Supporting Information for

Development of an Orally Available and Central Nervous System (CNS)-Penetrant *Toxoplasma gondii* calcium-dependent protein kinase 1 (*Tg*CDPK1) Inhibitor with Minimal Human Ether-à-go-go-Related Gene (hERG) Activity for the Treatment of *Toxoplasmosis*

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Table of Contents

Supplementary Table 1. IC_{50} values of compounds **32** and **33** for 20 representative human kinases.

	32 IC_{50} (nM)	**33** IC_{50} (nM)
ACVR1	>10000	>10000
**AKT1	>10000	>10000
*Alk	>10000	>10000
**Aur1	9300	>10000
**BRAF	>10000	>10000
CAMK1D^	>10000	>10000
CAMK2A^	>10000	>10000
CAMKK2^	>10000	>10000
Ck1alpha1	>10000	>10000
**EGFR	8600	>10000
**Erk2	9200	>10000
**Flt1	>10000	>10000
*Jak3	>10000	>10000
**Kdr	1700	7400
*MAP3K10	>10000	>10000
MEK1	1500	>10000
p38alpha	>10000	>10000
Prkcn	120	280
**Rock1	>10000	>10000
*Zipk	>10000	>10000

^denotes kinases that are most similar to *Tg*CDPK1

**Denotes kinases (**) or close relatives (*) whose inhibition causes cardiotoxicity

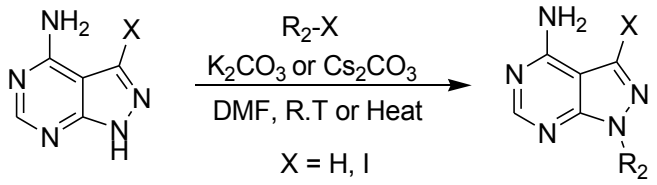
Supplementary Table 2. Crystallographic data and refinement statistics

	*Tg*CDPK + (33)
PDB entry	**4tzr**
Space Group	**$P2_1$**
Unit Cell (a b c Å)	**48.24 73.09 66.24**
(α β γ °)	**90.0 100.38 90.0**
Resolution (Å)	**48.7-2.00 (2.05-2.00)**
Total unique reflections	**30683 (2248)**
Replicate cc(1/2)	**0.998 (0.102)**
Redundancy	**7.4 (7.2)**
Completeness (%)	**100 (99)**
Refinement resolution (Å)	**48.7-2.00**
R /R_{free}	**0.211 / 0.247**
RMSD bonds (Å)	**0.015**
RMSD angles (°)	**1.63**
Protein atoms	**3751**
Non-protein atoms	**162**
TLS groups	**5**
Mean B_{eq} protein atoms ($Å^2$)	**50.1**
Mean B_{eq} ligand atoms ($Å^2$)	**42.3**
Local Ligand Density (LLDF)	**-0.15**

General synthetic procedures

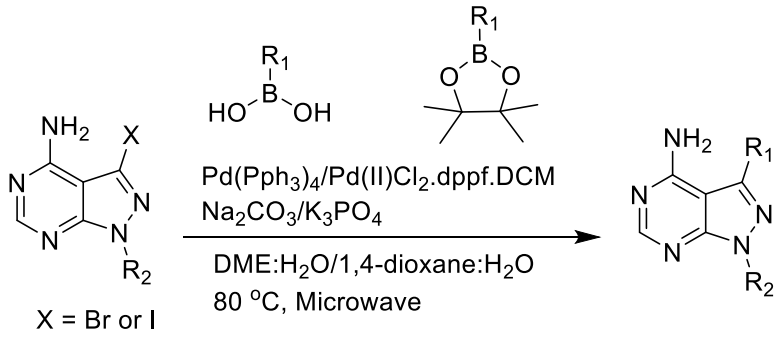
Syntheses and compound characterization data for all intermediates were described below. We reported many synthetic protocols in previous publications[1, 2].

General R_2 alkylation procedure:



Pyrazolopyrimidine (1 equiv.), K_2CO_3 or Cs_2CO_3 or K_2CO_3:NaH_2PO_4 (1.5-2 equiv.), and an alkylhalide (1.1 equiv.) or alkylmesylate (1.1 equiv.) were stirred in dry DMF at room temperature or 80 °C. The reaction was monitored by thin layer chromatography. After completion, ethyl acetate and water were added and the organic phase was separated. The water phase was extracted with ethyl acetate. The combined organic phases were washed with brine, dried over Na_2SO_4 and evaporated under reduced pressure. The crude product was then purified *via* flash chromatography over silica, eluting with either a hexanes/EtOAc or CH_2Cl_2/MeOH gradient. If necessary, further purification was performed with preparatory RP-HPLC.

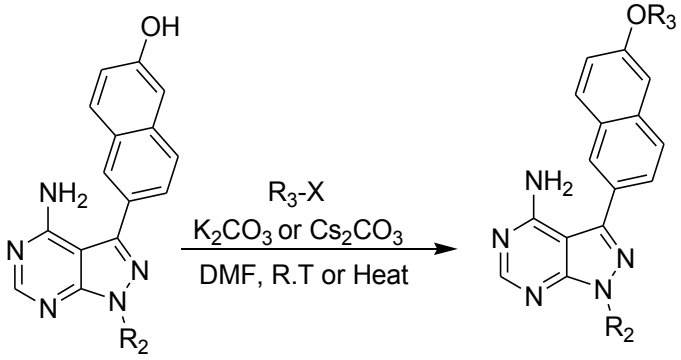
General Suzuki coupling procedure:



3-Iodopyrazolopyrimidines or 3-Bromopyrazolopyrimidines (1 equiv.), Na_2CO_3 or K_3PO_4 (2-4 equiv.), $Pd(PPh_3)_4$ or Pd(II)Cl_2dppf.DCM, (0.05 equiv.), and boronic acids or boronate pinacol esters (1-2 equiv.) were dissolved in a mixture of dimethoxyethane (1.5 mL)

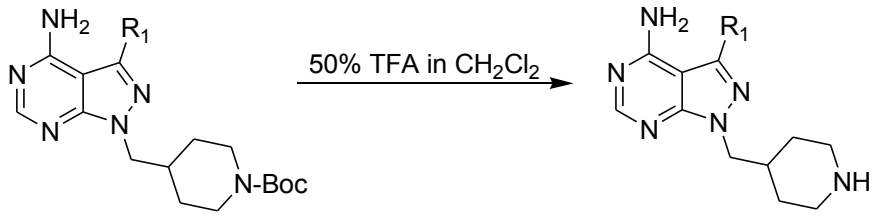
and water (0.5 mL) and then heated in a microwave at 80 °C for one hour. After cooling, ethyl acetate and water were added and the organic phase was separated. The water phase was extracted with ethyl acetate. The combined organic phases were washed with brine, dried over Na_2SO_4 and evaporated under reduced pressure. The crude product was then purified *via* flash chromatography over silica, eluting with either a hexanes/EtOAc or CH_2Cl_2/MeOH gradient. If necessary, further purification was performed with preparatory RP-HPLC.

General naphthol alkylation procedure:



6-Hydroxy-2-naphthalene pyrazolopyrimidines (1 equiv.), K_2CO_3 or Cs_2CO_3 (1.5-2 equiv.), and alkyl halides or epoxides (1.1 equiv.), NaH_2PO_4:K_2CO_3 (1:1 equiv.), were stirred in dry DMF at room temperature or 60-80°C and monitored by thin layer chromatography. After completion, ethyl acetate and water were added and the organic phase was separated. The water phase was extracted with ethyl acetate. The combined organic phases were washed with brine, dried over Na_2SO_4 and evaporated under reduced pressure. The crude product was then purified *via* flash chromatography over silica, eluting with either a hexanes/EtOAc or CH_2Cl_2/MeOH gradient. If necessary, further purification was performed with preparatory RP-HPLC.

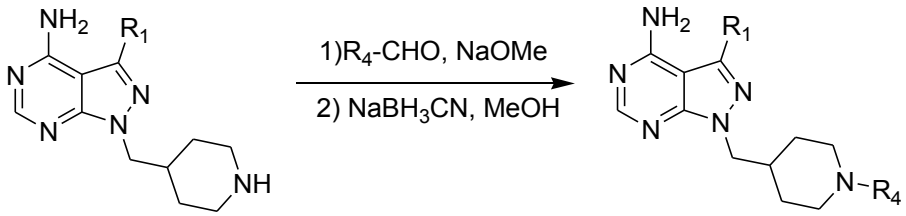
General boc-deprotection procedure:



Boc-amine-containing pyrazolopyrimidines was stirred in a TFA:CH_2Cl_2 (1:1) mixture for ~3 h. The reaction was then concentrated and purified *via* preparatory RP-HPLC. After

HPLC purification, the product was then re-concentrated from 1.25 M HCl in EtOH to afford the final, purified product as a bis-HCl salt.

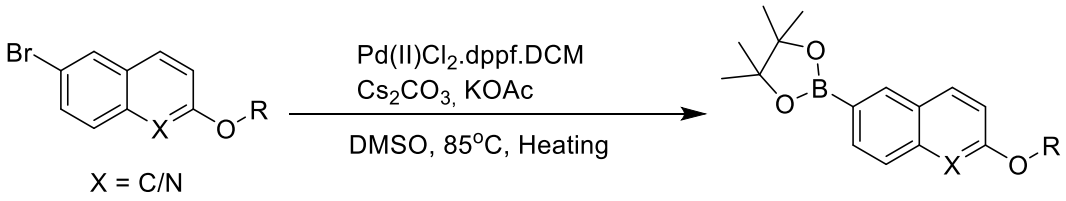
General reductive amination procedure:



Deprotected pyrazolopyrimidines (1 equiv.) were dissolved in methanol and neutralized with sodium methoxide. A solution containing 2% acetic acid and an aldehyde or ketone (5-10 equiv.) was stirred at room temperature for 10 min. Sodium cyanoborohydride (5 equiv.) was then added and the reaction was stirred until reaching completion, as determined by thin layer chromatography (typically ~2 h). The reaction crude was then purified *via* preparatory RP-HPLC. After HPLC purification, the residue was dissolved in a small amount of 2 M HCl in methanol and, after concentration *in vacuo*, the final product was obtained as an HCl salt.

Synthesis and spectral data of various intermediates

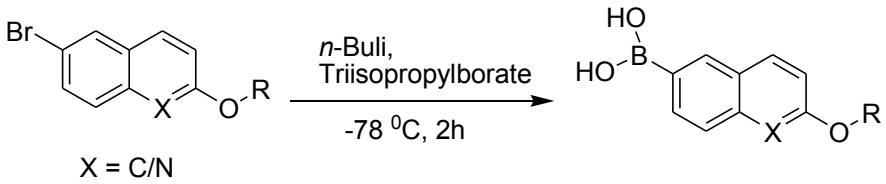
General pinacol ester formation procedure:



Alkylated naphthols or quinolones (1 equiv.), Cs_2CO_3 (1.5-2 equiv.), pinacolatodiborane (2.0 equiv.), Pd(II)Cl_2(dppf).DCM (0.05 equiv.), and KOAc (1 equiv.) in dry DMSO were heated at 85 °C for 5-8 h. After completion, ethyl acetate and water were added and the organic phase was separated. The water phase was extracted with ethyl acetate. The combined organic phases were washed with brine, dried over Na_2SO_4 and evaporated under reduced pressure. The crude

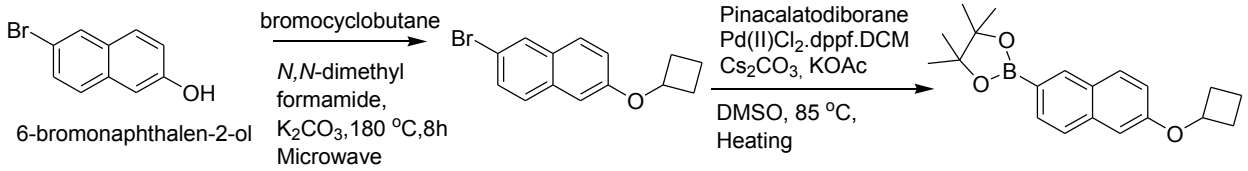
product was then purified *via* flash chromatography over silica, eluting with a hexanes/EtOAc solvent gradient.

General procedure for boronylation using triisopropylborate:



Aryl halides (1 equiv.) and triisopropylborate (1.5 equiv.) were dissolved in tetrahydrofuran:toluene (2:8), cooled to -78 ^{o}C, and *n*-Buli (1.7 equiv.) was added dropwise over 30-40 min. After addition, the reaction was stirred at -78 ^{o}C for 1 h. After 1 h, the reaction was allowed to warm to 0 ^{o}C and stirred for 15-25 min followed by addition of 2N HCl slowly. The organic layer was separated and concentrated *in vacuo* to afford the desired crude product as a white crystalline product or by collecting and washing with water the white crystalline solid that forms upon addition of 2N HCl.

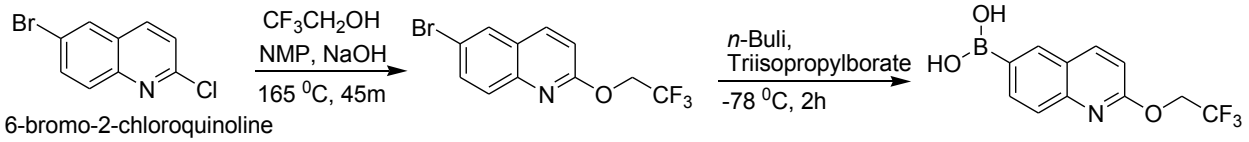
2-(6-Cyclobutoxynaphthalen-2-yl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (38)



2-Bromo-6-cyclobutoxynaphthalene: 6-bromonaphthalene (700 mg, 3.1 mmol), K_2CO_3 (2.140 g, 15.5 mmol), and bromocyclobutane (1.75 mL, 18.6 mmol) in dry DMF were heated at 180 °C in a microwave for 8 h. After completion, ethyl acetate and water were added and the organic phase was separated. The aqueous phase was extracted with ethyl acetate (2x10mL). The combined organic phases were washed with brine, dried over Na_2SO_4 and evaporated under reduced pressure. The crude product was then purified *via* flash chromatography over silica, eluting with a hexanes/EtOAc solvent gradient to afford 693 mg (80% yield) of pure product. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.88 (s, 1H), 7.65-7.43 (m, 3H), 7.10 (dd, 1H), 6.94 (s, 1H), 4.74 (m, 1H), 2.58-2.45 (m, 2H), 2.30-2.13 (m, 2H), 1.95-1.69 (m, 2H); MS (ESI) 278.5 *m/z* [MH^+], $C_{14}H_{14}BrO$ requires 278.2.

2-(6-Cyclobutoxynaphthalen-2-yl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane: 2-Bromo-6-cyclobutoxynaphthalene and pinacolatodiborane were subjected to ***General pinacol ester formation procedure*** to afford the desired pure product (631 mg, 60% yield); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.26 (s, 1H), 7.93-7.61 (m, 3H), 7.2-6.93 (m, 2H), 4.80 (m, 1H), 2.67-2.36 (m, 2H), 2.41-2.11 (m, 2H), 2.01-1.60 (m, 2H), 1.37 (s, 12H); MS (ESI) 325.1 *m/z* [MH^+], $C_{20}H_{26}BO_3$ requires 325.1.

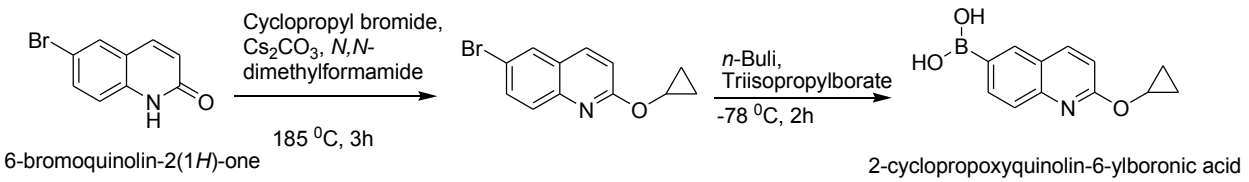
Synthesis of 2-(2,2,2-Trifluoroethoxy)quinolin-6-ylboronic acid (39)



6-Bromo-2-(2,2,2-trifluoroethoxy)quinolone: 6-bromo-2-chloroquinoline (1.00 g, 4.1 mmol), CF_3CH_2OH (0.95 mL, 12.3 mmol), *N*-methylmorpholine (12 mL), and NaOH (330 mg, 8.2 mmol) were taken in microwave tube and then heated at 165 ^{o}C for 45 min. After addition of water, the reaction mixture was extracted with ethyl acetate (3x20 mL). The organic layer was washed with brine, dried over sodium sulfate, and evaporated under reduced pressure. The crude compound was then taken to the next step without further purification.

Synthesis of 2-(2,2,2-Trifluoroethoxy)quinolin-6-ylboronic acid: 6-Bromo-2-(2,2,2-trifluoroethoxy) quinoline was subjected to the ***General procedure for boronylation using triisopropylborate*** to afford a white crystalline product (354 mg, 80% yield,). ^{1}H NMR (300 MHz, CD_3OD) δ 6.91 (s, 1H), 6.82 (d, J = 8.5 Hz, 1H,), 6.45 (s, 2H) 6.61 (d, J = 7.04 Hz, 1H), 3.5 (q, J = 8.7Hz, 2H,); MS (ESI) 272.4 *m/z* [MH+], $C_{11}H_{10}BF_3NO_3$ requires 272.2.

2-Cyclopropoxyquinolin-6-ylboronic acid (40)

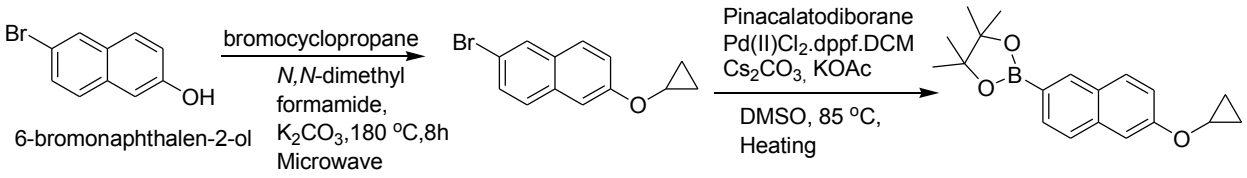


6-Bromo-2-cyclopropoxyquinoline: 6-Bromo-quinolin-2(1*H*)-one (1.00 g, 4.4 mmol, 1 equiv.), Cs_2CO_3 (5.08 g, 17.8 mmol), and bromocyclopropane (1.06 g, 13.3 mmol) in dry DMF (10 mL)

were heated at 180 °C in a microwave for 3 h. After completion, ethyl acetate and water were added and the organic phase was separated. The water phase was further extracted with ethyl acetate. The combined organic phases were washed with brine, dried over Na_2SO_4 and evaporated under reduced pressure. The crude product was then purified *via* flash chromatography over silica, eluting with a hexanes/EtOAc solvent gradient to afford 0.235 mg (20% yield) of pure product. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.89 (s, 1H), 7.87-7.82 (m, 1H), 7.78-7.65 (m, 2H), 6.88 (d, J = 8.91 Hz, 1H), 4.54-4.44 (m, 1H), 0.94-0.77 (m, 4H); MS (ESI) 265.5 *m/z* [MH^+], $C_{12}H_{11}BrNO$ requires 265.2.

2-Cyclopropoxyquinolin-6-ylboronic acid: 6-Bromo-2-cyclopropoxyquinoline (2.01 g, 7.95 mmol, 1 equiv.) and triisopropylborate (2.05 mg, 13.5 mmol, 1.69 equiv.) were subjected to ***General procedure for boronylation using triisopropylborate*** to afford the desired pure product (1.05 g, 80% yield); ^{1}H NMR (300 MHz, DMSO) δ 8.37-8.28 (m, 2H), 8.10-8.04 (m, 1H), 7.77 (d, J = 8.5 Hz, 1H), 7.06 (d, J = 8.9 Hz, 1H), 4.55-4.45 (m, 1H), 0.91-0.81 (m, 2H), 0.80-0.73 (m, 2H); MS (ESI) 230.2 *m/z* [MH^+], $C_{12}H_{13}BNO_3$ requires 230.2.

2-(6-Cyclopropoxynaphthalen-2-yl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (41)

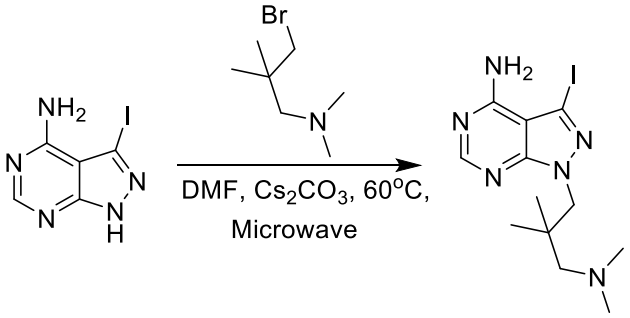


2-Bromo-6-cyclopropoxynaphthalene: 6-Bromonaphthalen-2-ol (3.00 g, 13.0 mmol), Cs_2CO_3 (1.29 g, 39.6 mmol) and bromocyclopropane (4.07 g, 39.0 mmol) were taken in a microwave tube and heated at 180 ^{o}C for 30 min. After completion, ethyl acetate and water were added and the organic phase was separated. The water phase was further extracted with ethyl acetate. The combined organic phases were washed with brine, dried over Na_2SO_4 and evaporated under reduced pressure. The crude product was then purified *via* flash chromatography over silica, eluting with a hexanes/EtOAc solvent gradient to afford 2.50 g (71% yield) of pure product. ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 7.91 (m, 1H), 7.66-7.58 (dd, J = 8.9, 4.6 Hz, 2H), 7.53-7.46

(dd, *J* = 8.7, 1.9 Hz, 1H), 7.39 (d, *J* = 2.1 Hz, 1H), 7.18-7.12 (dd, *J* = 8.9, 2.3 Hz, 1H), 3.83 (m, 1H), 0.87-0.78 (m, 4H); MS (ESI): 264.2 *m/z* [MH+], $C_{13}H_{12}BrO$ requires 264.2.

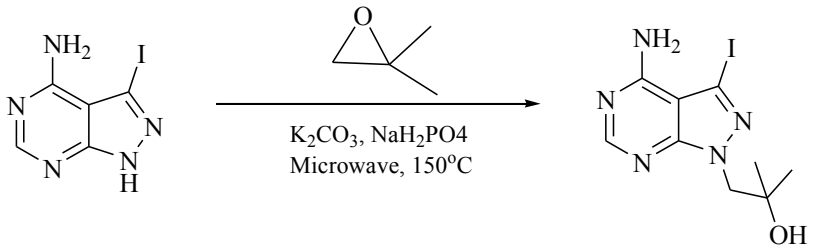
2-(6-Cyclopropoxynaphthalen-2-yl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane: 2-Bromo-6-cyclopropoxynaphthalene was subjected to the ***General pinacol ester formation procedure*** to afford 1.01 g, (65% yield) of a white crystalline product. ^{1}H NMR (300 MHz, $CDCl_3$): δ ppm 8.27 (s, 1H), 7.77 (m, 3H) 7.45 (d, *J* = 2.3 Hz, 1H), 7.14 (dd, *J* = 8.9, 2.48 Hz, 1H), 3.88 (m, 1H), 1.40 (s, 12H), 0.87 (m, 2H), 0.82 (m, 2H); MS (ESI): 311.5 *m/z* [MH+], $C_{19}H_{24}BO_3$ requires 311.2.

1-(3-(Dimethylamino)-2,2-dimethylpropyl)-3-iodo-1H-pyrazolo[3,4-d]pyrimidin-4-amine (42)



42 was generated with 3-iodo-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine[1] and 3-bromo-*N,N*,2,2-tetramethylpropan-1-amine using the ***General R_2 alkylation procedure,*** to afford **42** as pale yellow solid, (286 mg, 40% yield); ^{1}H NMR (300 MHz, MeOD4) δ 8.38 (s, 1H), 4.46 (s, 2H), 3.22 (s, 2H), 3.06 (s, 6H), 1.16 (s, 6H); MS (ESI) 375.2 [MH+], $C_{12}H_{20}IN_6$ requires 375.2.

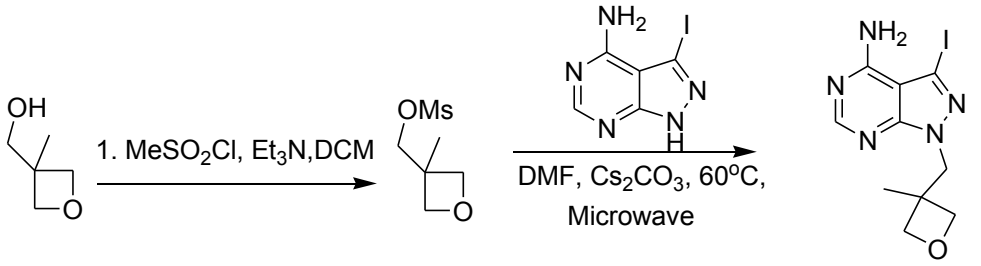
1-(4-Amino-3-iodo-1H-pyrazolo[3,4-d]pyrimidin-1-yl)-2-methylpropan-2-ol (43)



43 was generating using the ***General R_2 alkylation procedure*** using 3-iodo-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine[1,2] (500 mg, 1.92 mmol), 2,2-dimethyloxirane (0.276 mg, 3.80 mmol), and K_2CO_3:NaH_2PO_4 (0.262 mg, 1.90 mmol) in 3 mL of a acetonitrile:water (8.5:1.5) mixture. The reaction was stirred at 150 °C for 3 h in a microwave, affording **43** (150 mg, yield, 23.4% yield)

as a white solid after purification using a methanol/dichloromethane solvent gradient. ^{1}H NMR (300 MHz, CD_3OD) δ 8.26 (s, 1H), 4.37 (s, 2H), 1.29 (s, 6H). MS (ESI) *m/z* 334.2 [MH^+], $C_9H_{13}IN_5O$ requires 334.1.

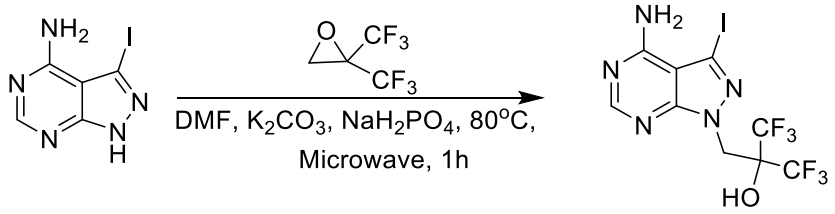
3-Iodo-1-((3-methyloxetan-3-yl)methyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (44)



Methanesulfonyl chloride (7.54 mL, 97.9 mmol) was added slowly at 0 ^{o}C to a solution of (3-methyloxetan-3-yl)methanol (5.00 mg, 48.9 mmol) and triethylamine (13 mL, 97 mmol) in dichloromethane (20 mL). The reaction was stirred for 5 h at room temperature. After completion of the reaction, dichloromethane was removed by reduced pressure and the reaction was diluted with ethyl acetate. The ethyl acetate was washed with $NaHCO_3$ (25 mL), 1N HCl, brine (50 mL), dried over sodium sulfate, and concentrated. The crude product was subjected to flash chromatography using a hexane/ethyl acetate solvent gradient to afford pure 3-methyloxetan-3-yl)methyl methanesulfonate (4.40 g, 50% yield). ^{1}H NMR (301 MHz, $CDCl_3$) δ 4.70-4.12 (m, 6H), 3.07 (s, 3H), 1.39 (s, 3H); MS (ESI) 181.1 [MH+], $C_6H_{13}O_4S$ requires 181.0.

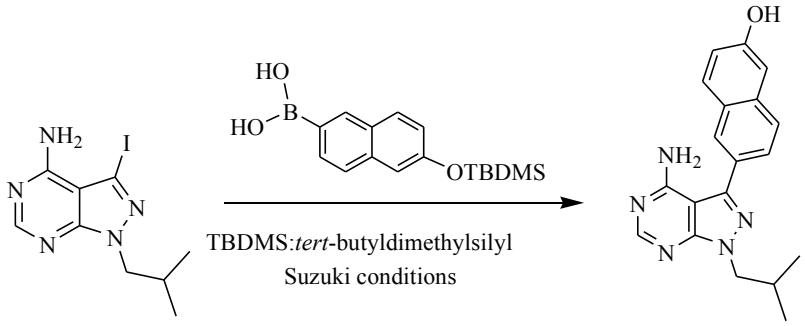
44 was generated by subjecting 3-iodo-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine[1,2] (150 mg, 0.60 mmol) and (3-methyloxetan-3-yl)methyl methanesulfonate (83 mg, 0.46 mmol) to the ***General R_2 alkylation procedure*** (110 mg, 55% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.33 (s, 1H), 5.94 (s, 2H), 4.79 (d, *J* = 6.4 Hz, 2H), 4.56 (s, 2H), 4.41 (d, *J* = 6.1 Hz, 2H), 1.28 (s, 3H); MS (ESI) *m/z* 346.2 [MH^+], $C_{10}H_{13}IN_5O$ requires 346.1.

2-((4-Amino-3-iodo-1H-pyrazolo[3,4-d]pyrimidin-1-yl)methyl)-1,1,1,3,3,3-hexafluoropropan-2-ol (45)



45 was generated by subjecting 3-iodo-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine[1,2] (250 mg, 0.96 mmol), 2,2-bis(trifluoromethyl)oxirane (0.17 mL, 1.44 mmol), and K_2CO_3:NaH_2PO_4 (198 mg, 1.44 mmol) to the ***General R_2 alkylation procedure*** (126 mg, 30% yield). ^{1}H-NMR (301 MHz, $CDCl_3$) δ 8.33 (s, 1H), 8.02 (s, 1H), 6.33 (s, 2H); MS (ESI) *m/z* 442.1 [MH^+], $C_9H_6F_6IN_5O$ requires 442.2.

6-(4-Amino-1-isobutyl-1H-pyrazolo[3,4-d]pyrimidin-3-yl)naphthalen-2-ol (46)



6-*Tert*-butyldimethylsilyloxy-2-naphthaleneboronic acid and 3-iodo-1-isobutyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine[2] were subjected to the ***General Suzuki coupling procedure.*** The crude product was purified by silica gel using dichloromethane/methanol gradient (note: deprotection of the *tert*-butyldimethylsilyloxy protecting group was observed after purification). ^{1}H NMR (300 MHz, CD_3OD) δ 8.27 (s, 1H), 8.03 (s, 1H), 7.82 (m, 2H), 7.71 (m, 1H), 7.18 (m, 2H), 4.21 (d, J = 4.2 Hz, 2H), 2.39 (m, 1H), 0.96 (d, J = 6.5 Hz, 6H); MS (ESI) 334.4 *m/z* [MH+], $C_{19}H_{19}N_5O$ requires 334.2.

Biological Procedrues

***Tg*CDPK1 Enzymatic Inhibition Assay.** A modified protocol from a previously reported study was used.[1] Inhibitors were evaluated in triplicate in eight-point dilutions (3-fold dilutions) during the enzymatic reactions. *Tg*CDPK1 enzymatic inhibition was determined with a coupled luciferase assay (Kinaseglo®). 2.1 nM *Tg*CDPK1 and 20 μM BioSyntide-2 (Biotin-C6-

PLARTLSVAGLPGKK (American Peptide Company, Inc. Sunnyvale, CA)) were incubated in 25 μL of buffer containing 1 mM EGTA (pH 7.2), 10 mM $MgCl_2$, 20 mM HEPES, pH 7.5 (KOH), 0.1% BSA, and 2 mM $CaCl_2$. The reaction was initiated with the addition of ATP at a 10 μM final concentration. After incubating at 30 °C for 90 min., changes in ATP concentration were determined by adding Kinaseglo® luciferase reagent (Promega, Madison, WI) and measuring luminescence with a MicroBeta2 multi-label plate reader (Perkin Elmer, Waltham, MA). Results were converted to percent inhibition, and IC_{50} values were calculated using nonlinear regression analysis in GraphPad Prism.

Src kinase enzymatic inhibition assay. A modified protocol from a previously reported study was used.[1] Inhibitors were evaluated in triplicate in eight-point dilutions (3-fold dilutions) during the enzymatic reactions. Chicken Src enzymatic inhibition was determined with a coupled luciferase assay (Kinaseglo®). 2 nM Src and 61 μM Src substrate peptide (sequence Ac-EIYGEFKKK, GenScript, Piscataway, NJ) were incubated in 25 μL of buffer containing 40 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM DTT, and 0.1% BSA. The reaction was initiated with the addition of ATP at a 10 μM final concentration. After incubating at 30 °C for 90 min., changes in ATP concentration were determined by adding Kinaseglo® luciferase reagent (Promega, Madison, WI) and measuring luminescence with a MicroBeta2 multi-label plate reader (Perkin Elmer, Waltham, MA). Results were converted to percent inhibition, and IC_{50} values were calculated using nonlinear regression analysis in GraphPad Prism.

Human Cell Growth Inhibition Assay. A modified protocol from a previously reported study was used.[1] CRL-8155 human lymphocytic cells (ATCC, WIL2-NS) were cultured in RPMI-1640 growth medium supplemented with 10% heat inactivated fetal bovine serum, 10 mM HEPES, 1

mM sodium pyruvate, and 1 mM L-glutamine. The Alamar Blue® assay (Invitrogen, Grand Island, NY), which measures *General* cellular metabolism, was used to quantify cell growth. Mid-log cells were seeded in 96-well flat-bottom plates (Corning, Corning, NY) at a density of $3x10^5$ cells/mL containing test compounds at six final concentrations (40 µL, 20 µL, 10 µL, 5 µL, 2.5 µL, and 1.25 µL) in quadruplicate and grown at 37°C for 48 hours in a 5% CO_2 humidified incubator. A 1/10th volume of Alamar Blue® developing reagent was added to each well and incubated for an additional 3 hours and fluorescence was measured at the respective excitation and emission wavelengths of 560 nm and 590 nm in a FLx800 microplate reader (Biotek, Winooski, VT). Percent growth inhibition by test compounds was calculated based on DMSO vehicle and positive controls (50 µL quinacrine), which corresponded to 0% and 100% growth inhibition, respectively.

***T. gondii* growth inhibition assay.** The *T. gondii* growth inhibition assay was performed according to a previously reported procedure.[1] Briefly, a dilution series of an inhibitor (diluted in DMSO) was added to DMEM (final DMSO = 0.5%). *T. gondii* expressing a β-galactosidase reporter were added to the DMEM and incubated briefly before adding them to fibroblast monolayers in 96 well plates. Plates were visually inspected for evidence of cytotoxic effects on fibroblasts. After 44 h, β-galactosidase was assayed using chlorophenol red β-galactopyranose (Sigma-Aldrich, St. Louis, MO) as a substrate. Each dilution series of inhibitor was tested at least twice in triplicate. For assays with drug resistant *T. gondii*, the same procedure was followed except cell lines expressing HA-*Tg*CDPK1 or HA-Gly128Met *Tg*CDPK1 in a "wild type" background were tested.

***In vivo* efficacy against acute *T. gondii* infection in mice.** Infection and drug administration were performed as previously reported (performed under IACUC ACORP number is 3452-14).[3] Mice were infected with type 1 RH strain *T. gondii* expressing a yellow fluorescent protein. *T.gondii* were harvested from human foreskin fibroblasts, passed through a 3-μm-pore filter, and 10^5 tachyzoites were inoculated in a volume of 100 μl of phosphate-buffered saline (PBS) intraperitoneally (i.p.) into 4- to 5-week-old, 25-g female CF-1 mice. The compounds were dissolved in polyethylene glycol (PEG) 400 and administered by oral gavage 48 h after inoculation. The control group received PEG 400 only. Groups consisted of 4 mice. After mice were euthanized on the eighth day, the brain and spleen were collected from the mice and peritoneal lavage was performed with 3 ml of PBS (pH 7.4).

In vivo efficacy was evaluated with quantitative real-time PCR for *T. gondii* DNA from the brain and spleen, and quantification of peritoneal *T. gondii* infection as previously described.[15,35] A sample of 10 μl of peritoneal lavage fluid was examined in a hemocytometer using fluorescence microscopy (excitation/emission 480/535 nm). Yellow-fluorescent tachyzoites were quantified per mL of fluid. After the mice were euthanized, the entire brain and spleen were collected and homogenized. DNA was isolated with a DNA purification kit (Qiagen, Germantown, MD). 300 ng of total DNA from the brain homogenate and 200 ng of total DNA from the spleen homogenate were analyzed per mouse. A standard curve was generated from DNA purified from *T. gondii* tachyzoites in 10-fold dilutions from 160 ng to 1.6 fg of DNA. Quantitative real-time PCR was performed in duplicate using a 7300 Real-Time PCR System (Applied Biosystems, Grand Island, NY) with iTaq SYBR GREEN PCR Supermix and primers for the *T. gondii* 529-bp repeat element (sense 5'-AGG AGA GAT ATC AGG ACT GTA G-3' and anti-sense 5'-GCG TCG TCT CGT CTA GAT CG-3').[15] Results were quantified as *T. gondii* DNA per total DNA.

Analysis was performed with GraphPad Prism 5.0 software. This protocol was approved by the institutional animal care and use committee of the Portland Veterans Administration Medical Center.

Treatment of Latent *Toxoplasma* Infections in Mice. A modified protocol from a previously reported study was used.[5] 65 seven-wk-old CBA/J strain mice (Jackson Laboratory) were injected i.p. with 200 μL of sterile PBS containing 18 cysts of ME49 strain *T. gondii* (type II genotype) from infected CBA/J donor mice. Five weeks post-infection, mice were treated by oral gavage daily for 14 days with 100 μL of vehicle (polyethylene glycol (PEG) 400), by oral gavage daily for 14 days with 100 μL of 30 mg/kg **32** dissolved in PEG 400, or by oral gavage twice (days 1 and 7) with 100 μL of 105 mg/kg **32** dissolved in PEG 400. Two weeks after the final injection mice were euthanized humanely. Mouse brains were placed in 1 mL of sterile PBS and individually minced with scissors, vortexed, and, homogenized by three or four passages through a 22-G needle and syringe. Three 10-μL samples (30 μL total) of each brain homogenate were mixed on a glass microscope slide, smeared, air dried, fixed with ethanol for 15 min, dried further, and then mounted on a coverslip in Eukitt mounting medium (Sigma-Aldrich). Penicillin and streptomycin (Gibco) (50 U/mL and 50 μg/mL final concentrations, respectively) were added to remaining brain homogenate for storage at 4 °C and resampling as necessary. Cysts were enumerated by phase-constrast microscopy without knowledge of the sample's treatment group. This protocol was approved by the Committee on the Use and Care of Animals of the University of Michigan.

Physiochemical procedures

Pharmacokinetic analysis in mice. For mouse oral PK studies, three female BALB/c mice (10 to 12 weeks old) were used in each group (performed under IACUC protocol number 2145-01

(UW, Seattle)). Each group received a test compound at a dose of 10 mg/kg body weight dissolved in 3% ethanol/7% Tween 80/90% normal saline by oral gavage. Blood samples were taken at the designated time points by tail bleeding and centrifuged to obtain plasma. The samples were frozen at -20 °C. The test compounds were extracted from the plasma samples using acetonitrile/0.1% formic acid with an internal standard. A standard mix of all test compounds was prepared for comparison and quantification. The compounds were quantified by LC/MS analysis. PK calculations were performed using Phoenix WinNonlin software (Pharsight).

Pharmacokinetic analysis in rats. Test compound was administered to Sprague-Dawley jugular canulated rats (Charles River) by either oral gavage or IV injection followed by blood sampling from the jugular vein at designated time points (performed under IACUC protocol number 2145-01 (UW, Seattle)). The oral dose was administered to each rat at 20 mg/kg for compound **32** and 5 mg/kg for compound **33** at time = 0 in a 1 mL volume of dosing solution (7% Tween 80, 3% EtOH, 5% DMSO, 0.9% saline.) IV injections were administered at 5 mg/kg from time = 0 to 3 minutes in a 1 mL volume of dosing solution, and blood was sampled at the same time points *via* the jugular vein. Experiments were performed with groups of 2 rats each for the oral and IV dosing. Plasma was separated and extracted with acetonitrile and quantified by LC/MS analysis. PK calculations were performed using Phoenix WinNonlin software (Pharsight).

Pharmacokinetic analysis in calves. Previously described procedures were used for calf enrollment, housing, management, and for test compound administration (performed under WSU IACUC # ASAF 04477 “Novel Therapeutics for Cryptosporidiosis and other Parasites: Bumped Kinase Inhibitors”).[6] Male Holstein calves were manually caught during parturition. Calves were

fed 4 L of ≥50 g IgG/L commercial colostrum replacer (Bovine IgG, Colostrum Replacement, Land O'Lakes Inc., St. Paul, MN) within the first 4 h of life *via* an oroesophageal feeding tube to ensure delivery of the entire volume. Adequacy of passive transfer of colostral IgG was determined *via* comparison serum total protein measurements at birth and 24 h after feeding of the colostrum replacer. Beginning at 12 h of age, calves were fed commercial 22% protein/20% fat non-medicated milk replacer (Nursing Formula NT Calf Milk Replacer, Land O'Lakes Inc.) with at least 0.62 kg of dry matter per day, split into 2 feedings (12 h apart), for the duration of the study. At 48 h of life, calves had a jugular catheter placed aseptically and 1.5 mL of blood was collected. After placement of the jugular catheter, **32** (10 mg/kg) or **33** (9.3 mg/kg) was delivered *via* an oral dosing syringe to ensure complete delivery of the test compounds. Following ingestion of drug, 1.5 mL of blood was collected from the jugular catheter at the following intervals: 0.5 h, 1 h, 1.5 h, 2 h, 4 h, 8 h, 12 h, 24 h, 72 h, 120 h, 168 h, and 288 h. Plasma was separated and extracted with acetonitrile and quantified by LC/MS analysis. PK calculations were performed using Phoenix WinNonlin software (Pharsight).

Pharmacokinetic analysis in dogs and monkeys. In each study, groups of three animals received a 1 mg/kg intravenous or oral dose of compound **32**. The dose was administered as a solution in PEG-400 containing 10% DMSO (0.5 ml/kg dose volume); the oral dose solution was placed in a capsule just prior to dosing. Blood samples for plasma concentration analysis were obtained from each animal for 24 hours after dosing. Plasma concentrations of parent drug were determined by LC-MS/MS. All studies involving vertebrate animals at AbbVie were approved by an animal ethics approval committee (IACUC).

Distribution of compounds between mouse plasma and brain. Mice were injected with **32** or **33** (5 mg/kg IP) and sacrificed at the indicated times for collection of plasma and brain (performed under IACUC protocol number 2145-01 (UW, Seattle)). Compound was dissolved in 0.4 mL of dosing solution (7% Tween 80, 3% ethanol, 5% DMSO, 0.9% saline) for IP injections. The brains were weighed and immediately frozen, then later homogenized in acetonitrile. Prior to animal studies, recovery of test compound was carried out by adding a known amount to a mouse brain in the test extraction solvent and performing the homogenization. Compound recovery was determined by liquid chromatography/tandem mass spectrometry analysis relative to a standard compound amount. Blood was taken from the same mice in heparinized capillary tubes for determination of compound concentration in plasma. The concentration of compound in the brain was obtained by dividing the moles of compound in the brain by the brain volume (obtained from the brain weight assuming 1 g is 1 mL) and correcting for the brain vasculature volume of 3% by weight.

Aqueous Solubility Assay. All compounds were tested in pH 6.5 PBS buffer. For the sample vial: 199 μL of pH = 6.5 PBS buffer was added to the vial, then 1 μL of 20 mM dimethyl sulfoxide stock solution was added. The vial was capped and vigorously shaken to mix the sample thoroughly, and then the sample was incubated at 25 °C overnight. The vial was centrifuged at 25 °C for 40 min at 13800 rpm. 100 μL of supernatant was transferred into another vial and diluted with 100 μL CH_3CN. The two fold diluted supernatant was analyzed by HPLC/ UV system. For the standard calibration curve: The 20 mM dimethyl sulfoxide stock solution was diluted to 2 μM, 5 μM, 10 μM, 20 μM and 50 μM with the solution of 50% CH_3CN in pH = 6.5 PBS buffer. The above solutions were analyzed by the HPLC/UV system (five-point standard calibration curve). The concentration of the two fold diluted supernatant was calculated from the

integrated peak area based on the five-point standard calibration cure. Solubility (μM) = [concentration of the two fold diluted supernatant] × 2.

Supplementary references

(1) Johnson, S. M.; Murphy, R. C.; Geiger, J. A.; DeRocher, A. E.; Zhang, Z.; Ojo, K. K.; Larson, E. T.; Perera, B. G.; Dale, E. J.; He, P.; Reid, M. C.; Fox, A. M.; Mueller, N. R.; Merritt, E. A.; Fan, E.; Parsons, M.; Van Voorhis, W. C.; Maly, D. J. Development of *Toxoplasma gondii* calcium-dependent protein kinase 1 (*Tg*CDPK1) inhibitors with potent anti-toxoplasma activity. *J. Med. Chem.* **2012**, *55*, 2416-2426.

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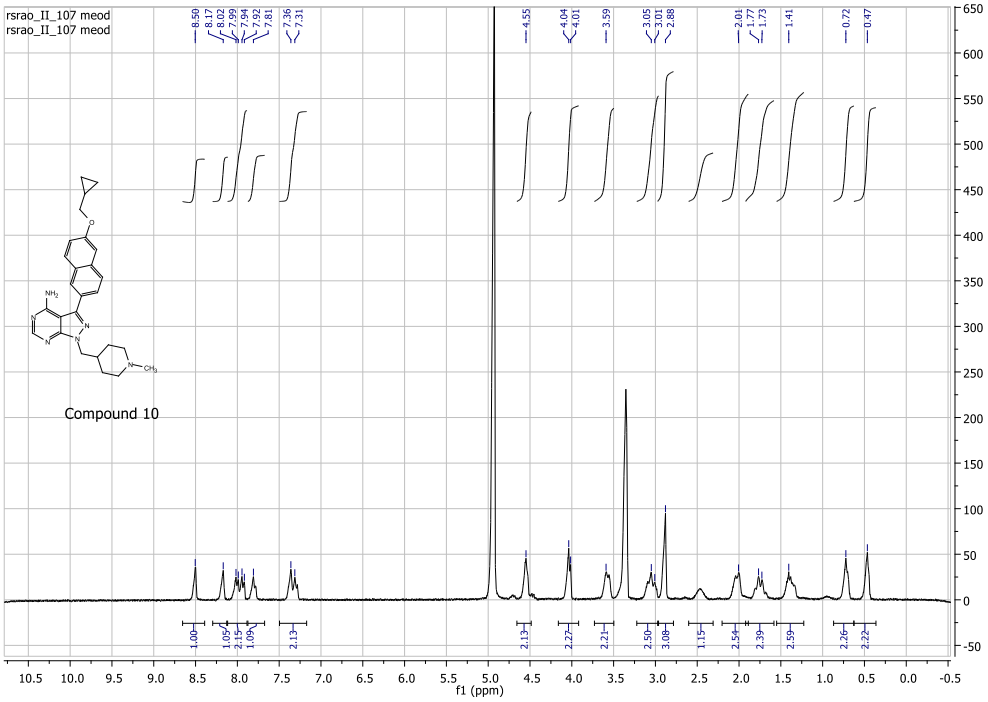
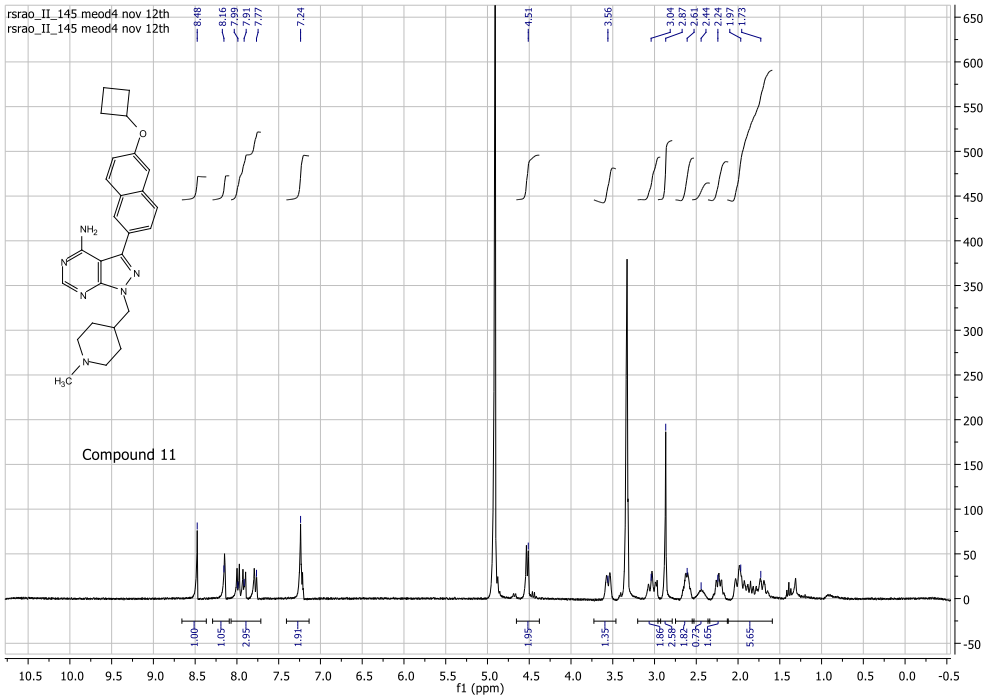
NMR spectra of Table 2

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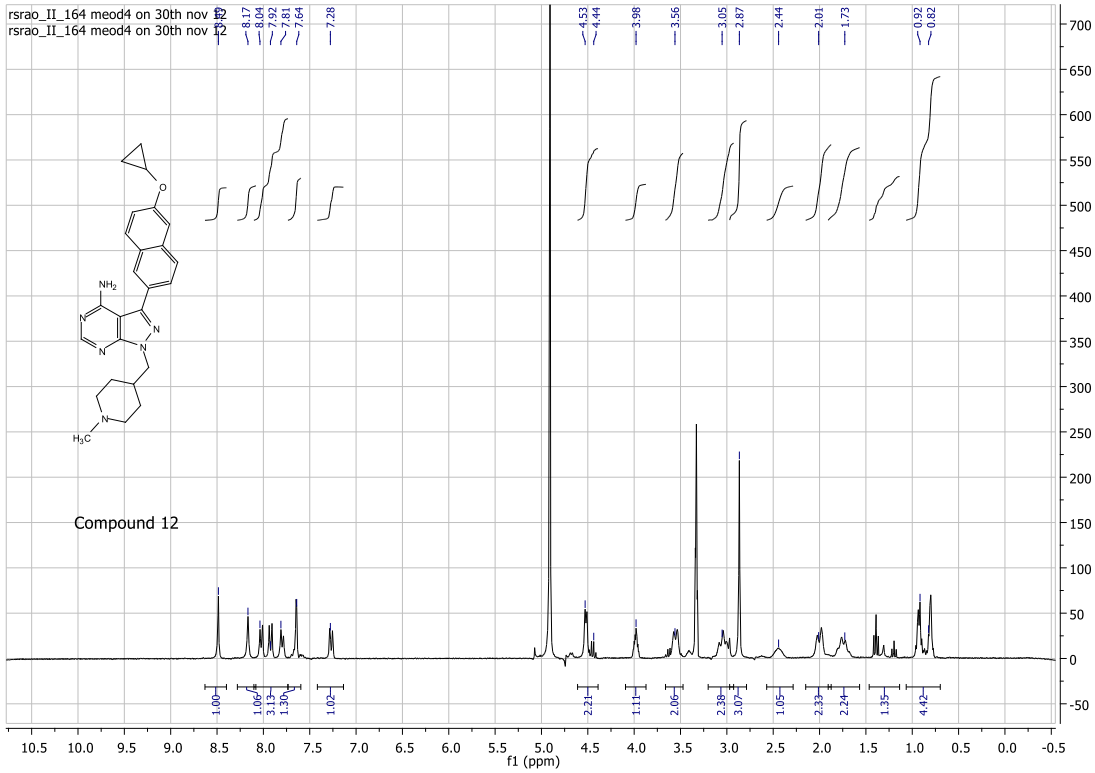
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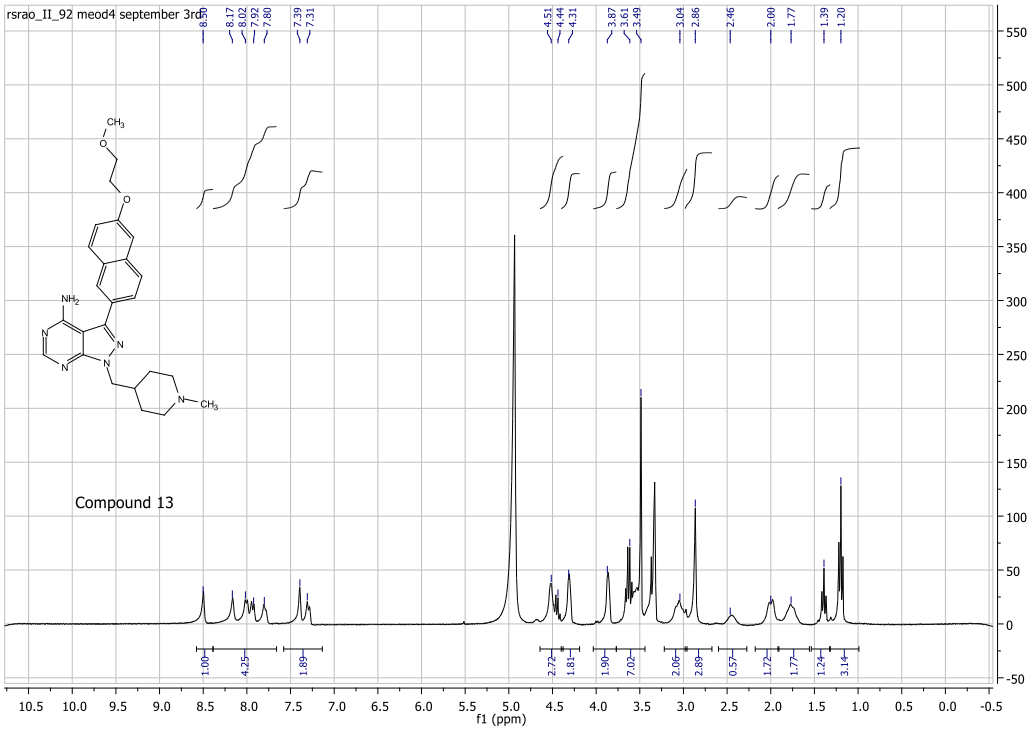
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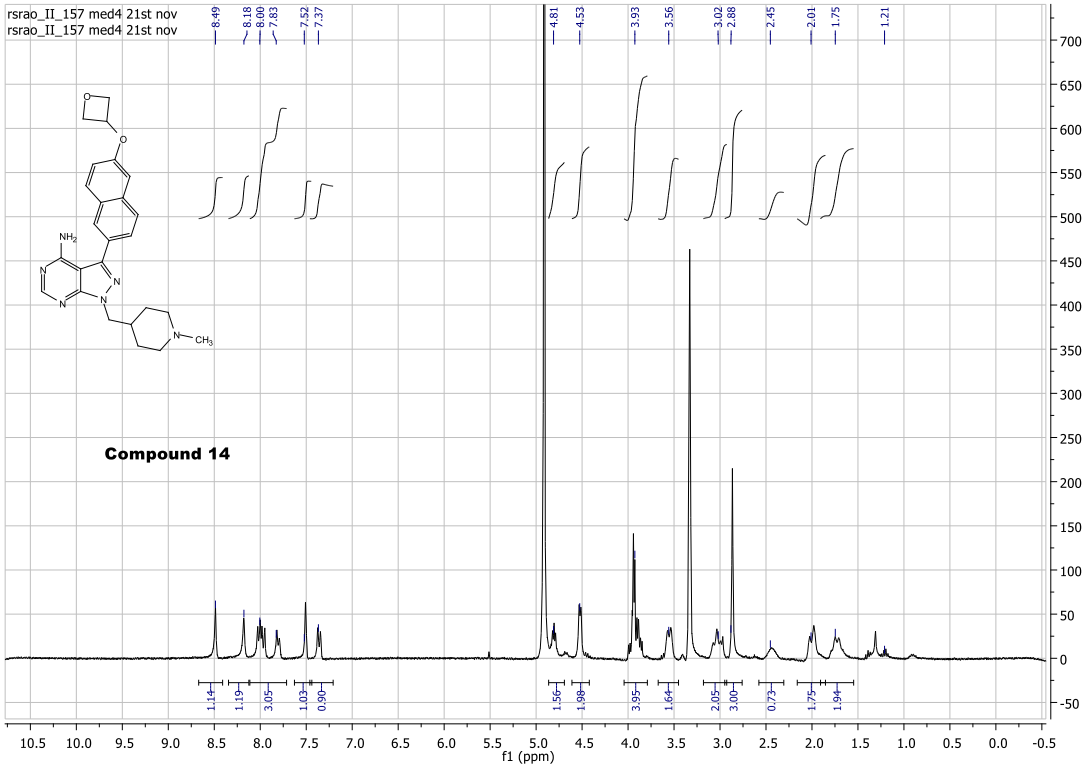
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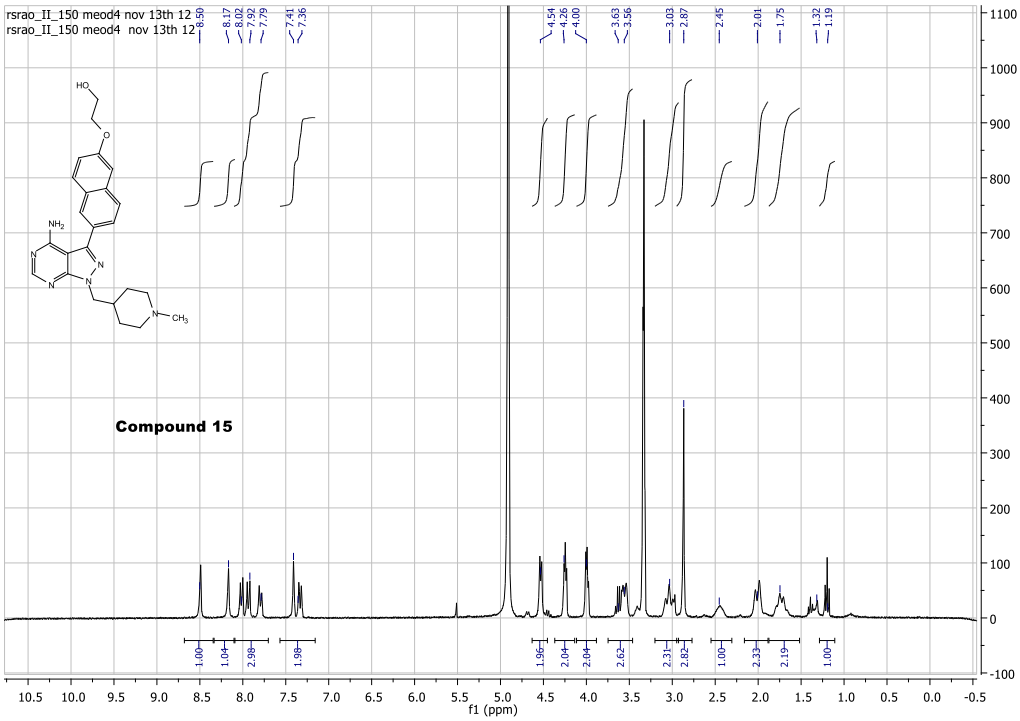
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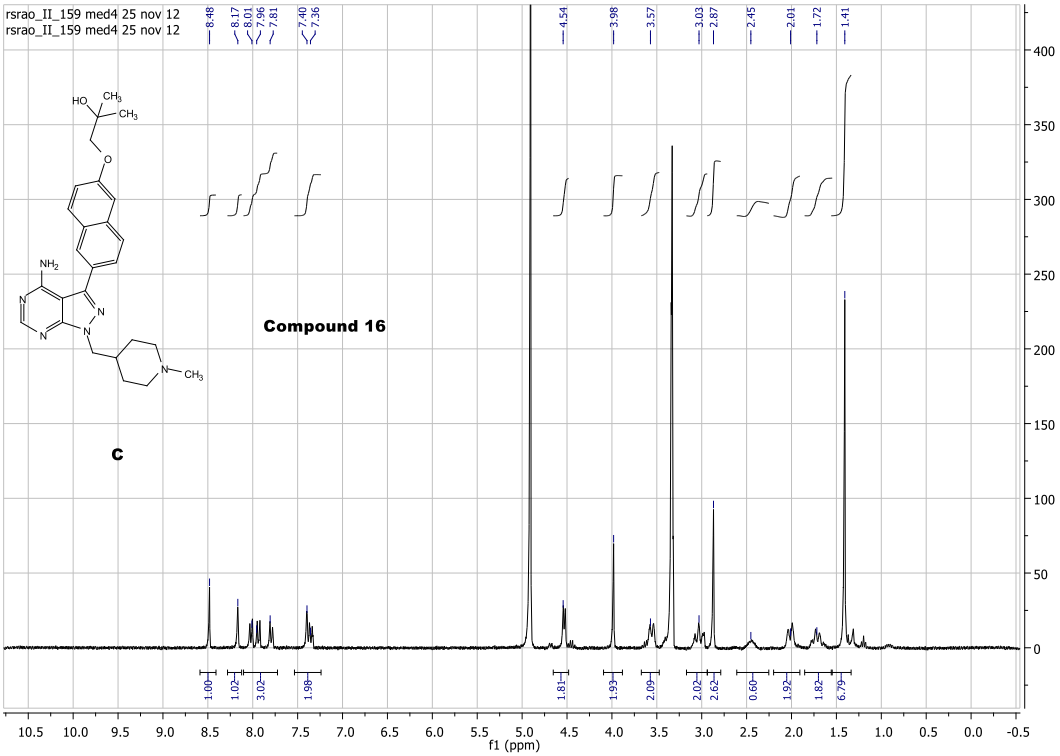
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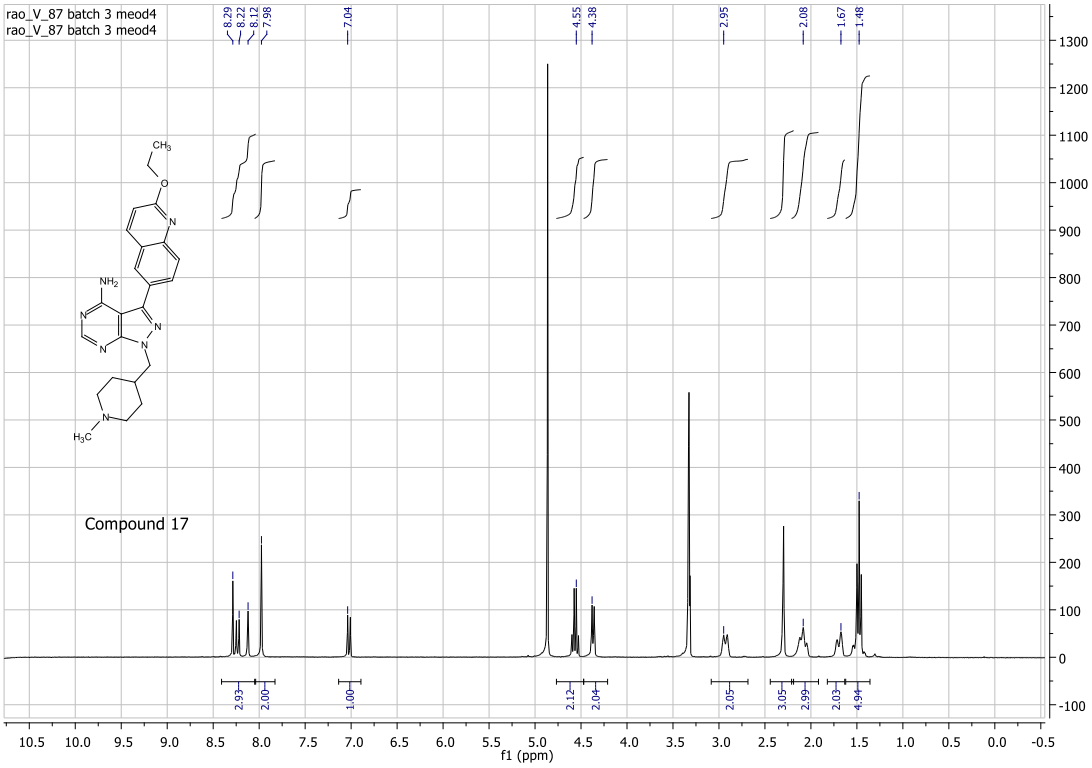
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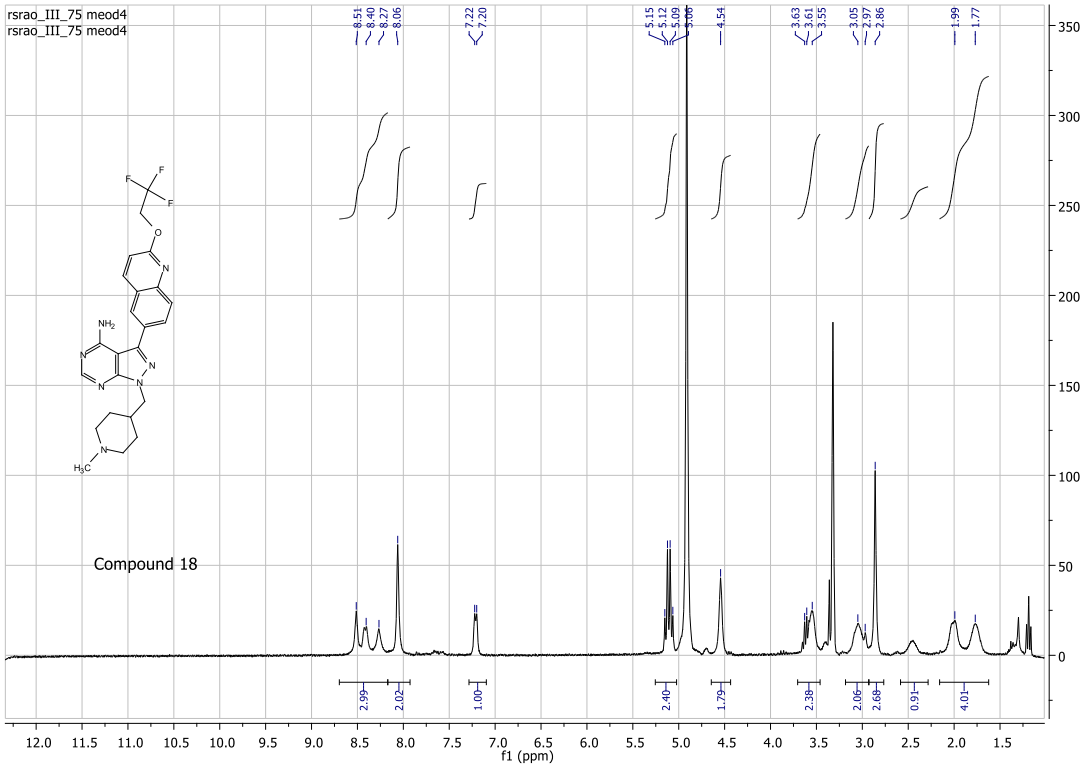
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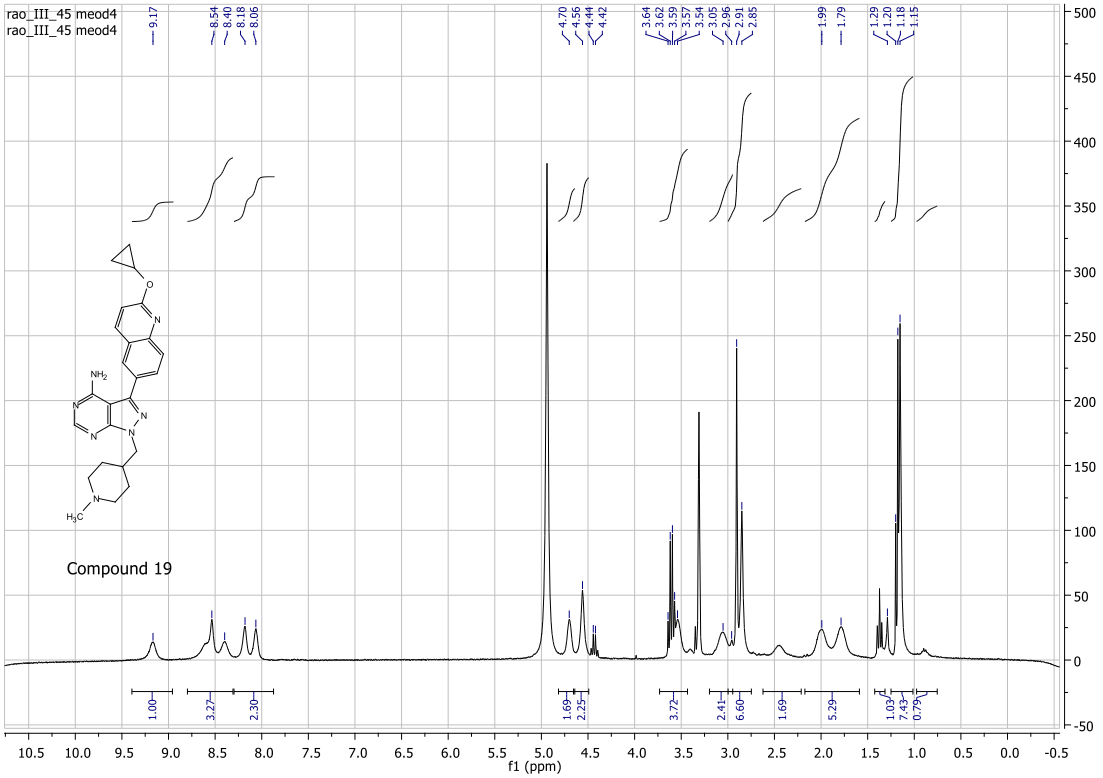
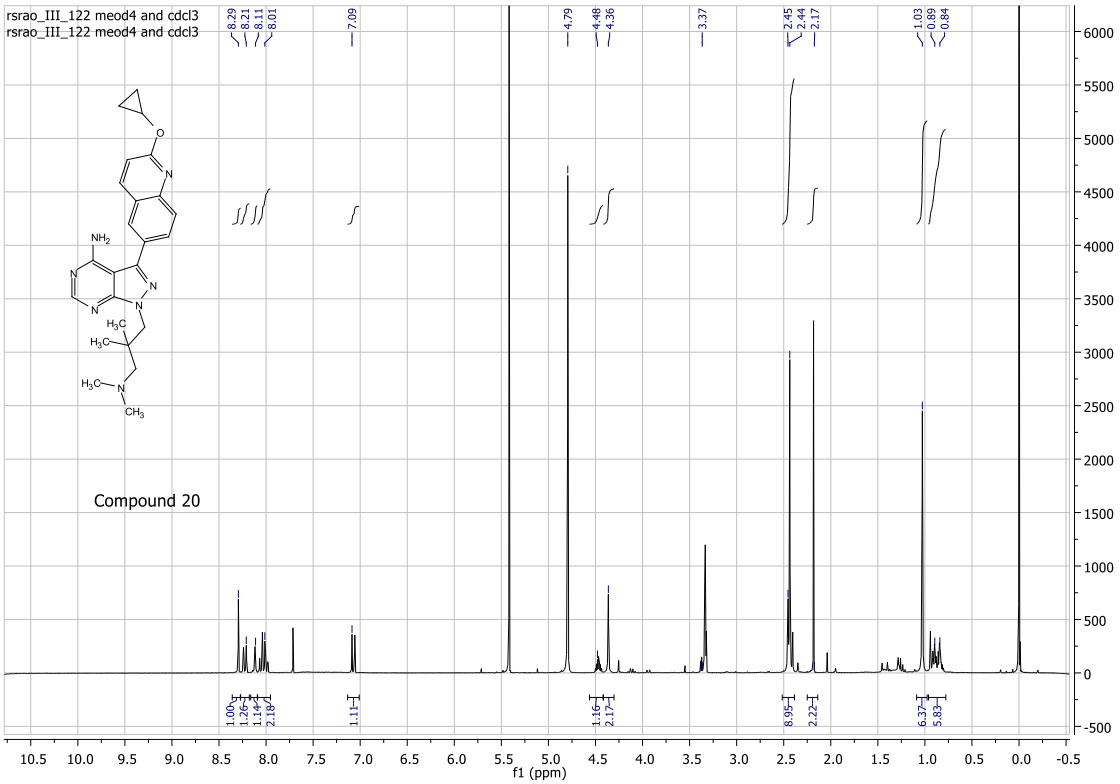


NMR spectra of Table 3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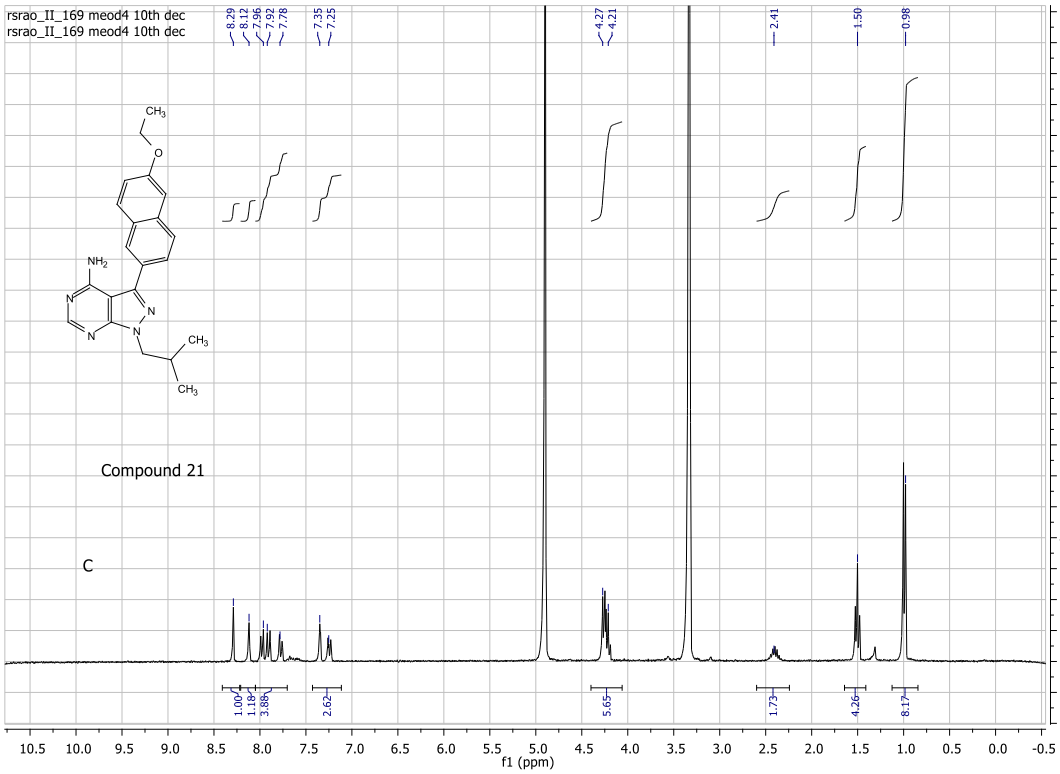
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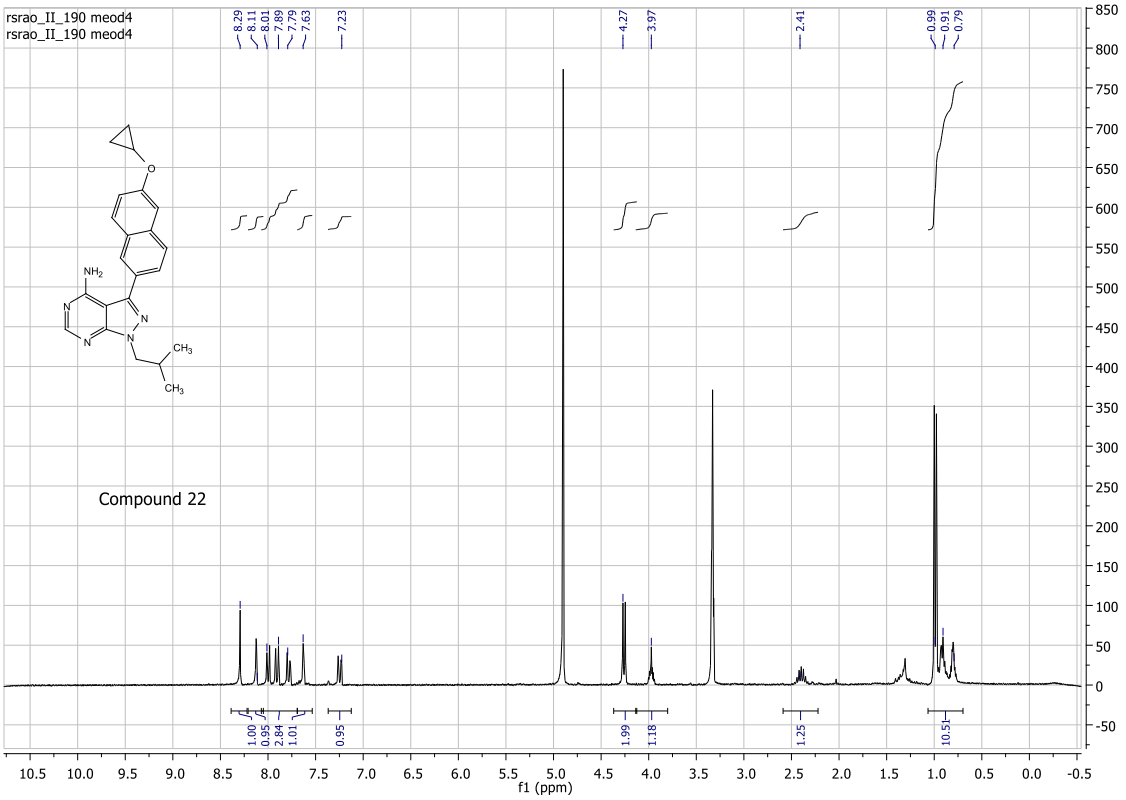
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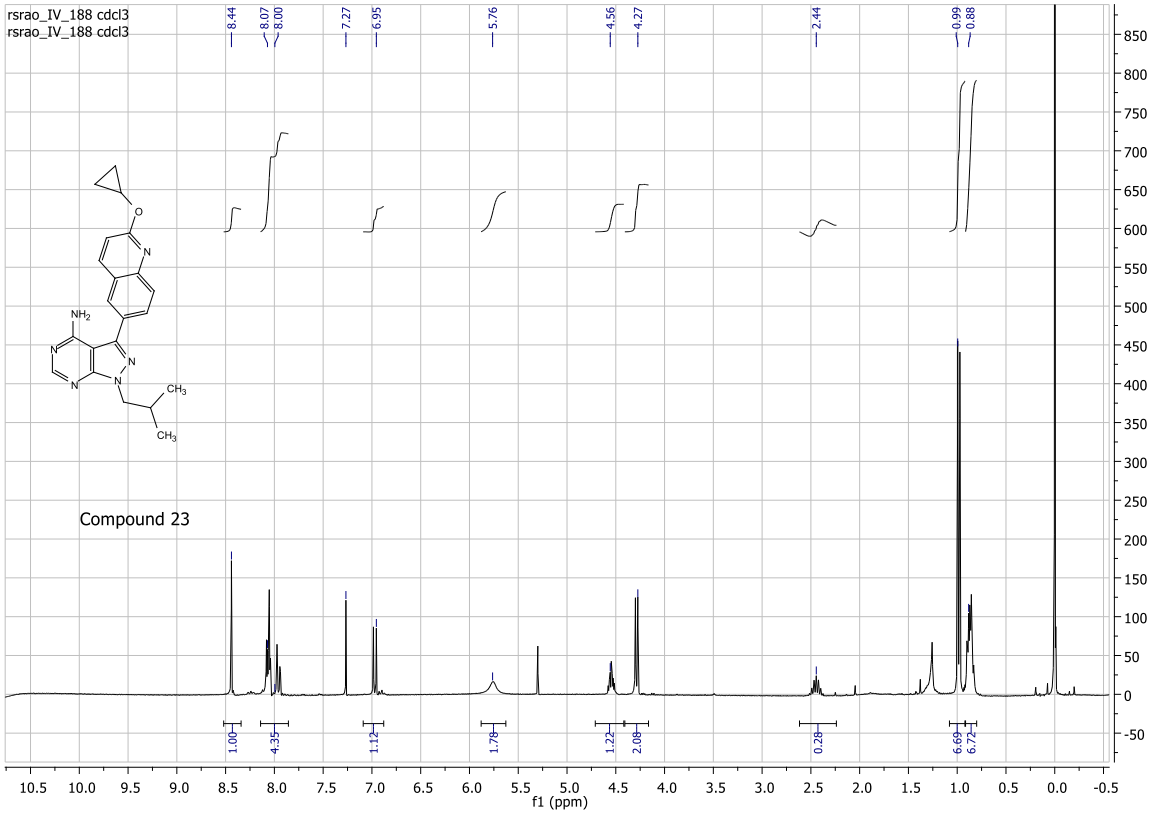
f1 (ppm)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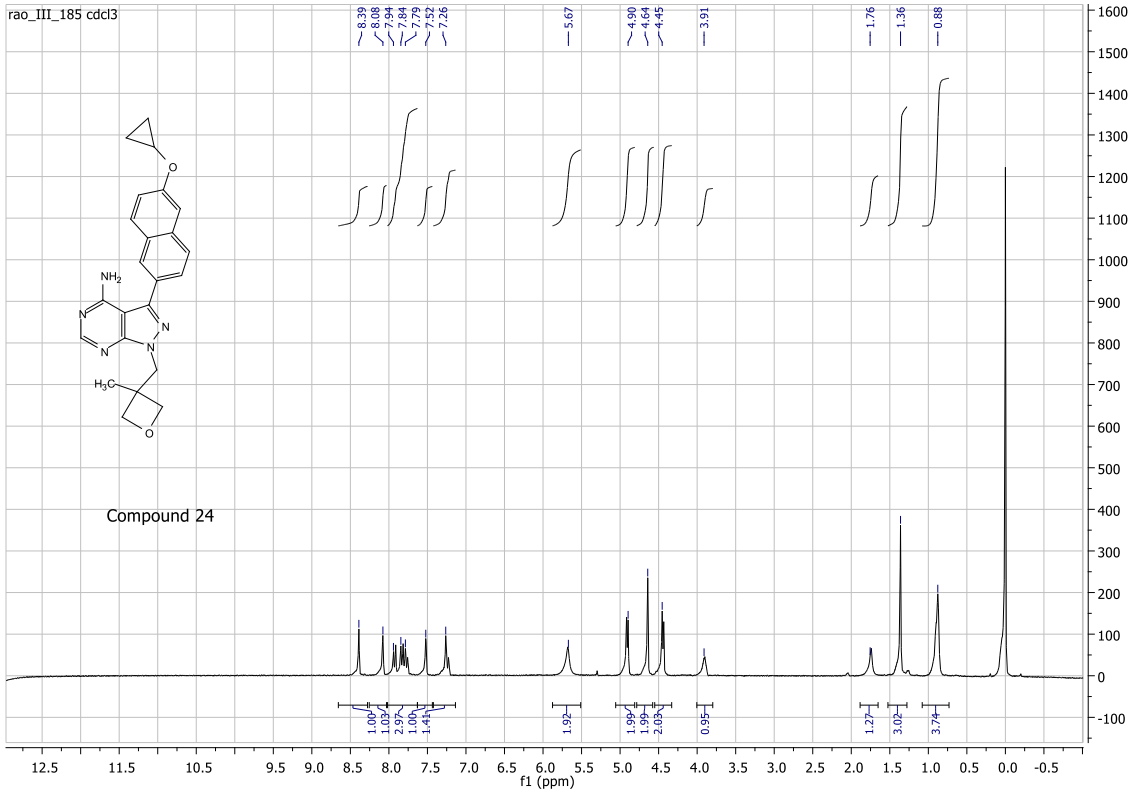
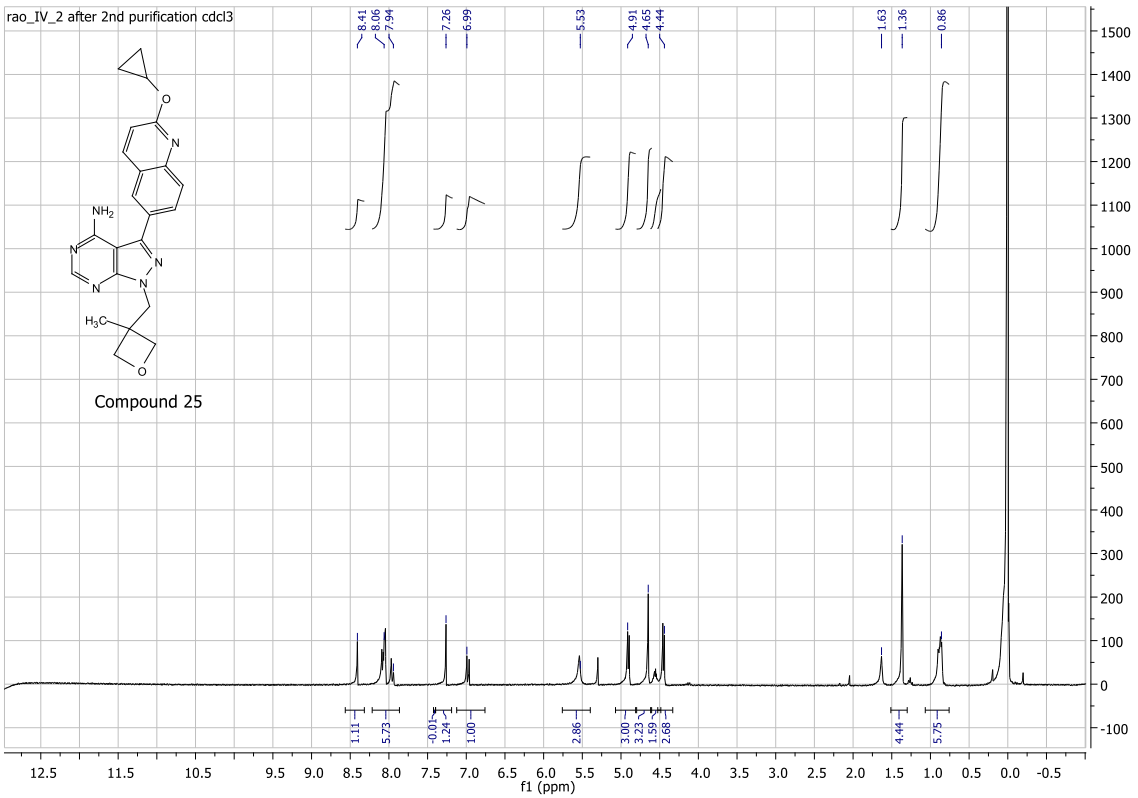


Compound 24



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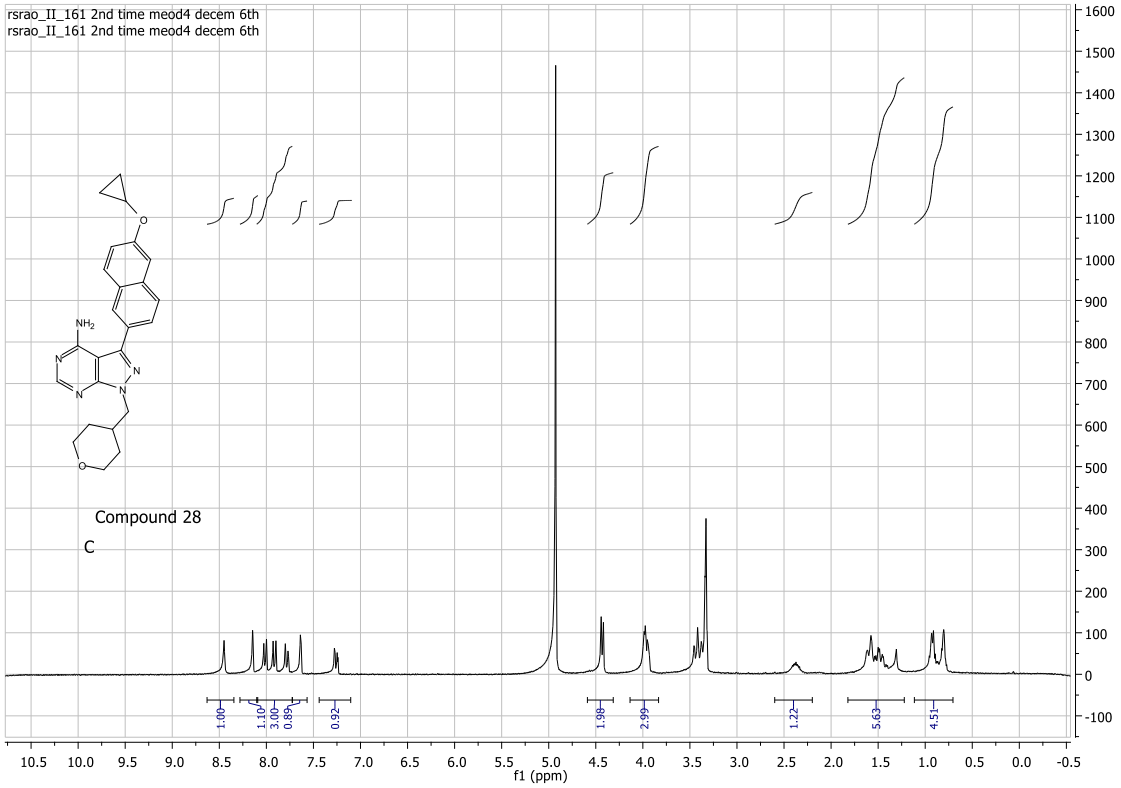
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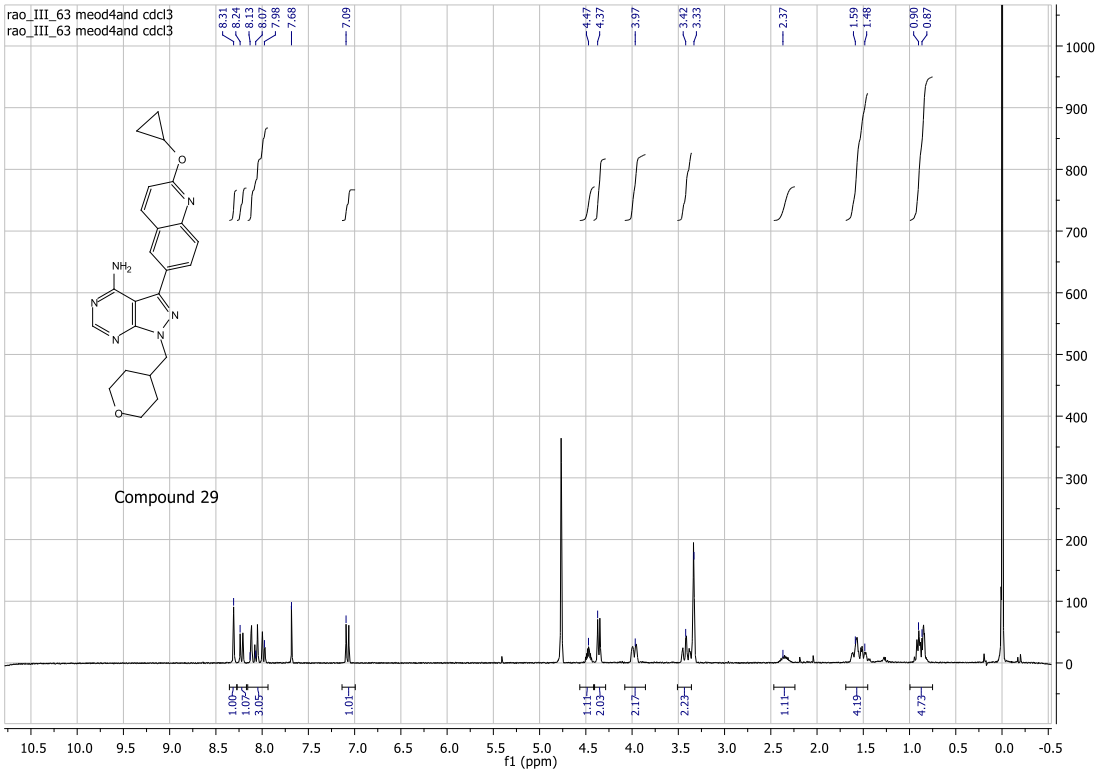
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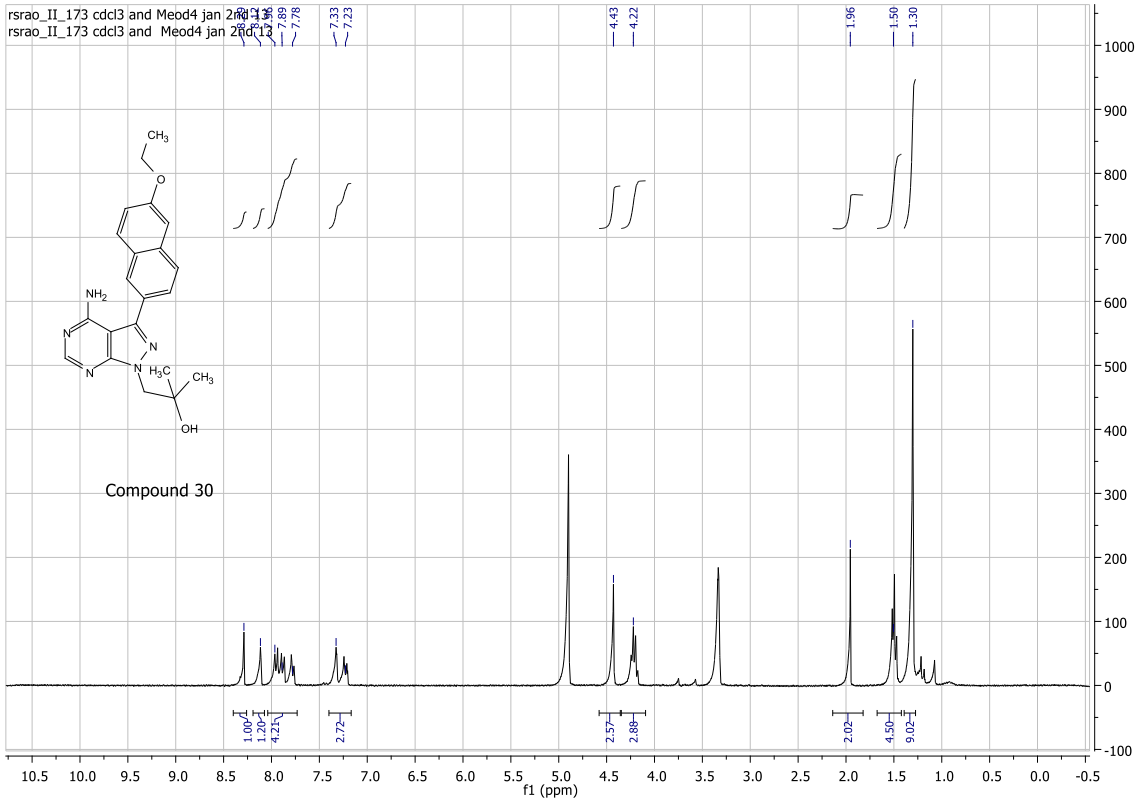
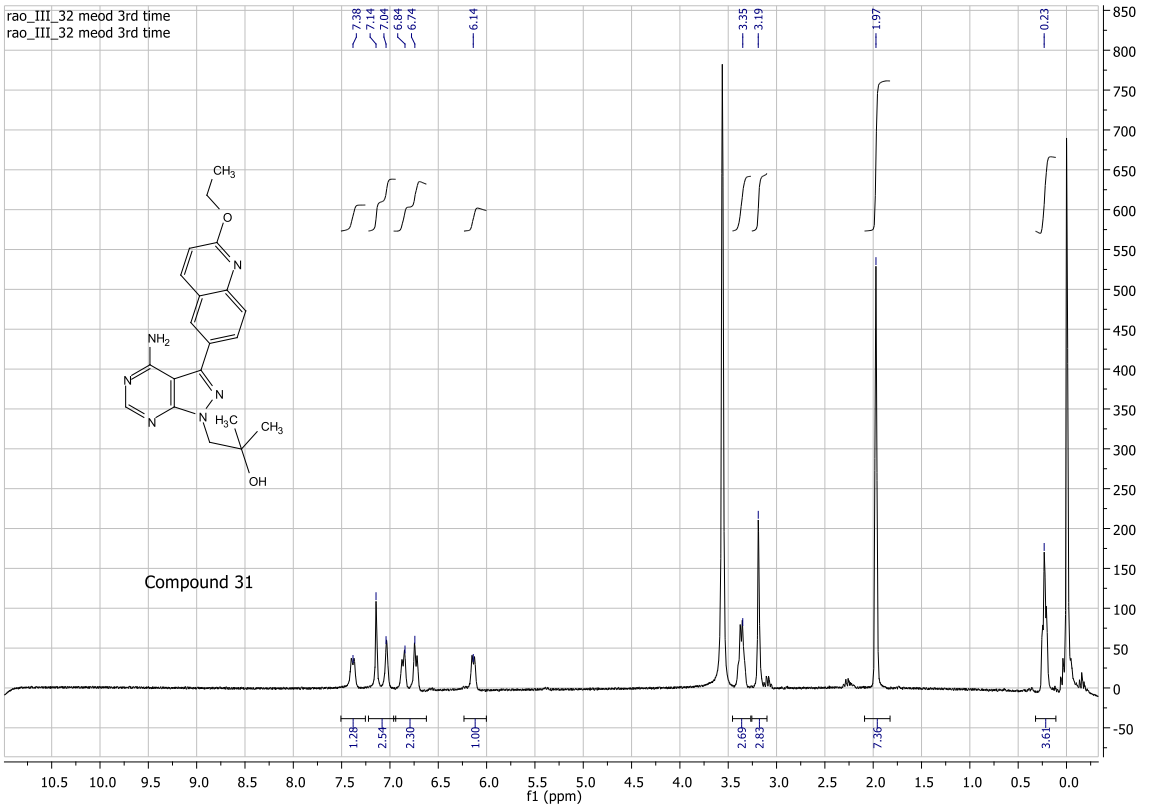
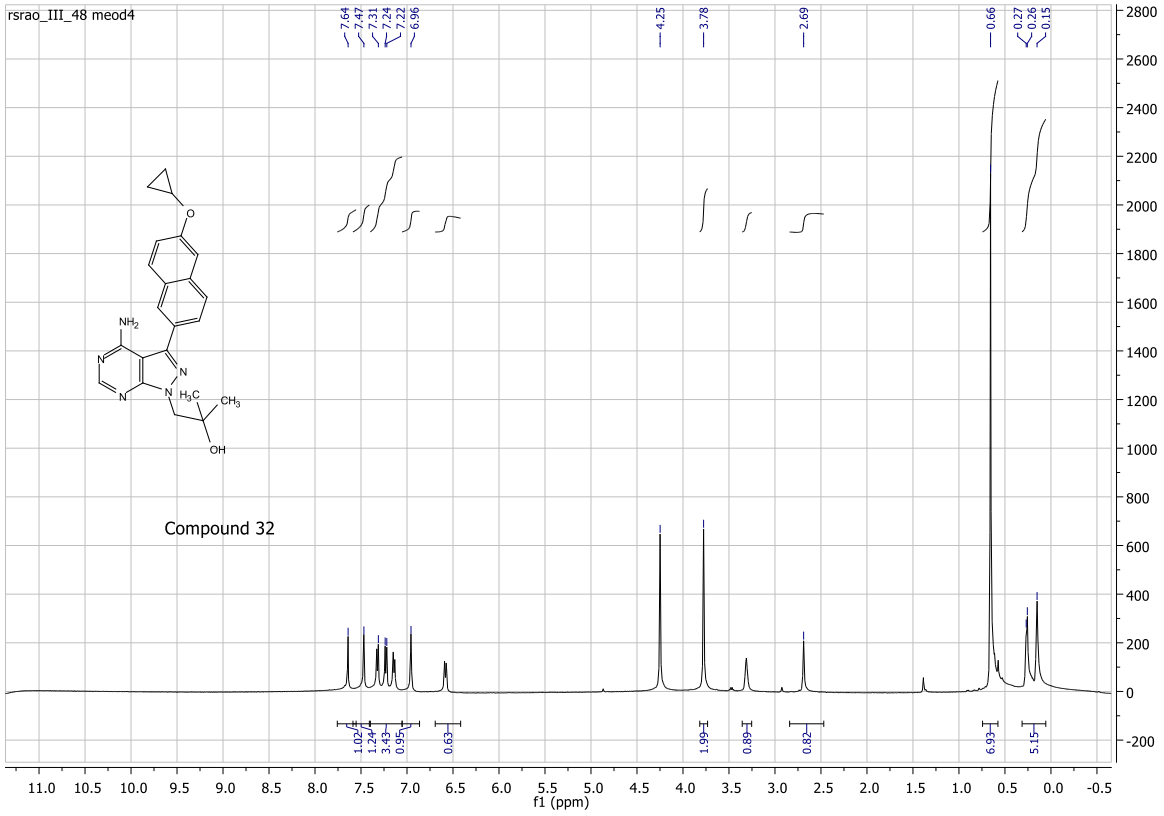
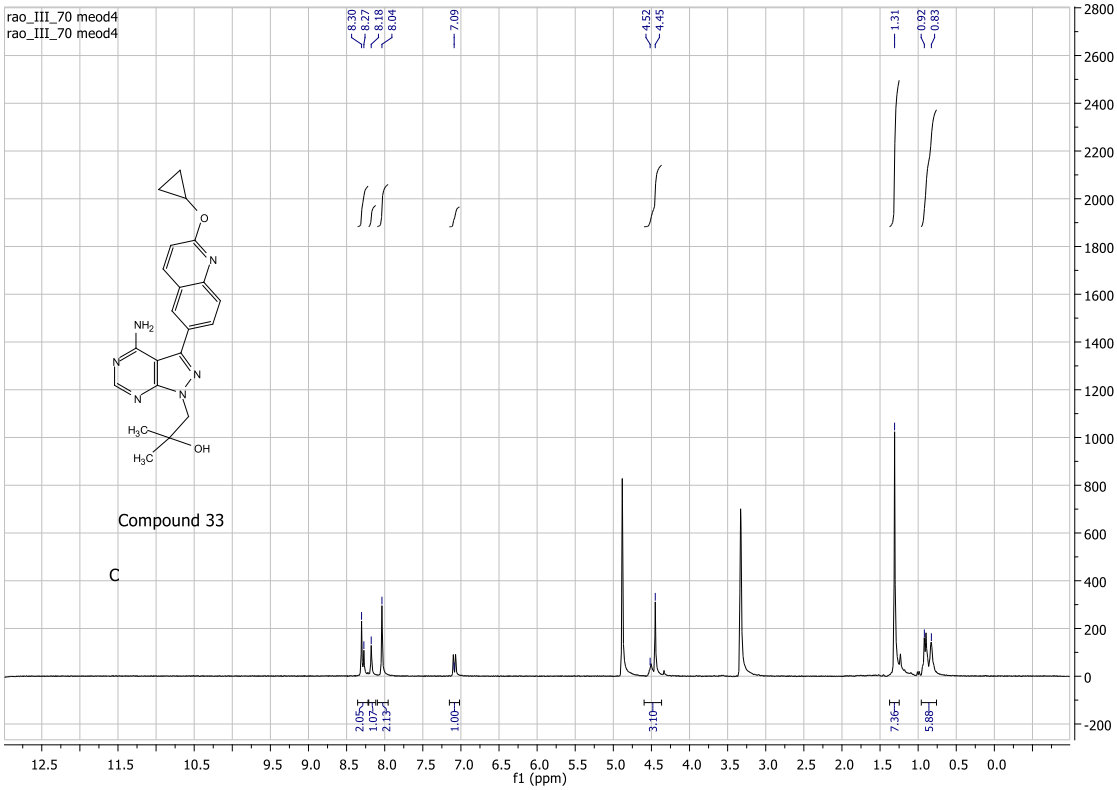
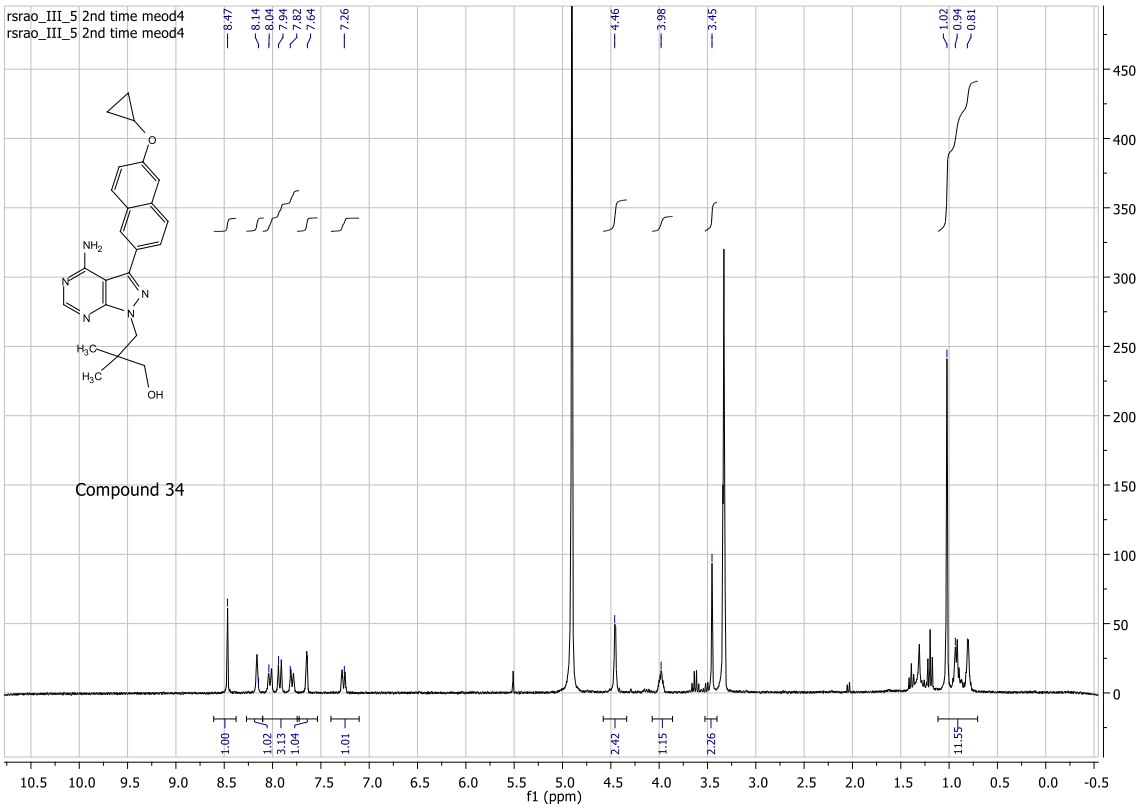
Compound 29
f1 (ppm)

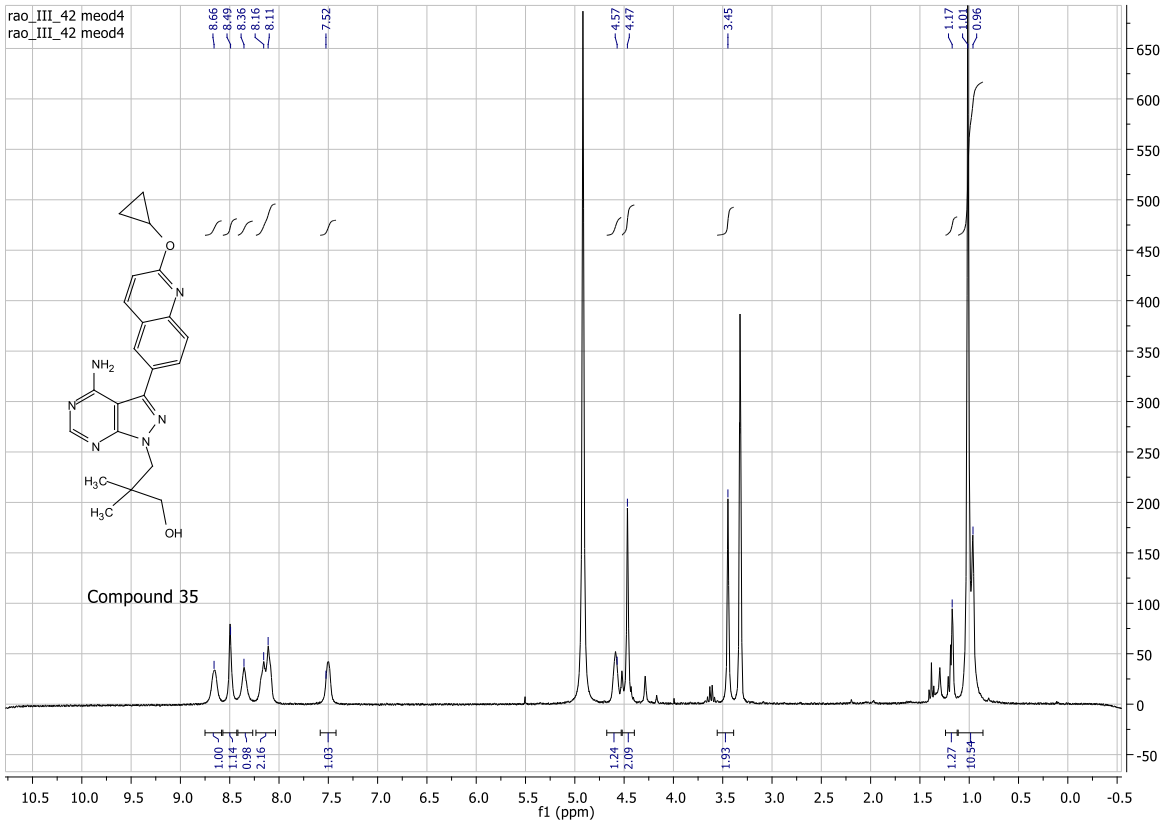

rsrao_II_173 cdcl3 and Meod4 jan 2nd 13
rsrao_II_173 cdcl3 and Meod4 jan 2nd 13
7.89
7.78
7.33
7.23
4.43
4.22
1.96
1.50
1.30
Compound 30
1.00
1.20
4.21
2.72
2.57
2.88
2.02
4.50
9.02
f1 (ppm)

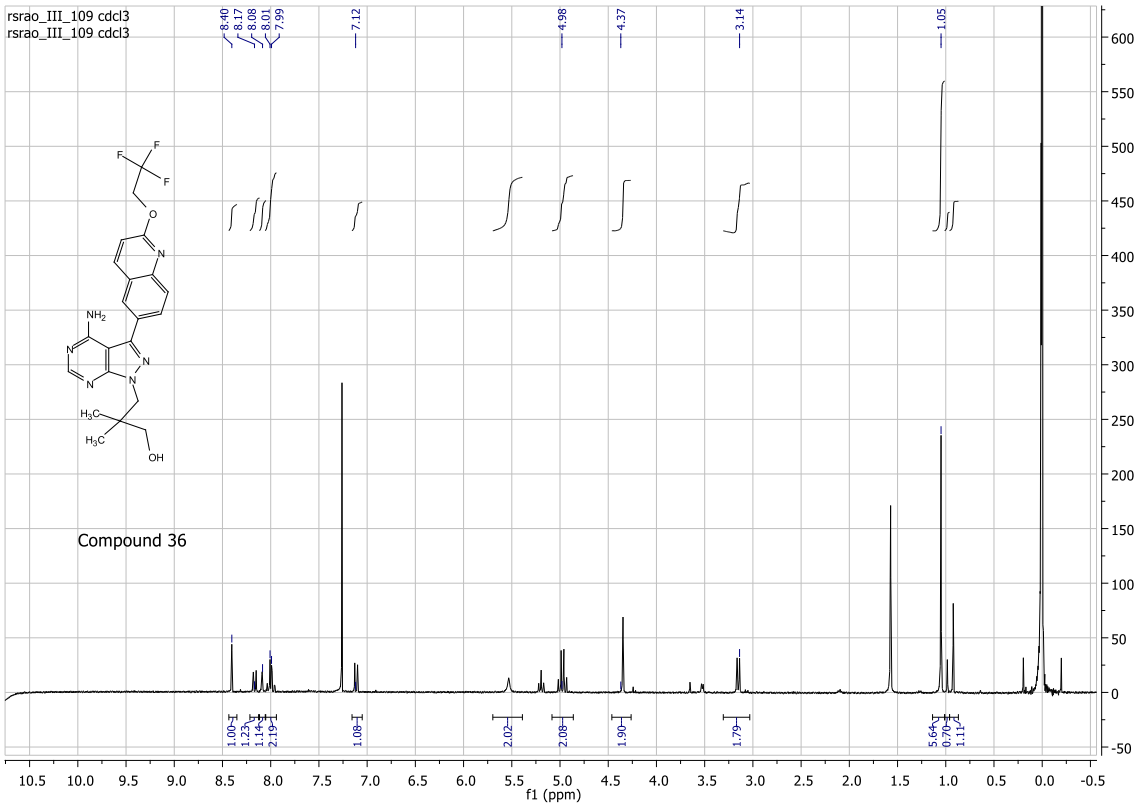

rao_III_32 meod 3rd time
rao_III_32 meod 3rd time
7.38
7.14
7.04
6.84
6.74
6.14
3.35
3.19
1.97
0.23
Compound 31
1.28
2.54
2.30
1.00
2.69
2.83
7.36
3.61
f1 (ppm)

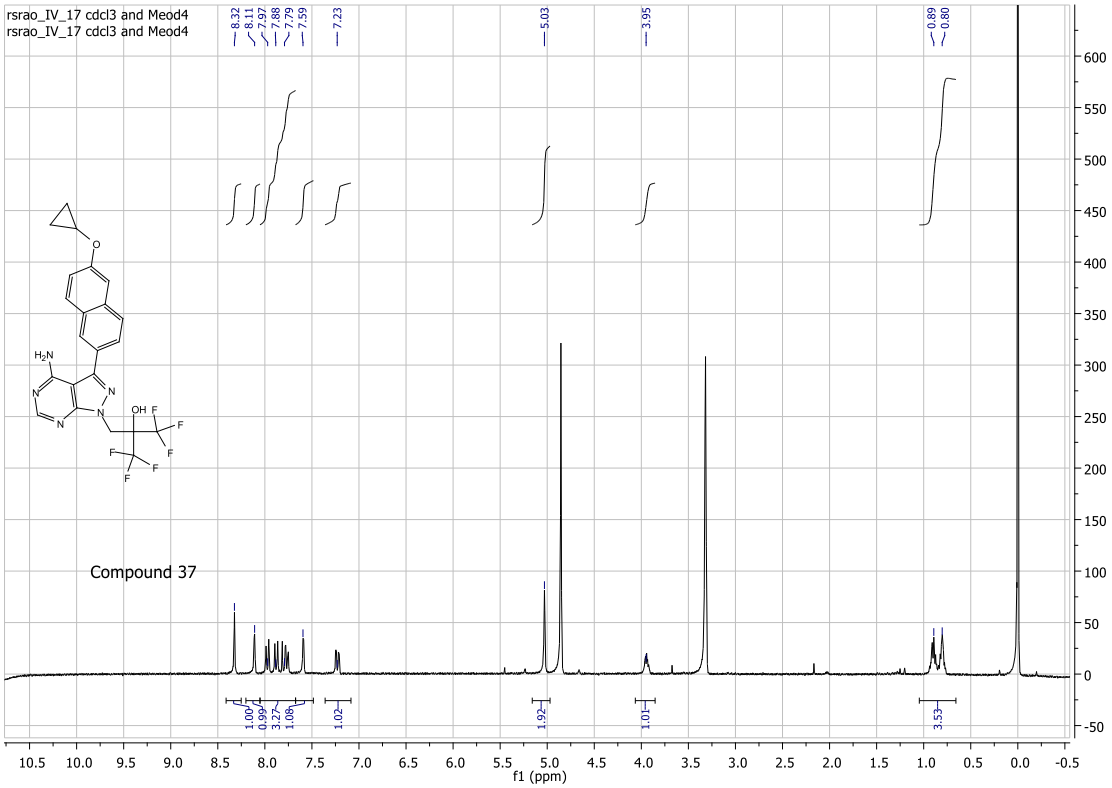

rsrao_III_48 meod4
Compound 32
f1 (ppm)


rao_III_70 meod4
rao_III_70 meod4
Compound 33
C
f1 (ppm)


rsrao_III_5 2nd time meod4
rsrao_III_5 2nd time meod4
8.47
8.14
8.04
7.94
7.82
7.64
7.26
4.46
3.98
3.45
1.02
0.94
0.81
Compound 34
f1 (ppm)


rao_III_42 meod4
rao_III_42 meod4
8.66
8.49
8.36
8.16
8.11
7.52
4.57
4.47
3.45
1.17
1.01
0.96
Compound 35
f1 (ppm)


rsrao_III_109 cdcl3
rsrao_III_109 cdcl3
8.40
8.17
8.08
8.01
7.99
7.12
4.98
4.37
3.14
1.05
Compound 36
f1 (ppm)


rsrao_IV_17 cdcl3 and Meod4
rsrao_IV_17 cdcl3 and Meod4
8.32
8.11
7.97
7.88
7.79
7.59
7.23
5.03
3.95
0.89
0.80
Compound 37
f1 (ppm)