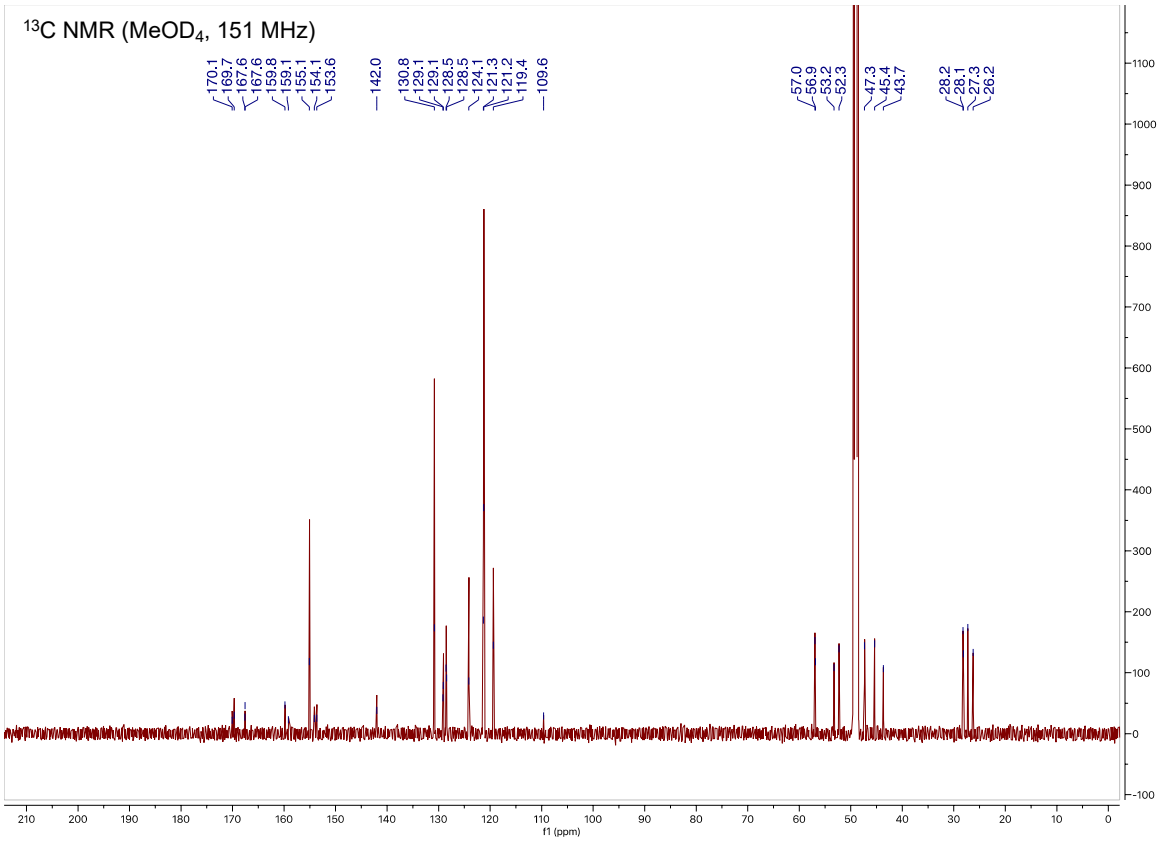
HSQC

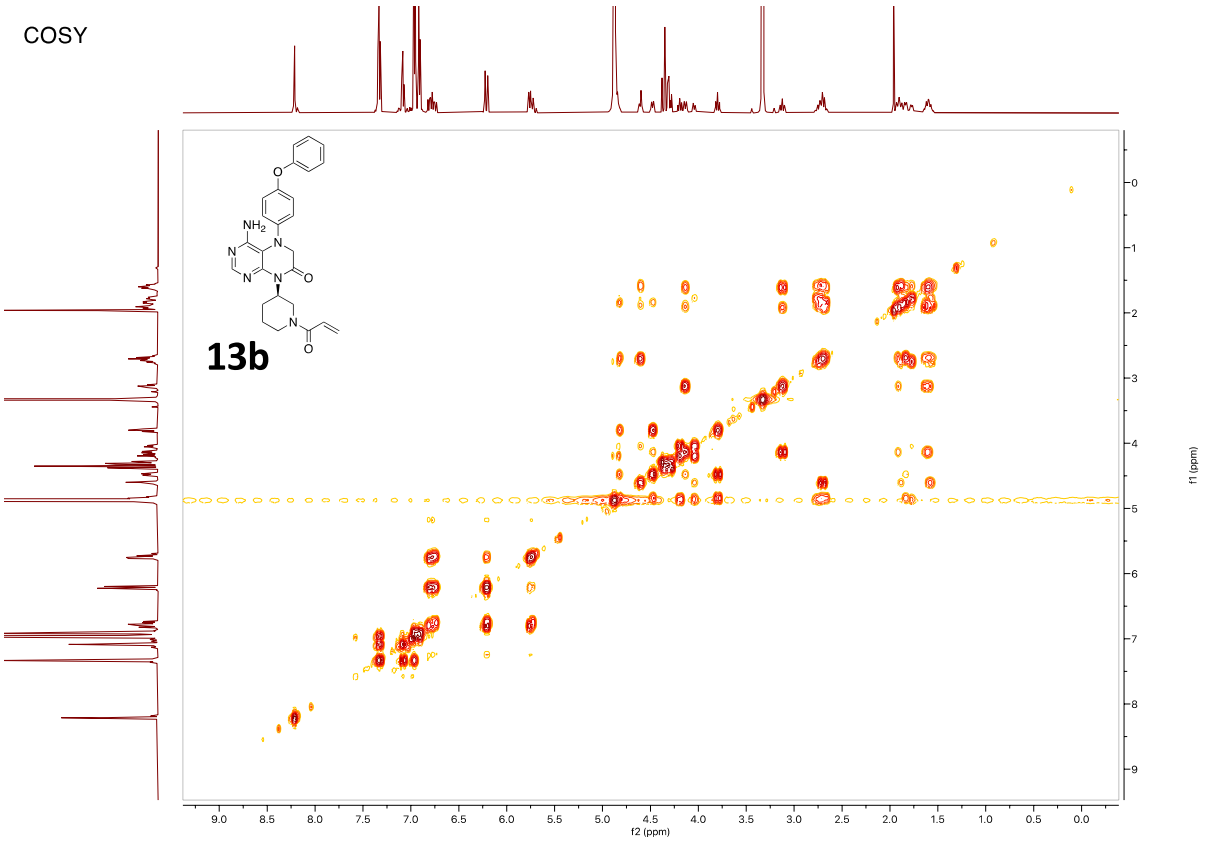


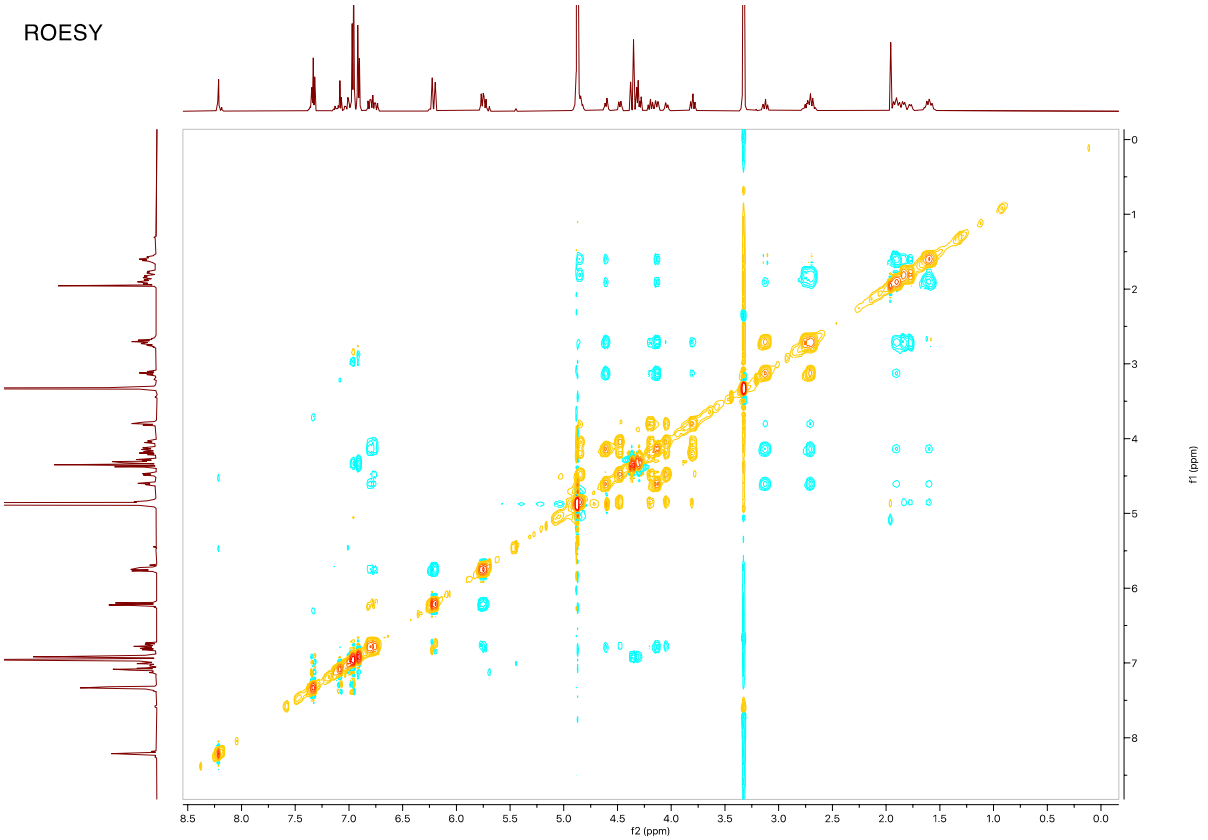
HMBC



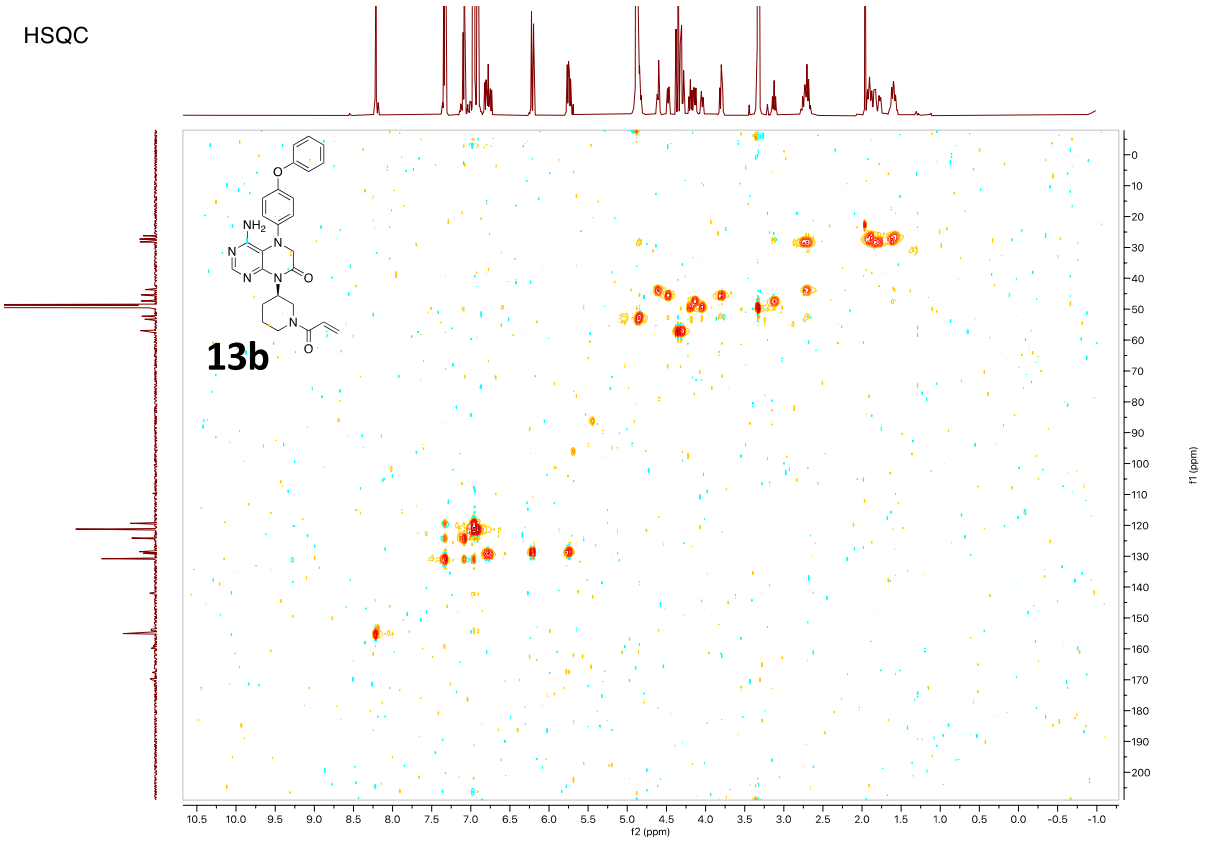

^{1}H NMR (MeOD$_4$, 600 MHz)
13b
Acetic acid
f1 (ppm)


^{13}C NMR (MeOD$_4$, 151 MHz)
f1 (ppm)

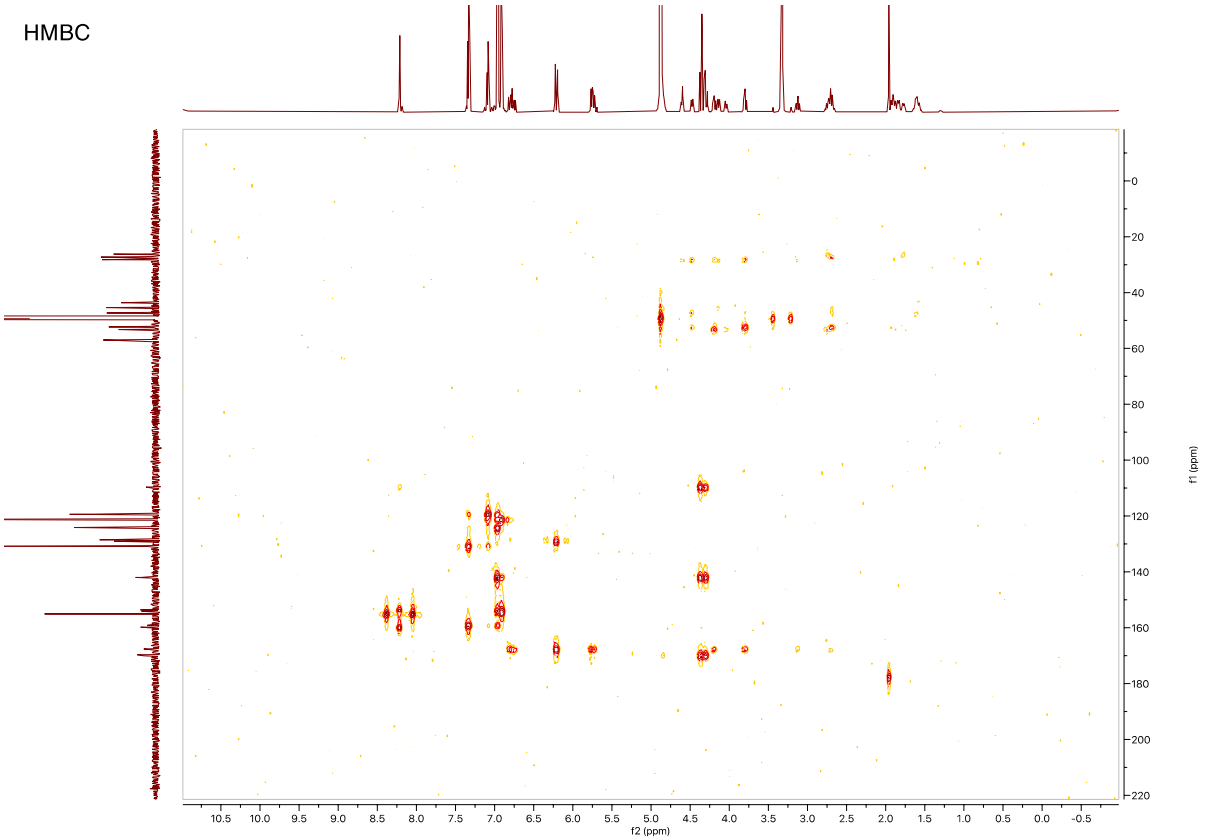

COSY
13b
f2 (ppm)
f1 (ppm)

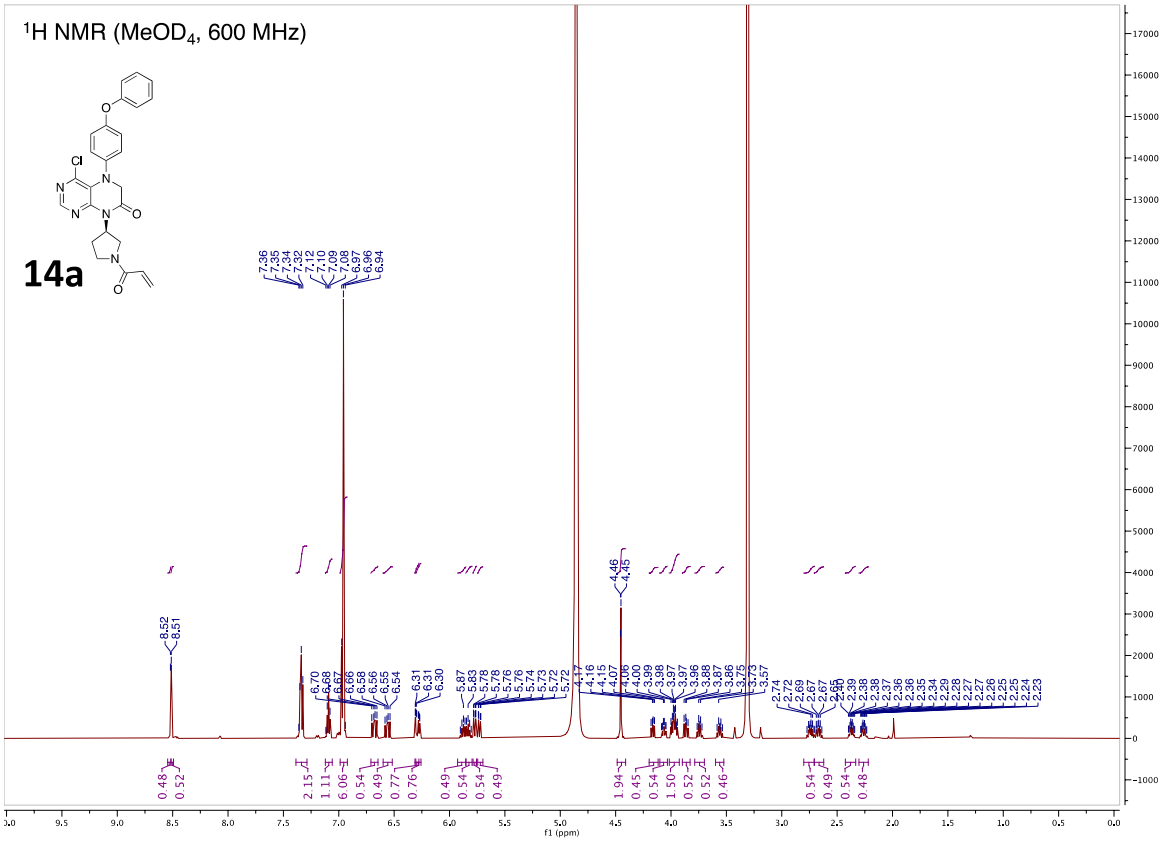

ROESY
f2 (ppm)
f1 (ppm)

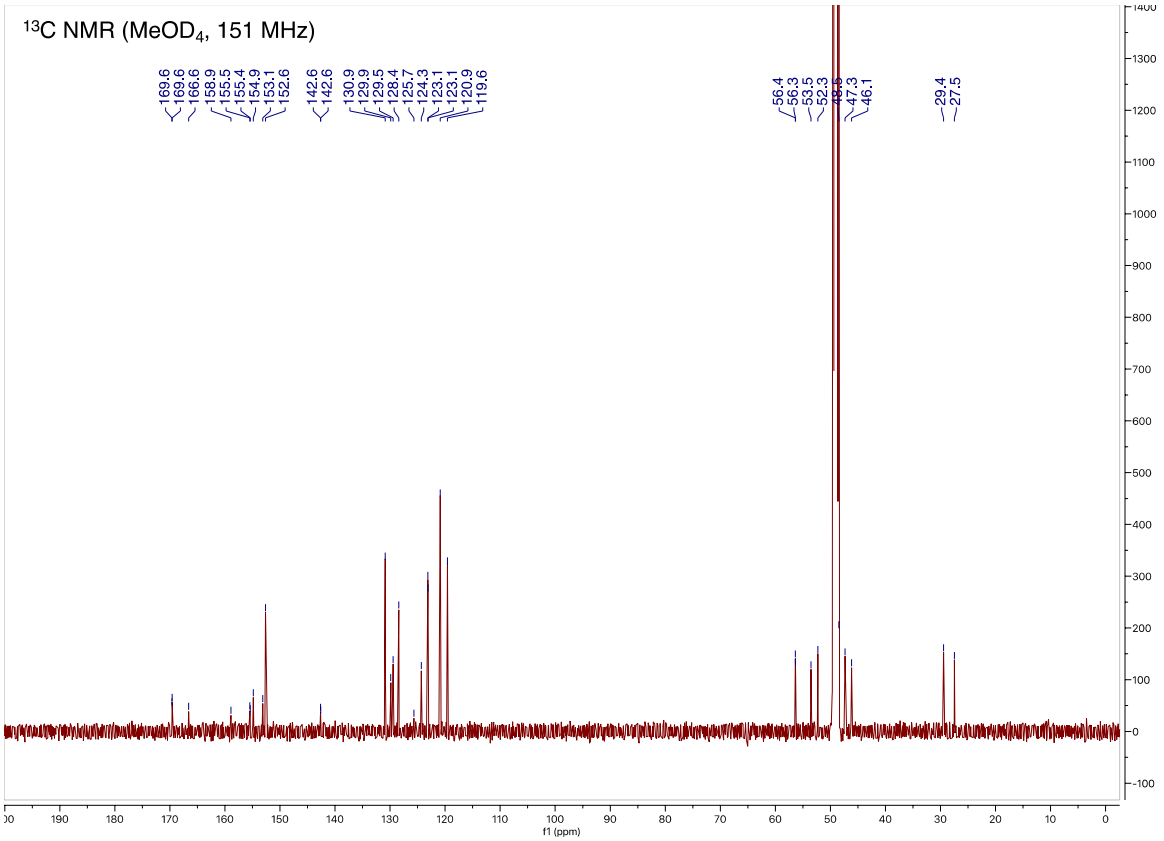
HSQC

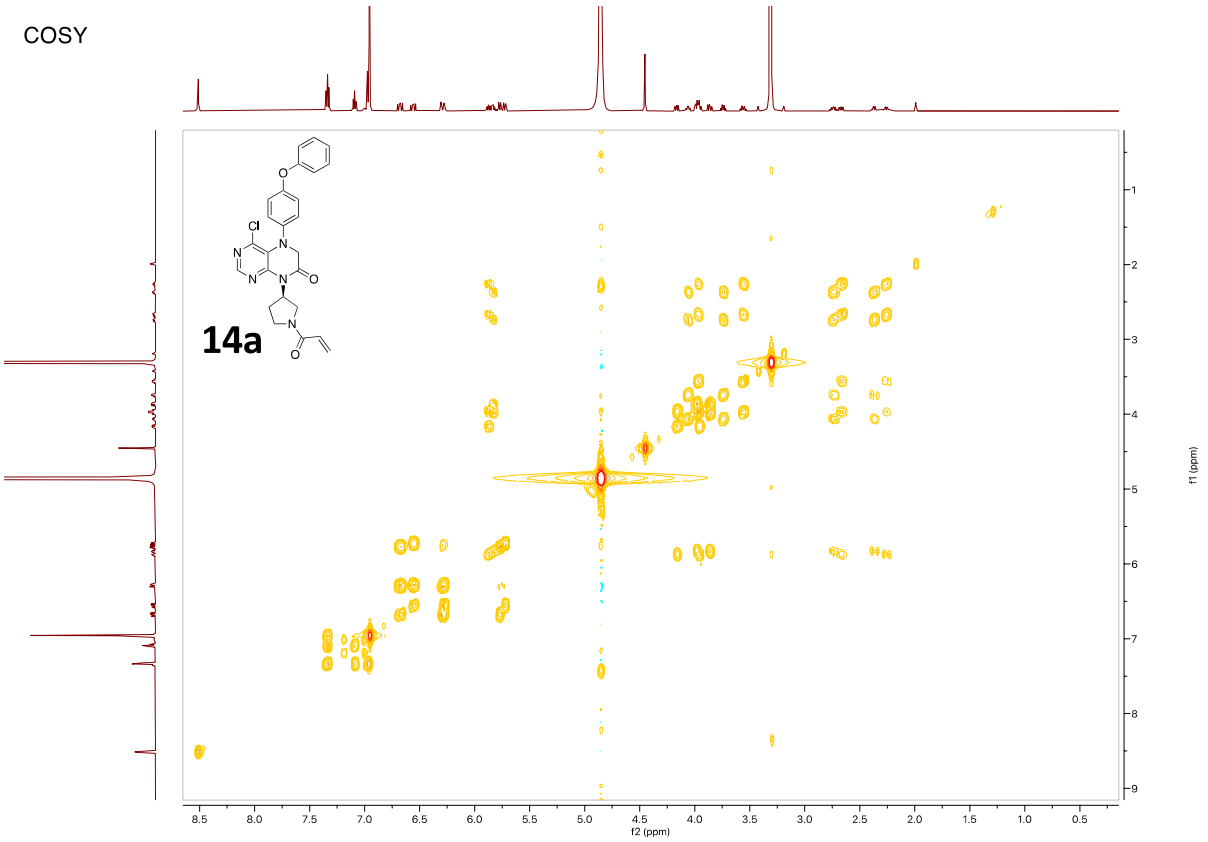


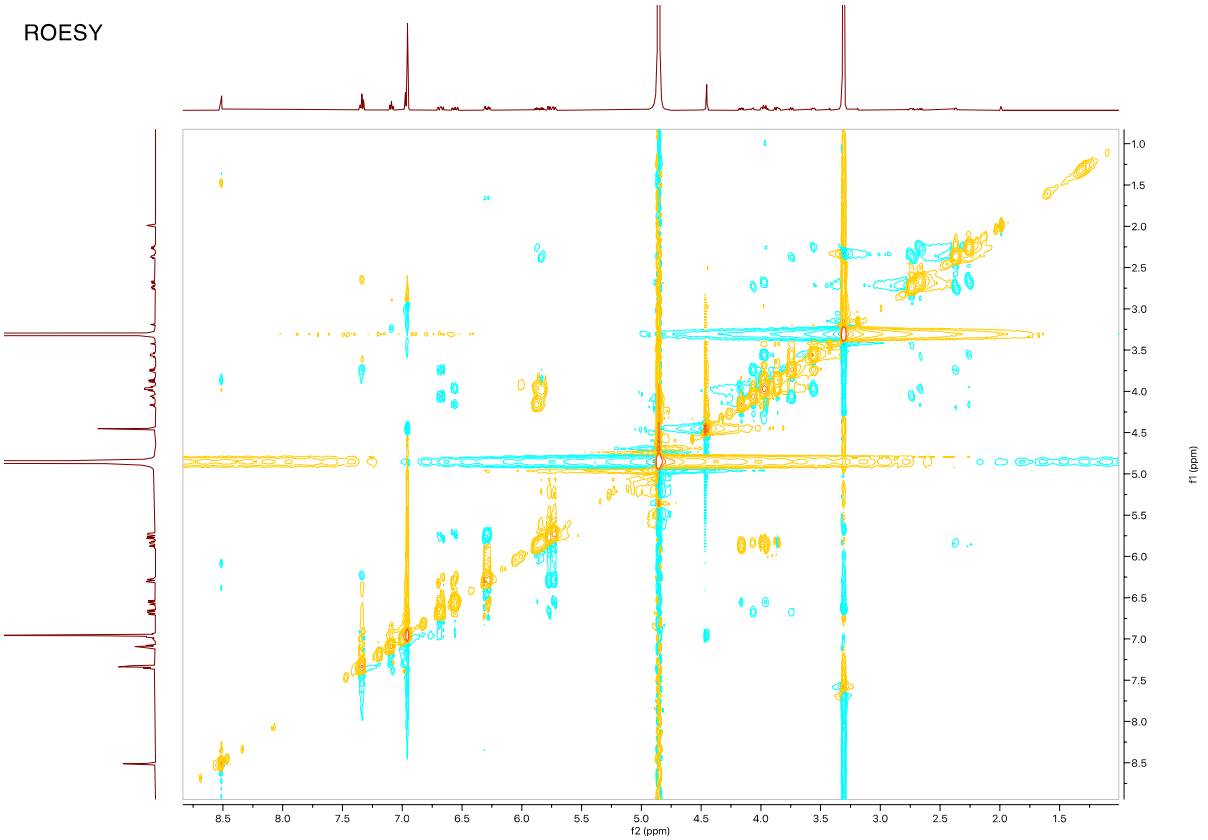
HMBC



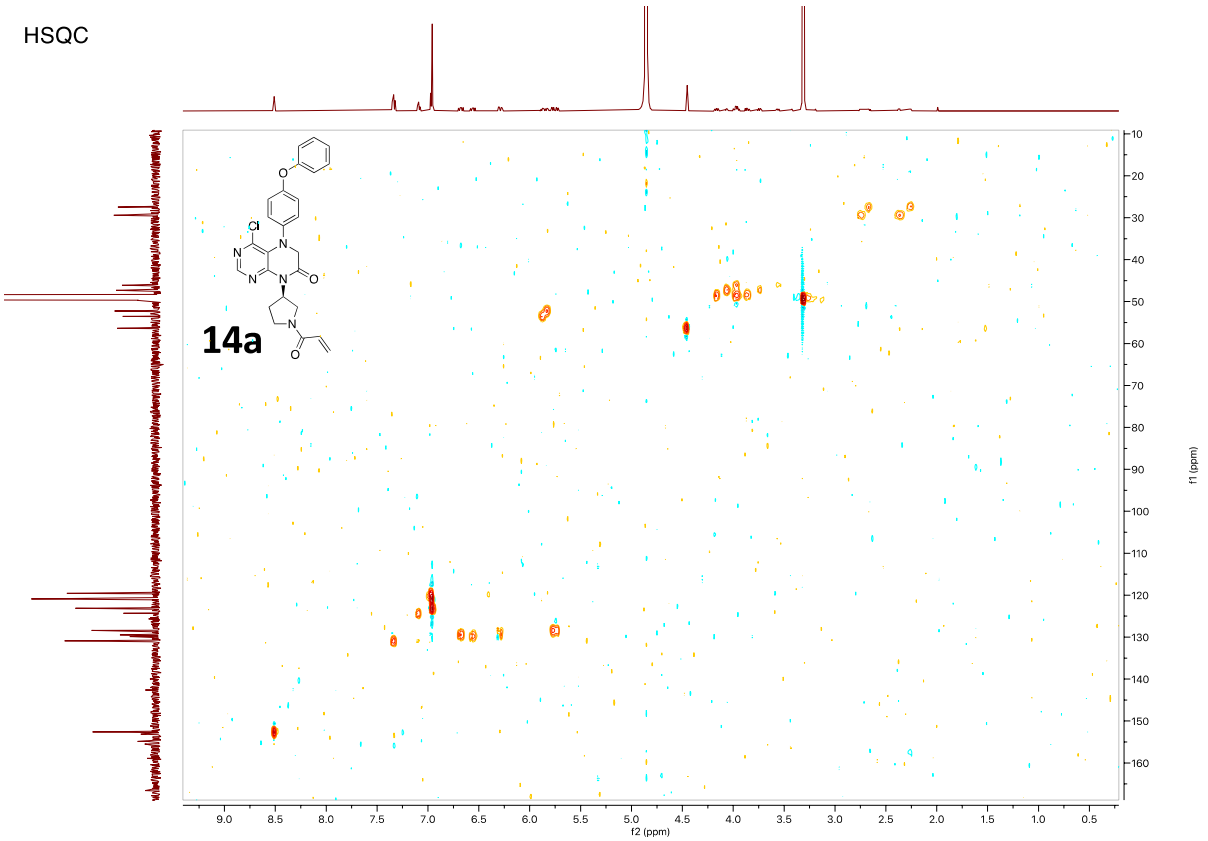

^{1}H NMR (MeOD$_4$, 600 MHz)
14a
f1 (ppm)


^{13}C NMR (MeOD$_4$, 151 MHz)
f1 (ppm)

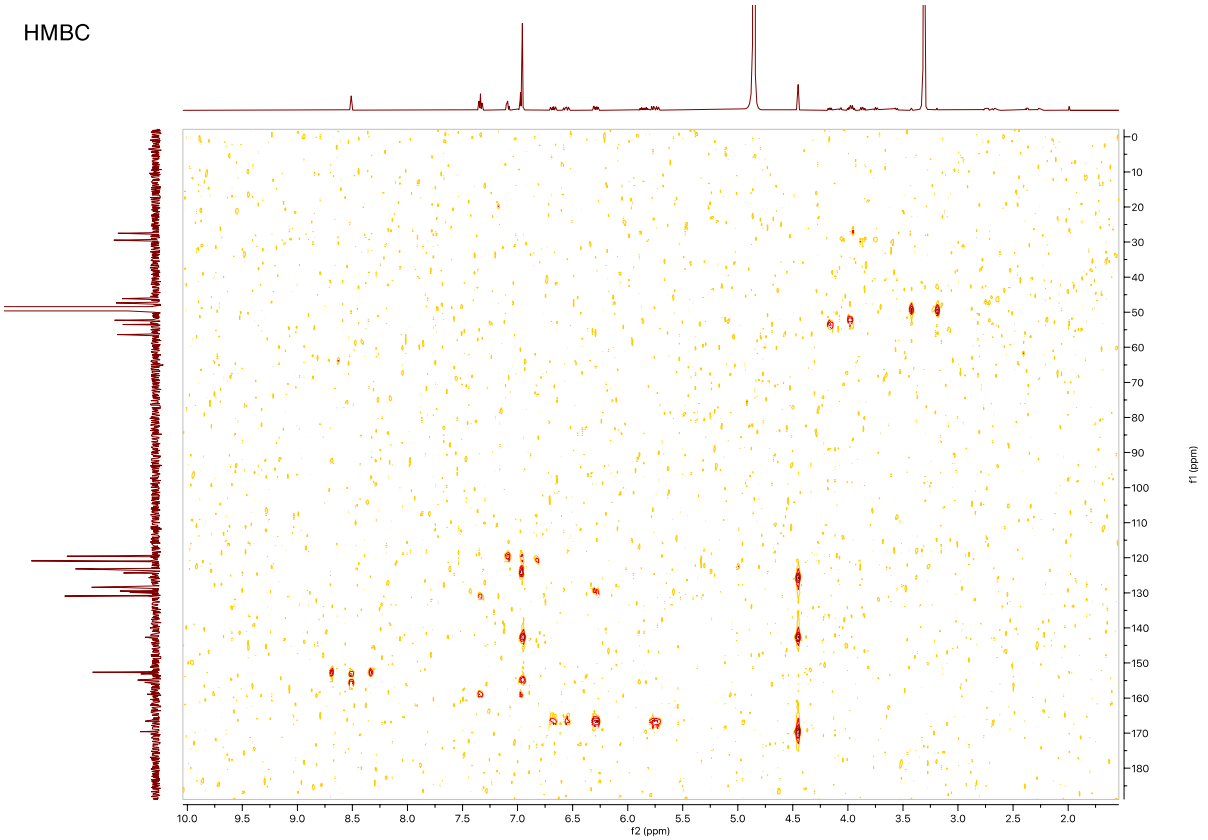

COSY
14a
f2 (ppm)
f1 (ppm)

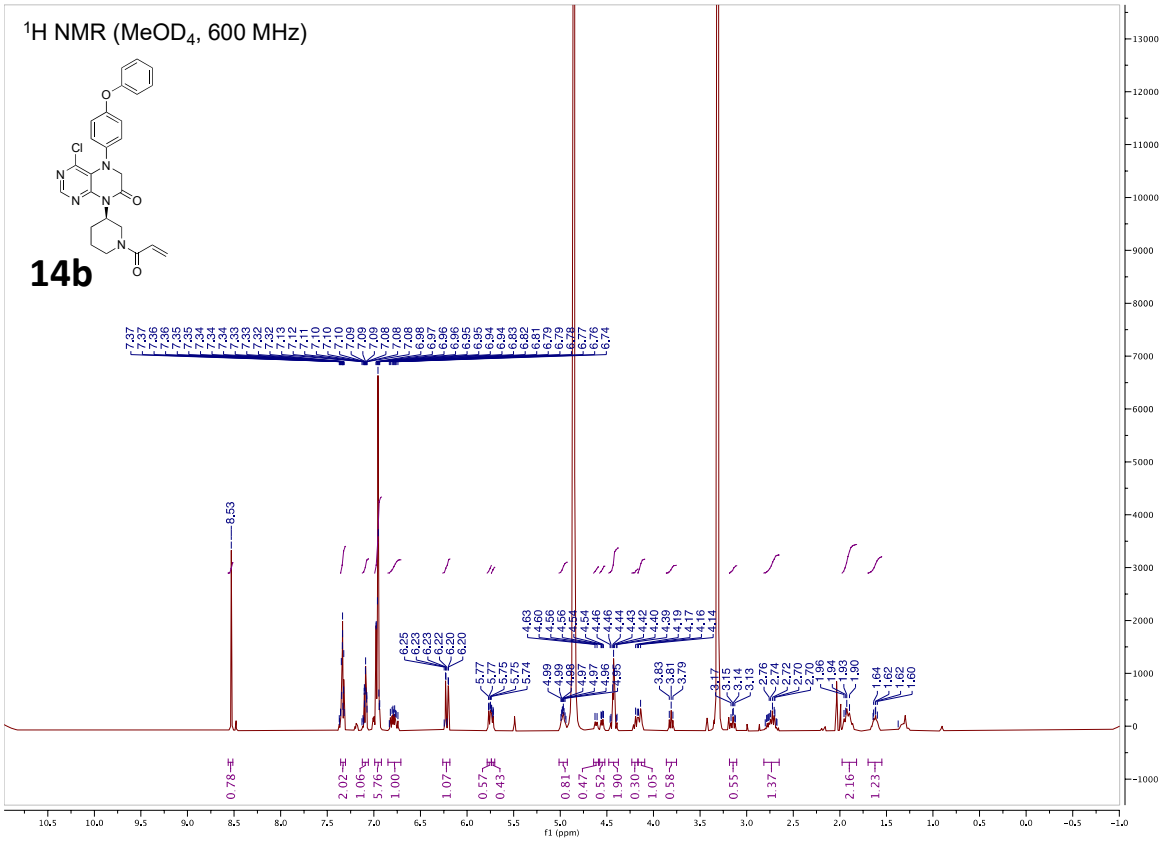

ROESY
f2 (ppm)
f1 (ppm)

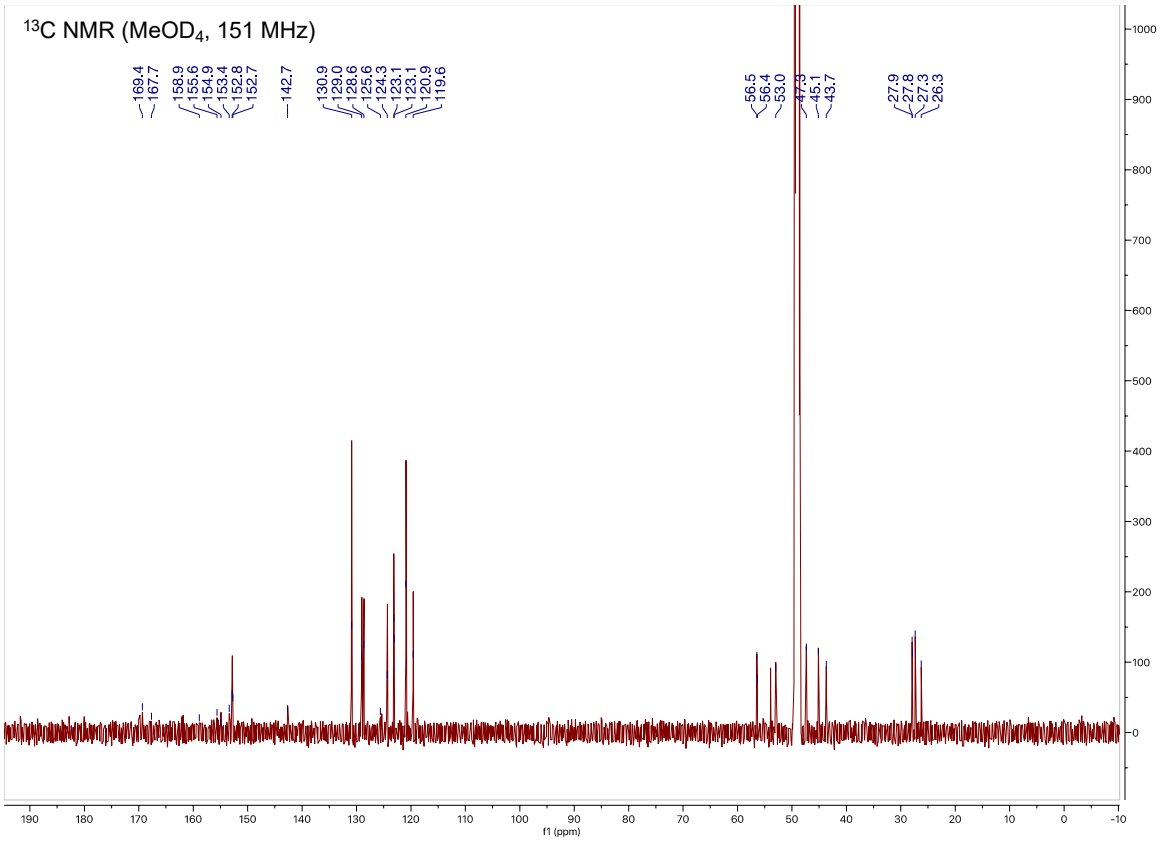
HSQC



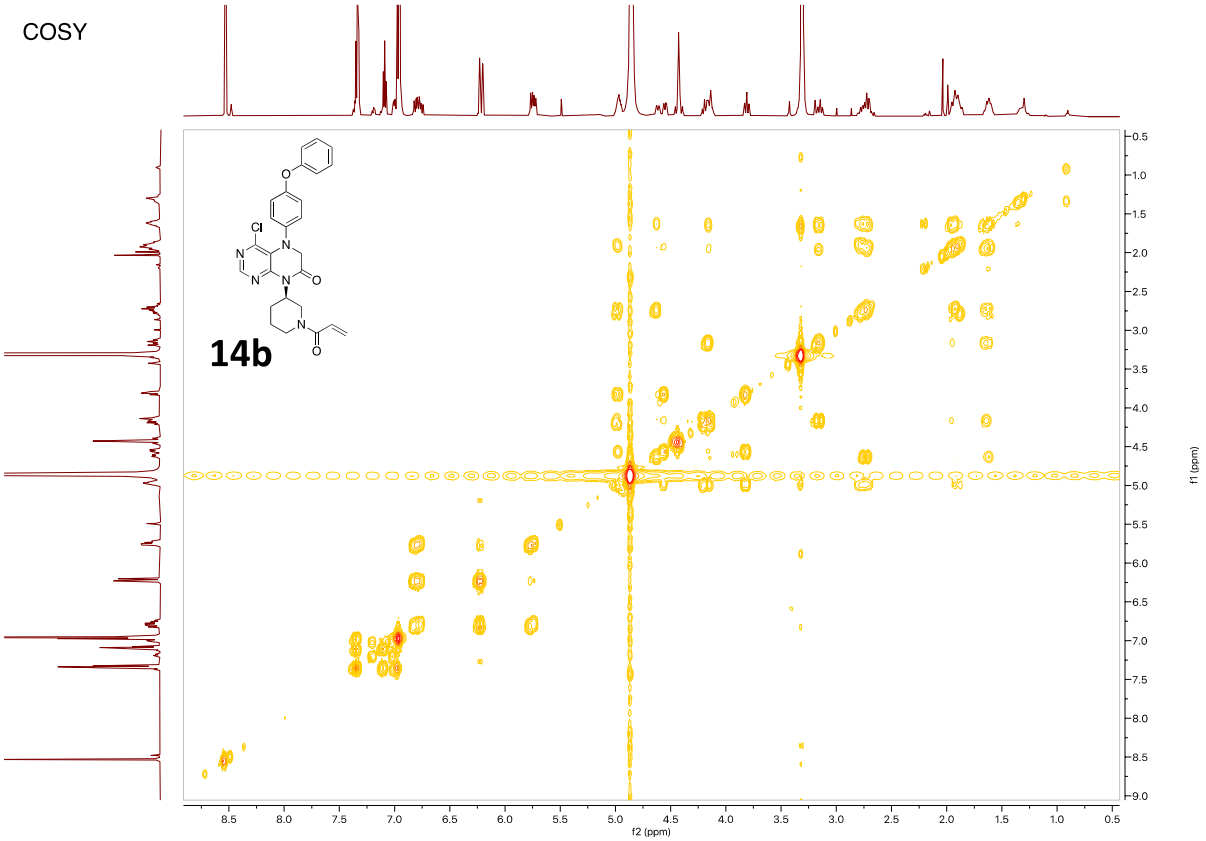
HMBC



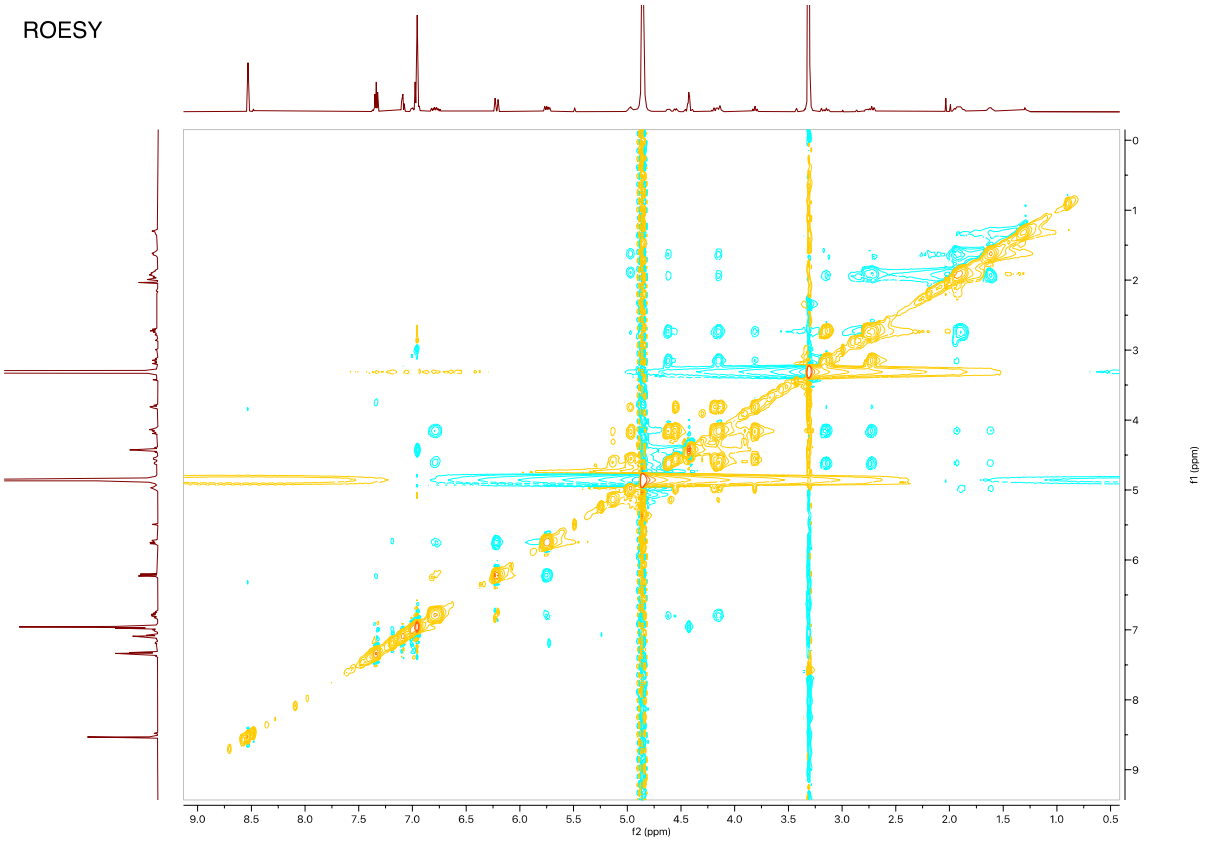

^{1}H NMR (MeOD$_4$, 600 MHz)
14b


^{13}C NMR (MeOD$_4$, 151 MHz)

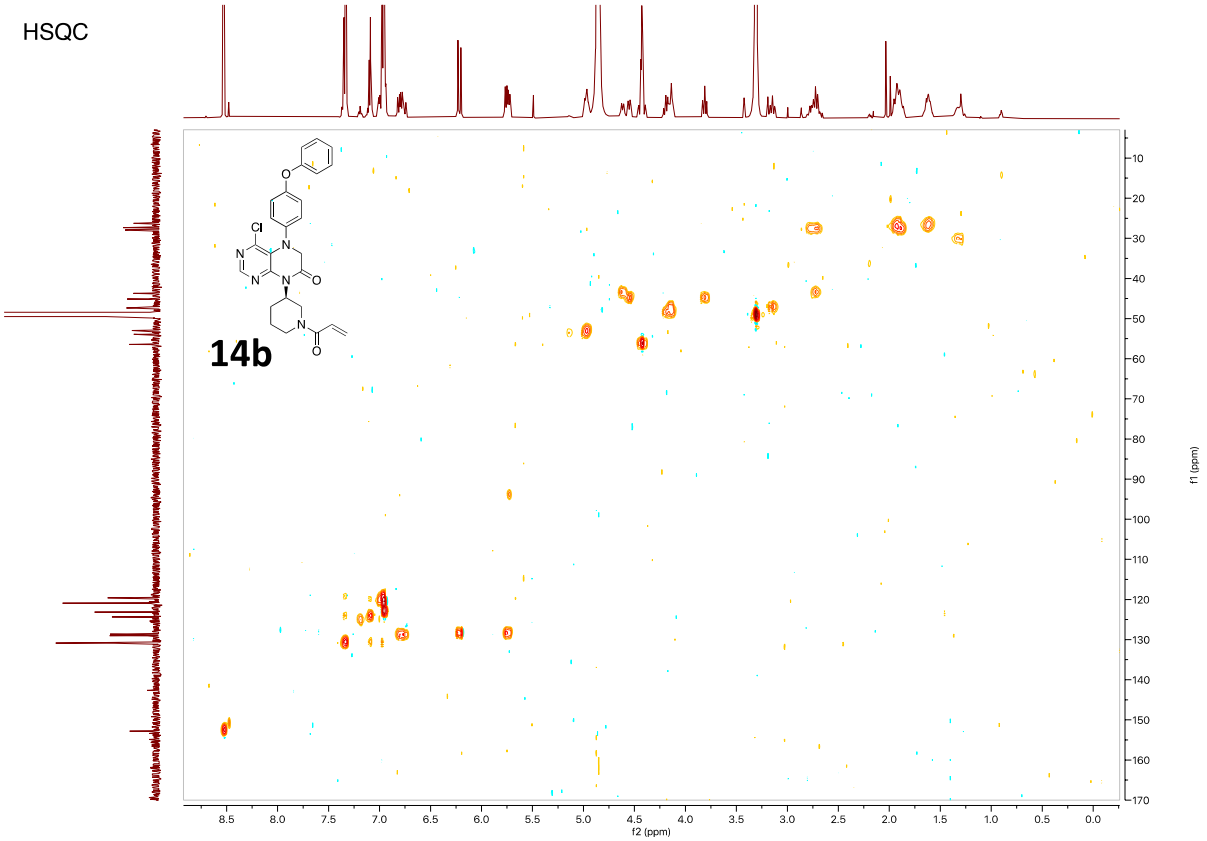
COSY



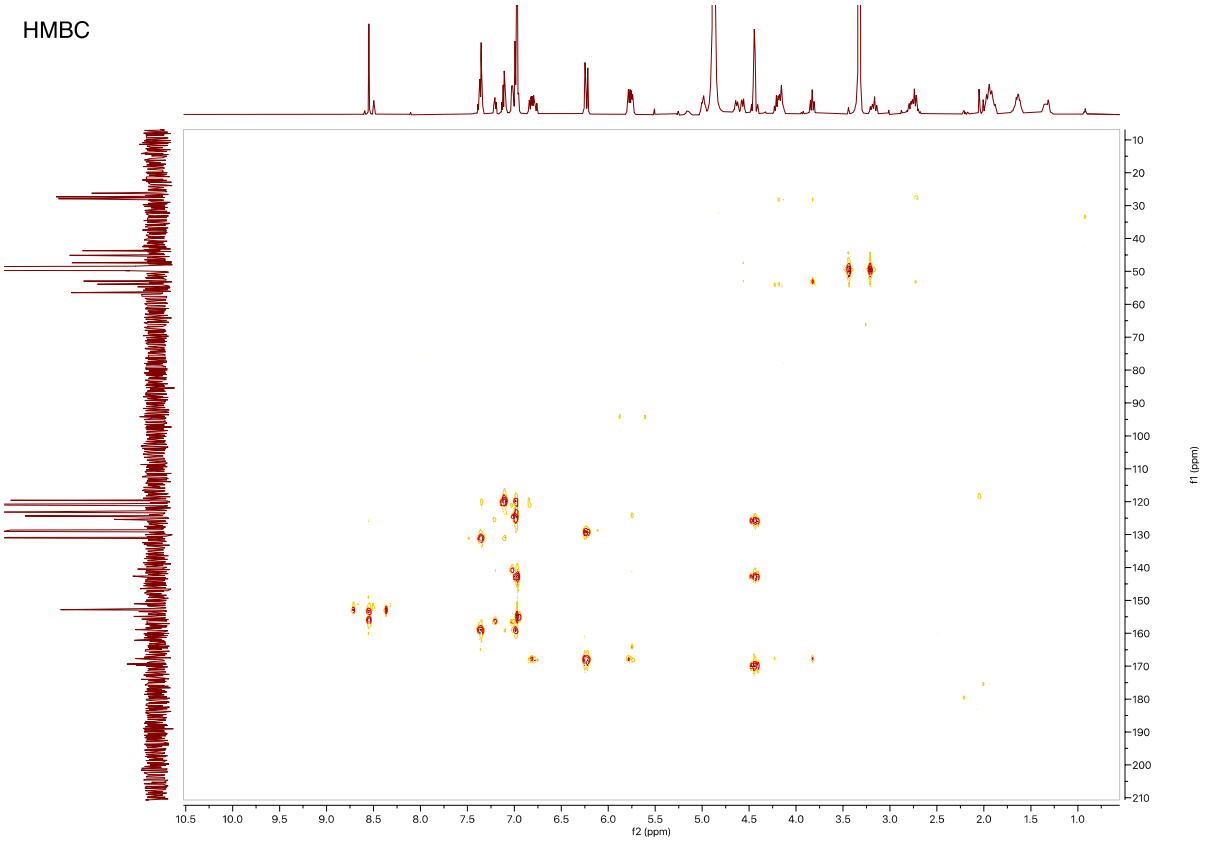
ROESY



HSQC



HMBC



REFERENCES

1. O. Trott and A. J. Olson, *J. Comput. Chem.*, 2009, DOI: 10.1002/jcc.21334, 455-461.
2. R. C. Coll, A. A. B. Robertson, J. J. Chae, S. C. Higgins, R. Muñoz-Planillo, M. C. Inserra, I. Vetter, L. S. Dungan, B. G. Monks, A. Stutz, D. E. Croker, M. S. Butler, M. Haneklaus, C. E. Sutton, G. Núñez, E. Latz, D. L. Kastner, K. H. G. Mills, S. L. Masters, K. Schroder, M. A. Cooper and L. A. J. O'Neill, *Nat. Med.*, 2015, **21**, 248.
3. D. E. O'Brien, F. Baiocchi, R. K. Robins and C. C. Cheng, *J. Med. Pharm. Chem.*, 1962, **5**, 1085-1103.
4. R. Suzuki, A. Hiramatsu, M. Tateyama and H. Sasahara; Ono Pharmaceutical Co., Ltd., US10239879 B2, 2019.