Supporting information

Discovery of BAY-390, a selective CNS penetrant chemical probe as Transient receptor potential ankyrin 1 (TRPA1) antagonist

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Analysis

Analytical LCMS methods

Method A: Instrument Waters Acquity UPLCMS SingleQuad; Column: Phenomenex Kinetix-XB C18 2.1 x 100mm, 1.7µm; eluent A: water + 0.1 vol % formic acid, eluent B: acetonitrile + 0.1 vol % formic acid; gradient: 0-5.3 min 5-100% B, 5.3-5.8 min 100% B; flow 0.6 ml/min; temperature: 40 ˚C; PDA scan: 210-420 nm.

Method B: Instrument Agilent G1312A with Waters PDA detector and Qtof-micro mass spectrometer or Shimadzu LCMS - LC 20-AB - LCMS 2010 MS detector; Column: Waters Atlantis dC18 2.1 x 100mm, 3µm; eluent A: water + 0.1 vol % formic acid, eluent B:

acetonitrile + 0.1 vol % formic acid; gradient: 0-5.0 min 5-100% B, 5.0-5.4 min 100% B; flow 0.6 ml/min; temperature: 40 ˚C; PDA scan: 210-420 nm.

Method C: Instrument Agilent G1312A with Waters PDA detector and ZQ mass spectrometer or Shimadzu LCMS - LC 20-AB - LCMS 2010 MS detector; Column: Kinetex Core-Shell C18, 2.1 x 50mm, 5µm; eluent A: water + 0.1 vol % formic acid, eluent B: acetonitrile + 0.1 vol % formic acid; gradient: 0-1.2 min 5-100% B, 1.2-1.3 min 100% B; flow 1.2 ml/min; temperature: 40 ˚C; PDA scan: 210-420 nm.

Method D:

Instrument: Waters Acquity UPLCMS SingleQuad; column: Acquity UPLC BEH C18 1.7 µm, 50 x 2.1mm; Eluent A: Water + 0.2 Vol-% aq. NH_3 (32%), Eluent B: Acetonitrile; Gradient: 0-1.6 min 1-99% B, 1.6-2.0 min 99% B; flow 0.8 ml/min; Temperature: 60 ˚C; DAD scan: 210-400 nm

Method E:

Instrument: Waters Acquity UPLC-MS SQD 3001; Column: Acquity UPLC BEH C18 1.7 µm, 50x2.1 mm; Eluent A: water + 0.2 vol % ammonia, Eluent B: acetonitrile; Gradient: 0-1.6 min 1-99% B, 1.6-2.0 min 99% B; Flow rate: 0.8 mL/min; Temperature: 60 ˚C; Injection: 2 µL; DAD scan: 210-400 nm; ELSD.

Method F:

Instrument: Waters Acquity UPLC-MS SQD 3001; Column: Acquity UPLC BEH C18 1.7 µm, 50x2.1 mm; Eluent A: water + 0.1 vol % formic acid , Eluent B: acetonitrile; Gradient: 0-1.6 min 1-99% B, 1.6-2.0 min 99% B; Flow rate: 0.8 mL/min; Temperature: 60 ˚C; Injection: 2 µL; DAD scan: 210-400 nm.

Method G: TOF/6500

Instrument: Agilent 1290 UPLCMS 6230 TOF; column: BEH C 18 1.7 µm, 50x2.1mm; Eluent A: water + 0.05 % formic acid (99%); Eluent B: acetonitrile + 0.05 % formic acid (99%); gradient: 0-1.7 2-90% B, 1.7-2.0 90% B; flow 1.2 ml/min; temperature: 60˚C; DAD scan: 190-400 nm.

Chiral separation methods and Preparative HPLC methods

Method SFC (Evo):

Instrument: Agilent: 1260, Aurora SFC-Module; column: Chiralpak IA 5µm 100x4.6mm; Eluent A: CO2, Eluent B: Ethanol; Isocratic: 5%B; flow 4.0 ml/min; Temperature: 37.5˚C; BPR: 100bar; UV 254 nm

Method SFC:

Instrument: Agilent: 1260, Aurora SFC-Module; column: Chiralpak IG 5µm 100x4.6mm; Eluent A: CO2, Eluent B: Ethanol; Isocratic: 15%B; flow 4.0 ml/min; Temperature: 37.5˚C; BPR: 100bar; MWD @ 254nm preparative Instrument: Sepiatec: Prep SFC100; column: Chiralpak IG 5µm 250x30mm; Eluent A: CO2, Eluent B: Ethanol; Isocratic: 15%B; flow 100.0 ml/min Temperature: 40˚C; BPR: 150bar; UV 254 nm

Method N1:

Instrument: Amy-C column: 4.6mm x 250mm, 5µm; Eluent A: MeOH; Eluent B: CO_2; Isocratic: 20%A+80%B; flow 4 ml/min; Temperature: 40 ˚C; UV 210-400 nm.

Preparative: Instrument: Amy-C column: 20mm x 250mm, 5µm; Eluent A: MeOH; Eluent B: CO_2; Isocratic: 20%A+80%B; flow 50 ml/min; Temperature: 40 ˚C; UV 210 nm.

Method POB:

Instrument: Agilent HPLC 1260; column: Chiralpak IG 3µ 100x4,6mm; Eluent A: Acetonitrile + 0.1 Vol-% Diethylamine (99%); Eluent B: Ethanol; Isocratic: 90%A+10%B; flow 1.4 ml/min; Temperature: 25 ˚C; DAD 254 nm; preparative Instrument: Labomatic HD5000, Labocord-5000; Gilson GX-241, Labcol Vario 4000, column: Chiralpak IG 5µ 250x30mm; Eluent A: Acetonitrile + 0.1 Vol-% Diethylamine (99%); Eluent B: Ethanol; Isocratic: 90%A+10%B; flow 50.0 ml/min; UV 254 nm

Method SFCB:

Instrument: Agilent: 1260, Aurora SFC-Modul; column: Chiralpak IF 5µm 100x4.6mm; Eluent A: CO2, Eluent B: Methanol + 0.2 Vol-% aq. NH_3 (32%); Isocratic: 8%B; flow 4.0 ml/min; Temperature: 37.5˚C; BPR: 100bar; MWD @ 254nm; preparative Instrument: Sepiatec: Prep SFC100; column: Chiralpak IF 5µm 250x20mm; Eluent A CO2, Eluent B:

Methanol + 0.2 Vol-% aq. NH_3 (32%); Isocratic: 8%B; flow 100.0 ml/min Temperature: 40˚C; BPR: 150bar; UV 254 nm

Method NP:

Instrument: Agilent HPLC 1260; column: Chiralpak IG 3µ 100x4,6mm; Eluent A: Hexane; Eluent B: Ethanol; Isocratic: 95%A+5%B; flow 1.4 ml/min; Temperature: 25 ˚C; DAD 220 nm; **preparative** Instrument: Labomatic HD5000, Labocord-5000; Gilson GX-241, Labcol Vario 4000, column: Chiralpak IG 5µ 250x30mm; Eluent A: Hexane; Eluent B: Ethanol; Isocratic: 95%A+5%B; flow 40.0 ml/min; UV 220 nm

Method NP2:

Instrument: Agilent: 1260, Aurora SFC-Module; column: Chiralpak IF 5µm 100x4.6mm; Eluent A: CO2, Eluent B: Methanol; isocratic: 5%B; flow 4.0 ml/min; Temperature: 37.5˚C; BPR: 100bar; UV 254 nm; **preparative** Instrument: Sepiatec: Prep SFC100; column: Chiralpak IF 5µm 250x30mm; Eluent A: CO2, Eluent B: Methanol; isocratic: 5%B; flow 100.0 ml/min Temperature: 40˚C; BPR: 150bar; UV 254 nm

Method NP3:

Instrument: Waters Acquity UPLCMS SingleQuad; column: Acquity UPLC BEH C18 1.7 µm, 50x2.1mm; Eluent A: water + 0.1 Vol-% formic acid (99%), Eluent B: Acetonitrile; Gradient: 0-1.6 min 1-99% B, 1.6-2.0 min 99% B; flow 0.8 ml/min; Temperature: 60˚C; DAD scan: 210-400nm; preparative Instrument: Waters Autopurificationsystem; column: Waters XBrigde C18 5µ 100x30mm; Eluent A: water + 0.1 Vol-% formic acid (99%), Eluent B: Acetonitrile; Gradient: 0.00–0.50 min 20% B (40->70mL/min), 0.51–5.50 min 40-60% B (70mL/min), DAD scan: 210-400 nm

Method H (basic)

Instrument: pump: Labomatic HD-5000 or HD-3000, head HDK 280, lowpressure gradient module ND-B1000; manual injection valve: Rheodyne 3725i038; detector: Knauer Azura UVD 2.15; collector: Labomatic Labocol Vario-4000; column: Chromatorex RP C-18 10 µm, 125x30mm; eluent A: water + 0.2 vol-% ammonia (32%), eluent B: acetonitrile;

LC-MS traces for key compounds 16-18, 34h (Anti-probe)

Figure S1. LC-MS trace of compound 16

No mass ion detected.

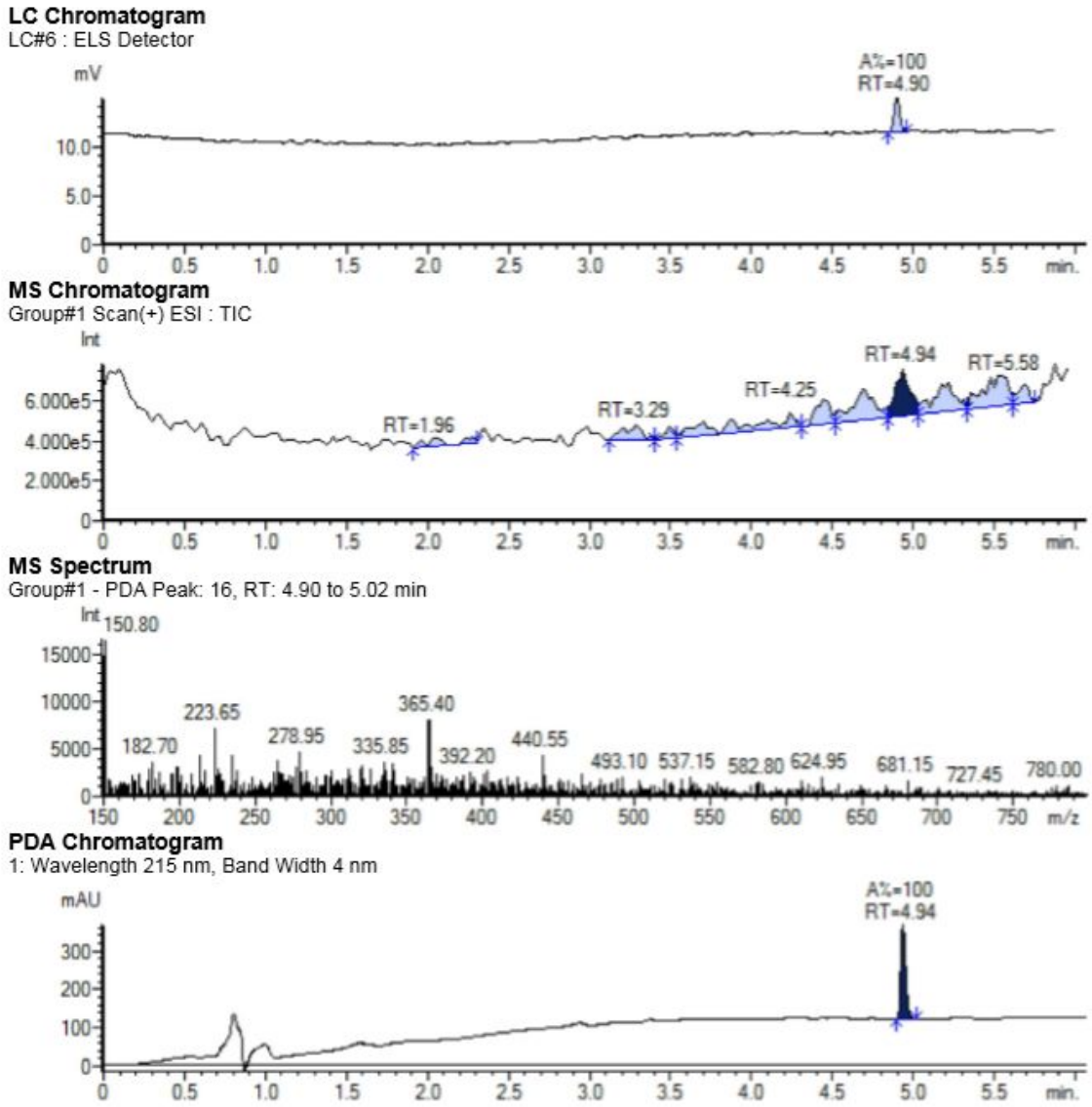


Figure S2. LC-MS trace of compound 17

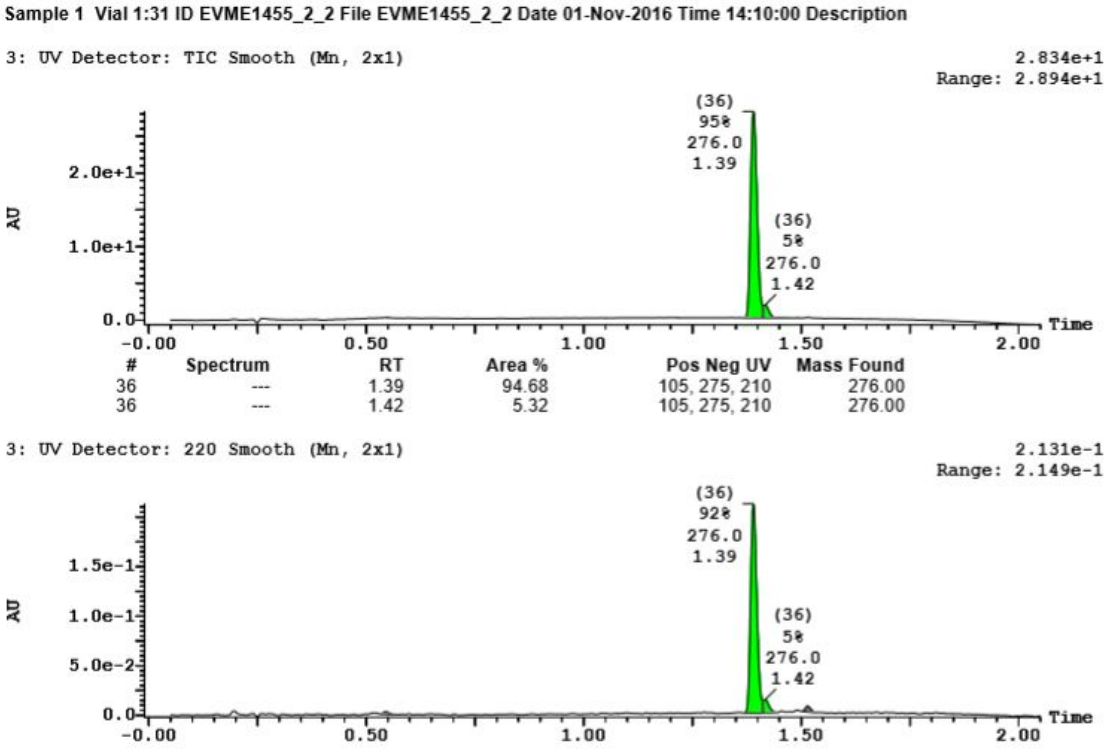


Figure S3. LC-MS trace of compound 18

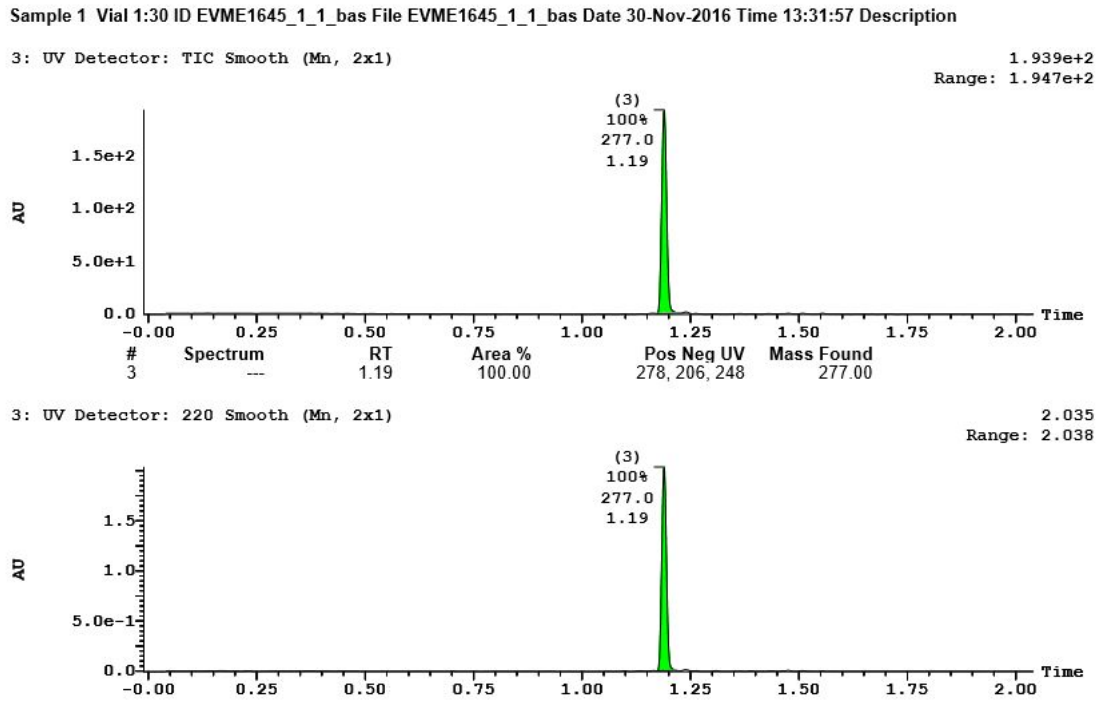
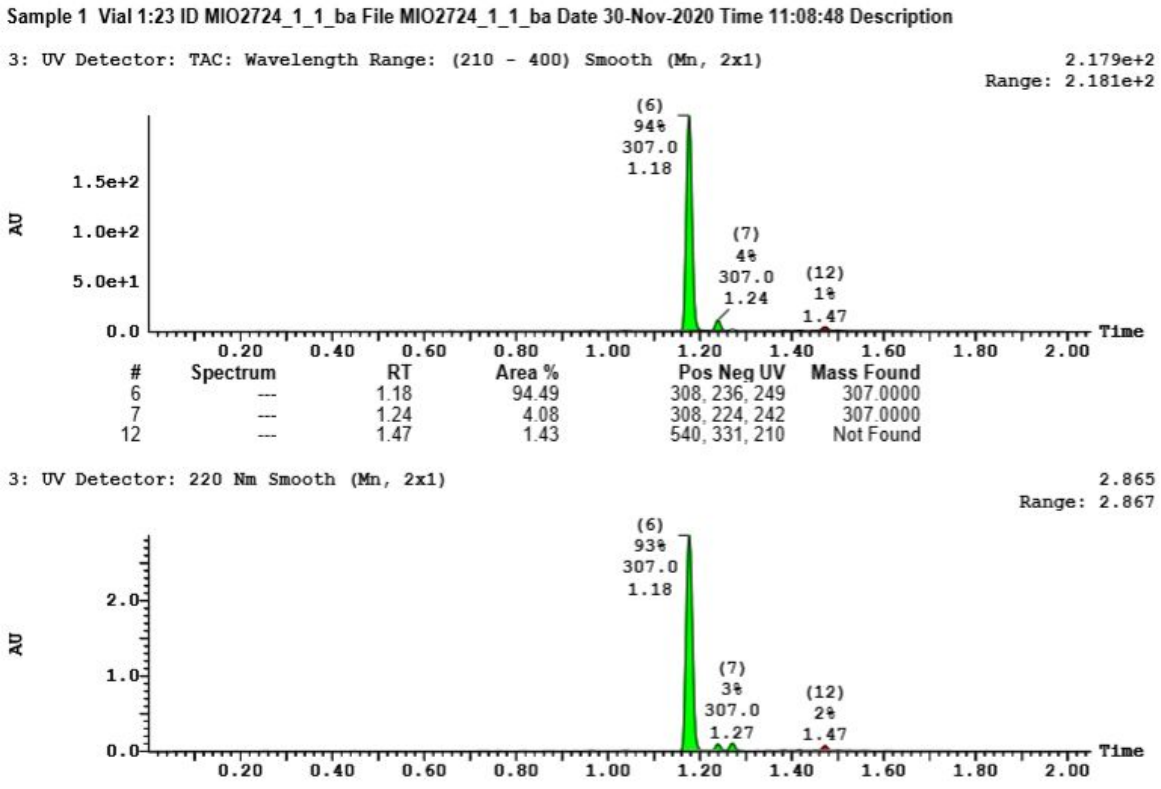


Figure S4. LC-MS trace of compound 34h



^{1}H-NMR and ^{13}C-NMR spectra of compound 18

Figure S5. ^{1}H-NMR spectrum of compound 18

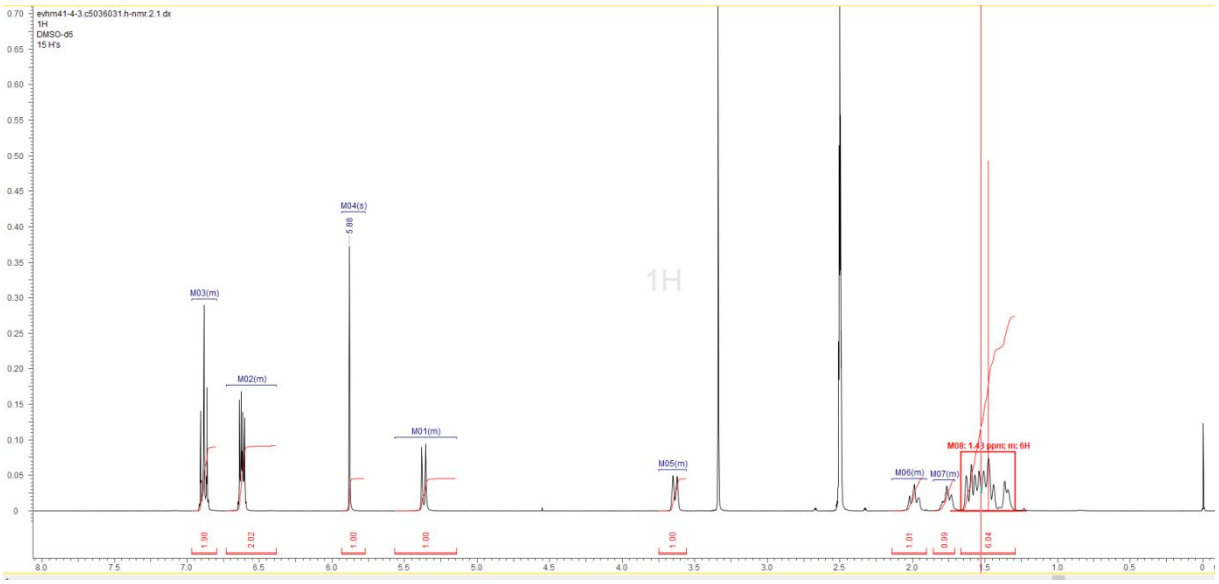
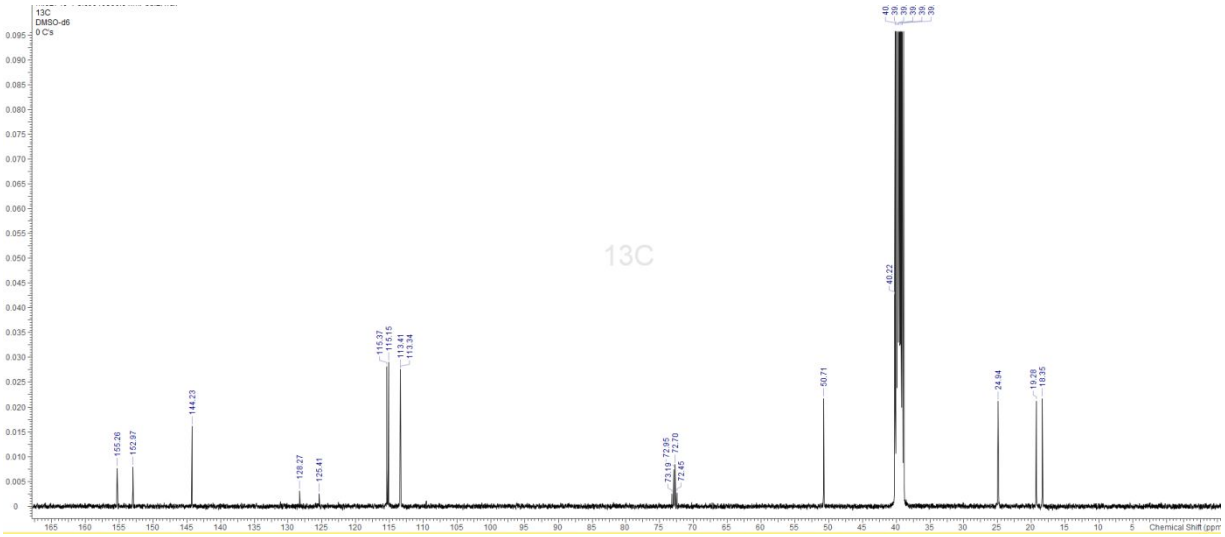


Figure S6. ^{13}C-NMR spectrum of compound 18



^{1}H-NMR spectra of compound 16, 24a-24h, 17, 27a-27h, 31, 32a-32f, 33, and 34a-34i

Figure S7. ^{1}H-NMR spectrum of compound 16

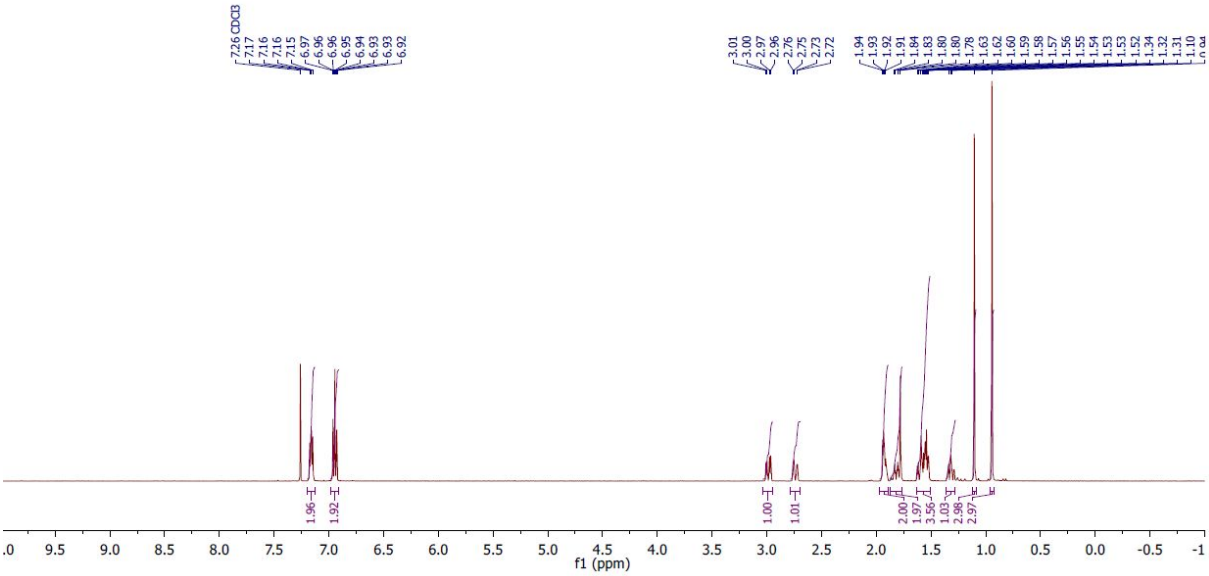


Figure S8. ^{1}H-NMR spectrum of compound 24a

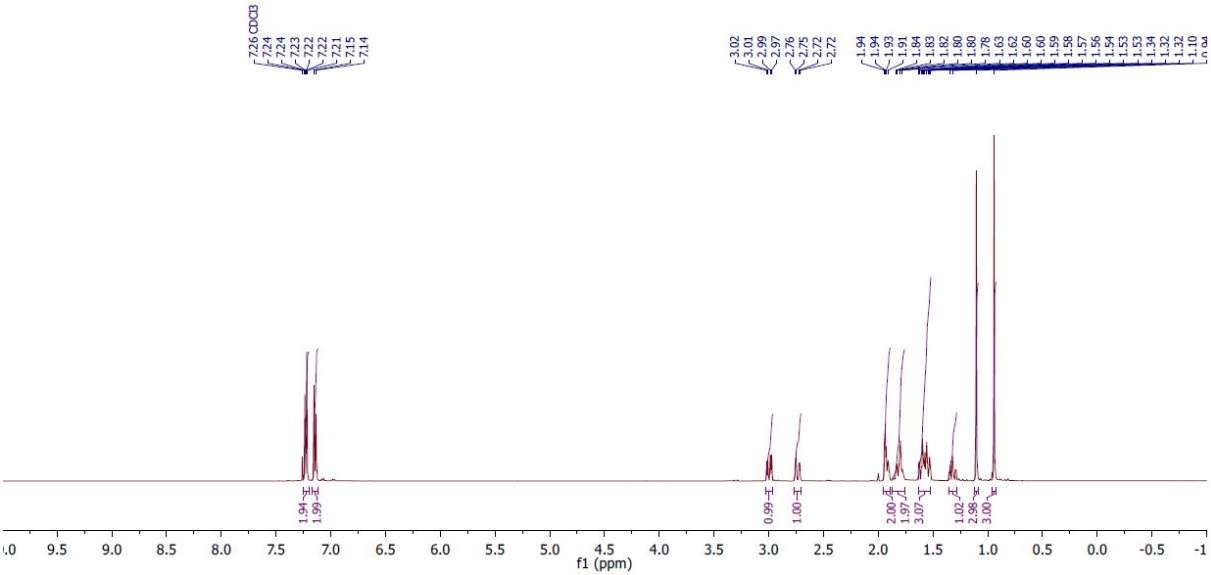


Figure S9. ^{1}H-NMR spectrum of compound 24b

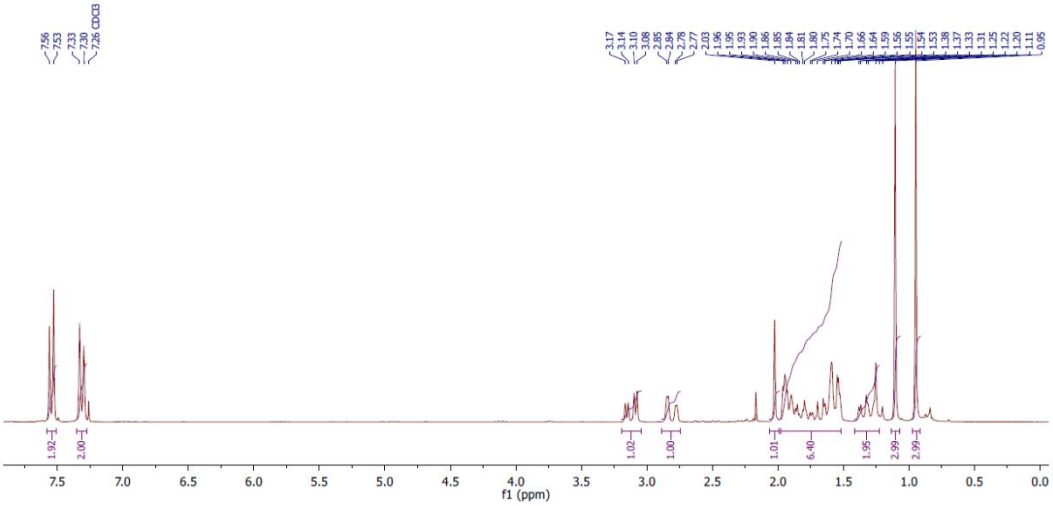


Figure S10. ^{1}H-NMR spectrum of compound 24c

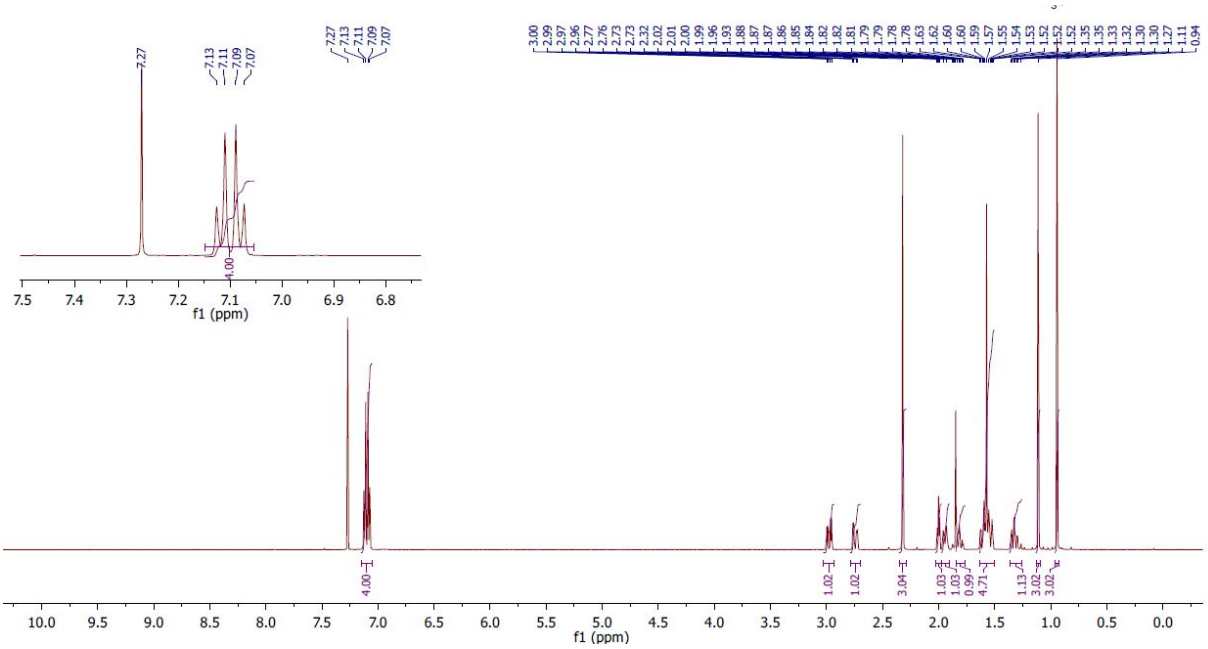


Figure S11. ^{1}H-NMR spectrum of compound 24d

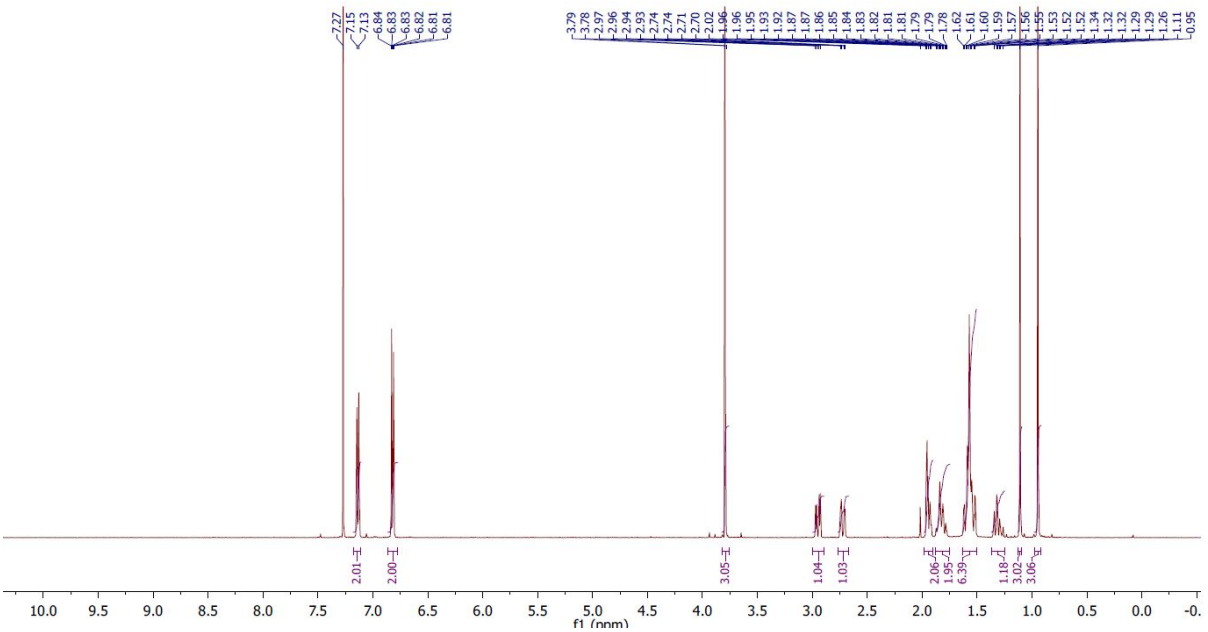


Figure S12. ^{1}H-NMR spectrum of compound 24e

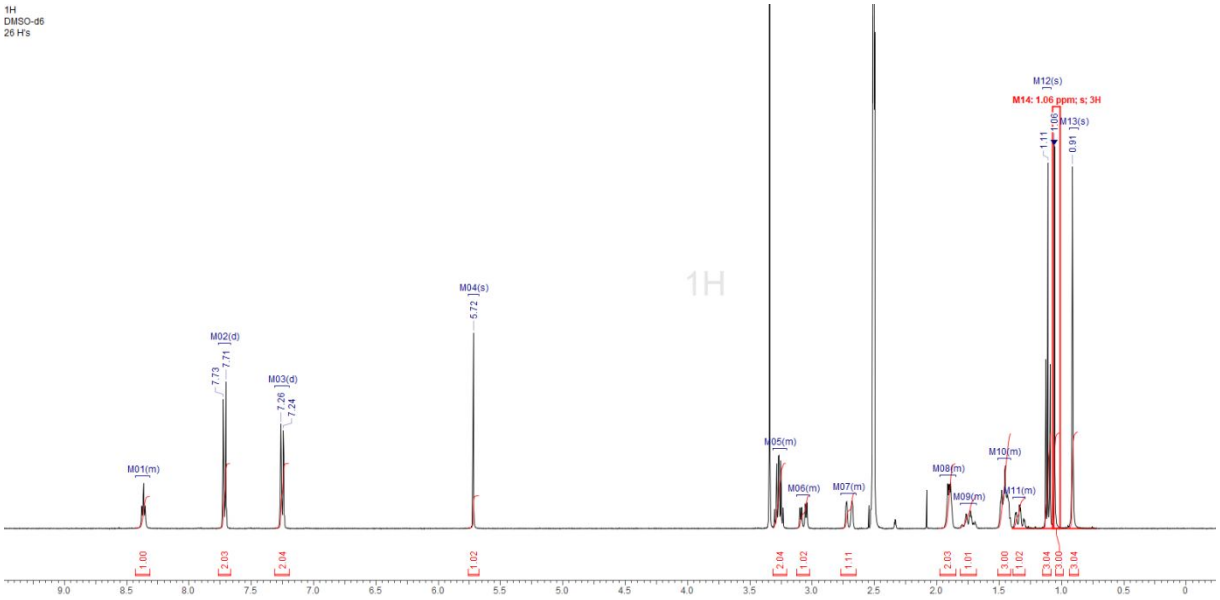


Figure S12. ^{1}H-NMR spectrum of compound 24f

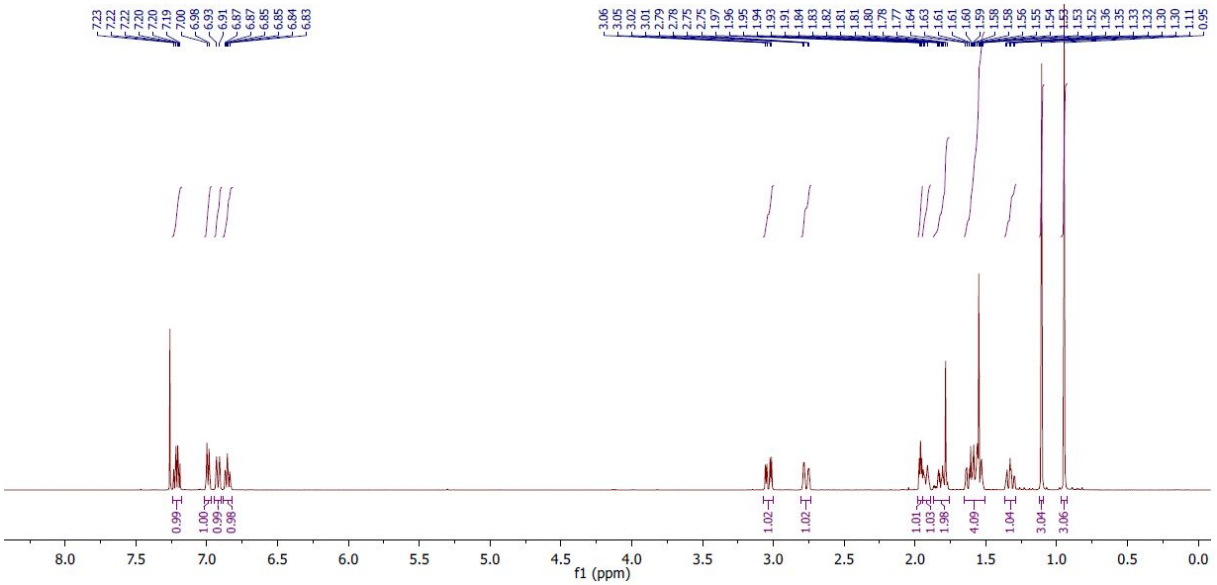


Figure S13. ^{1}H-NMR spectrum of compound 24g

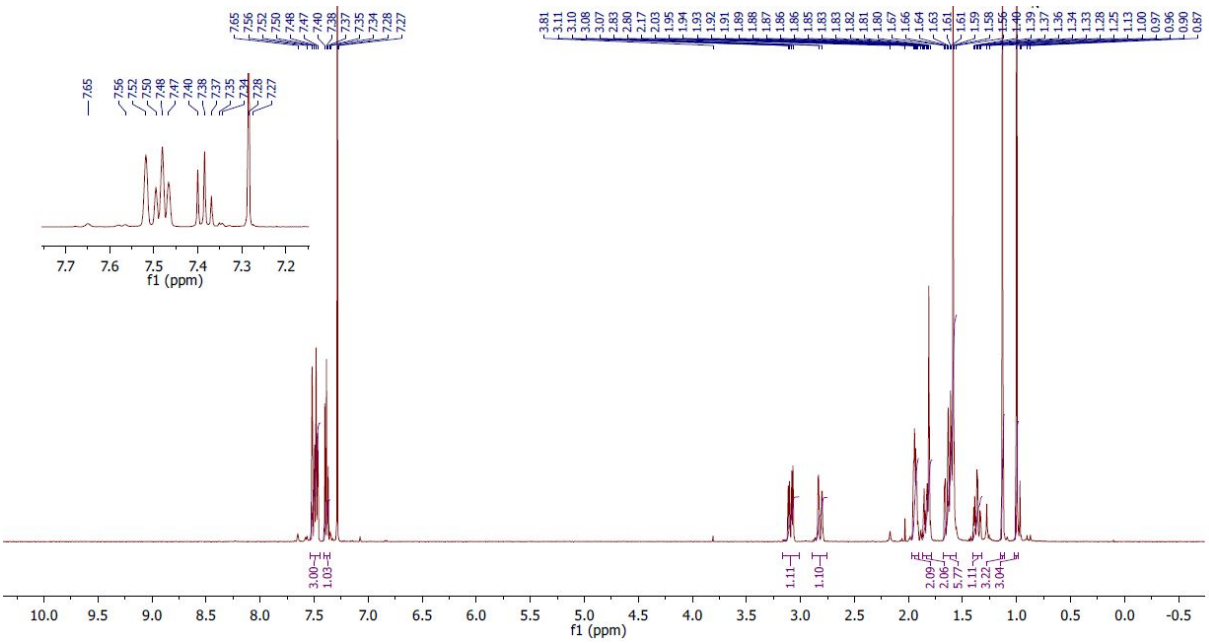


Figure S14. ^{1}H-NMR spectrum of compound 24h

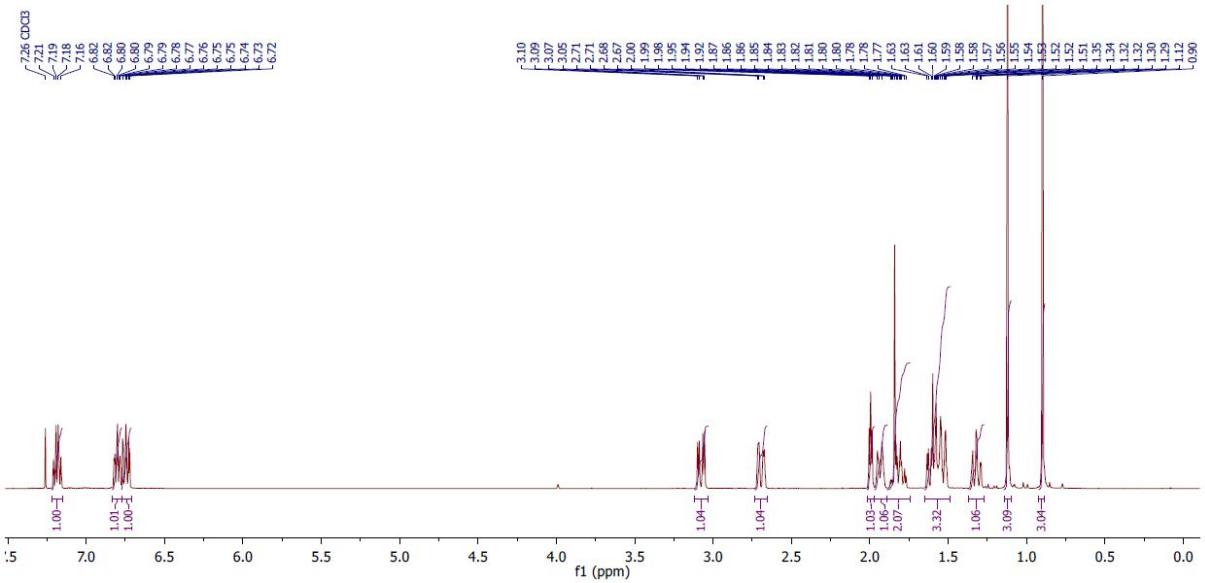


Figure S15. ^{1}H-NMR spectrum of compound 17

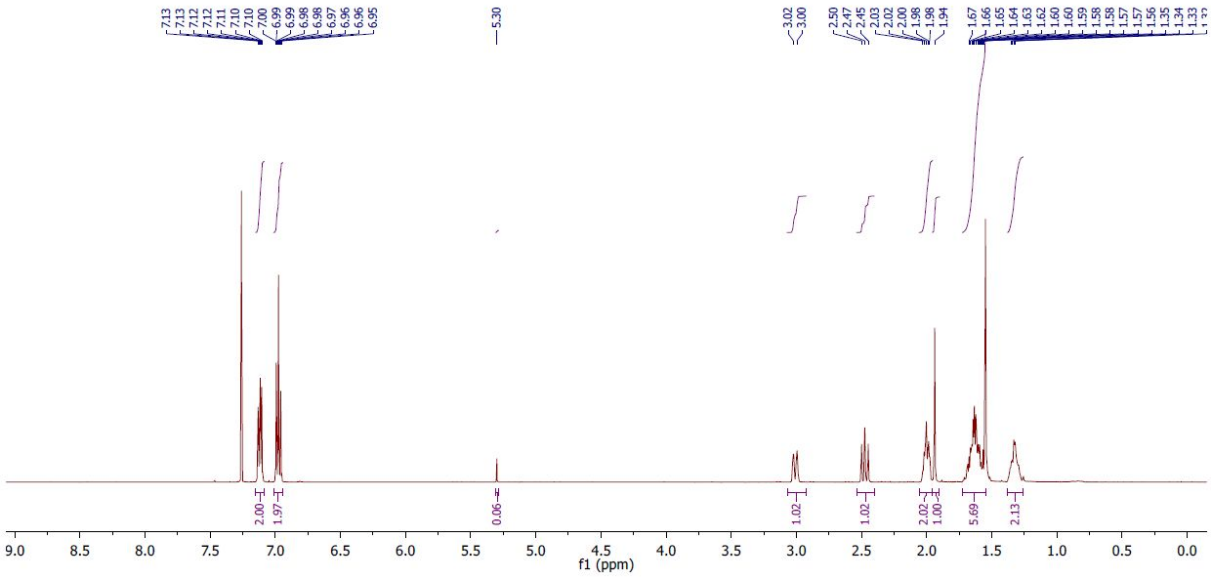


Figure S16. ^{1}H-NMR spectrum of compound 27a

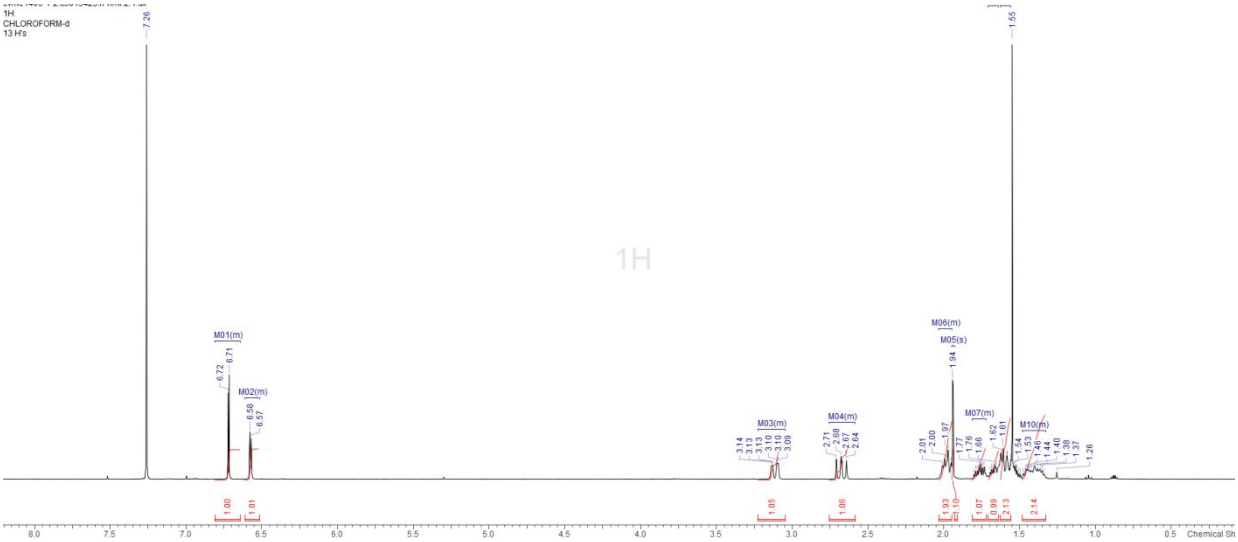


Figure S17. ^{1}H-NMR spectrum of compound 27b

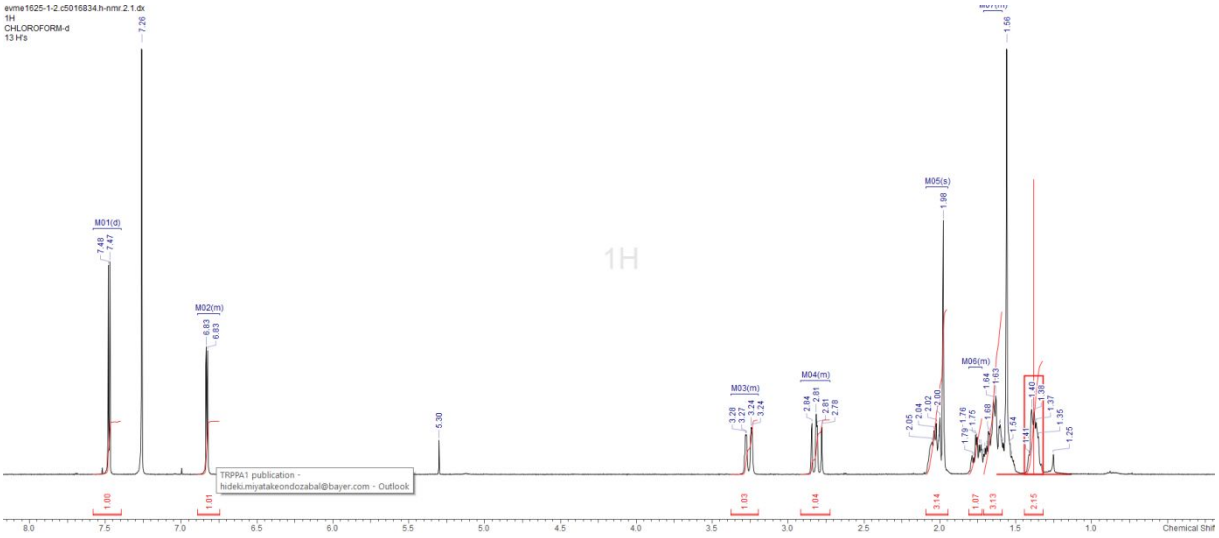


Figure S18. ^{1}H-NMR spectrum of compound 27c

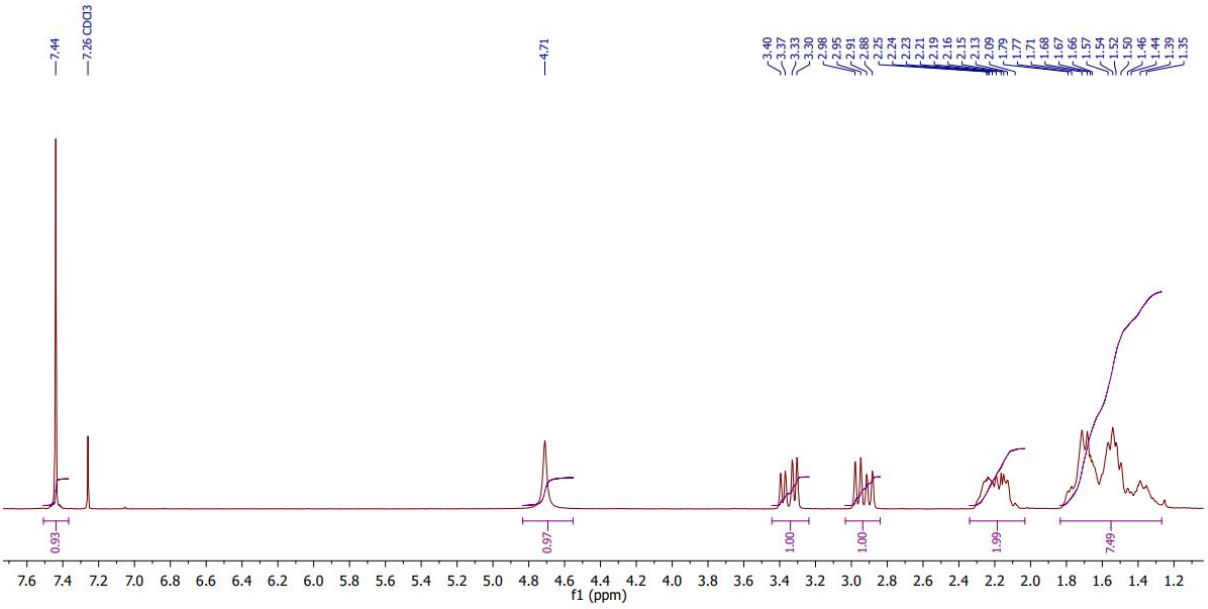


Figure S19. ^{1}H-NMR spectrum of compound 27d

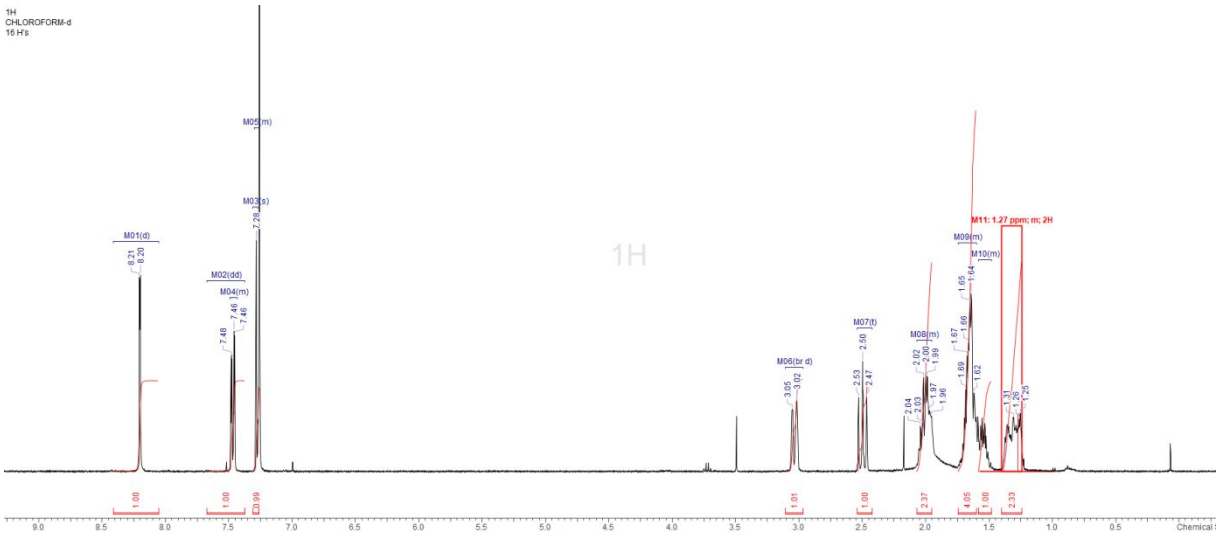


Figure S20. ^{1}H-NMR spectrum of compound 27e

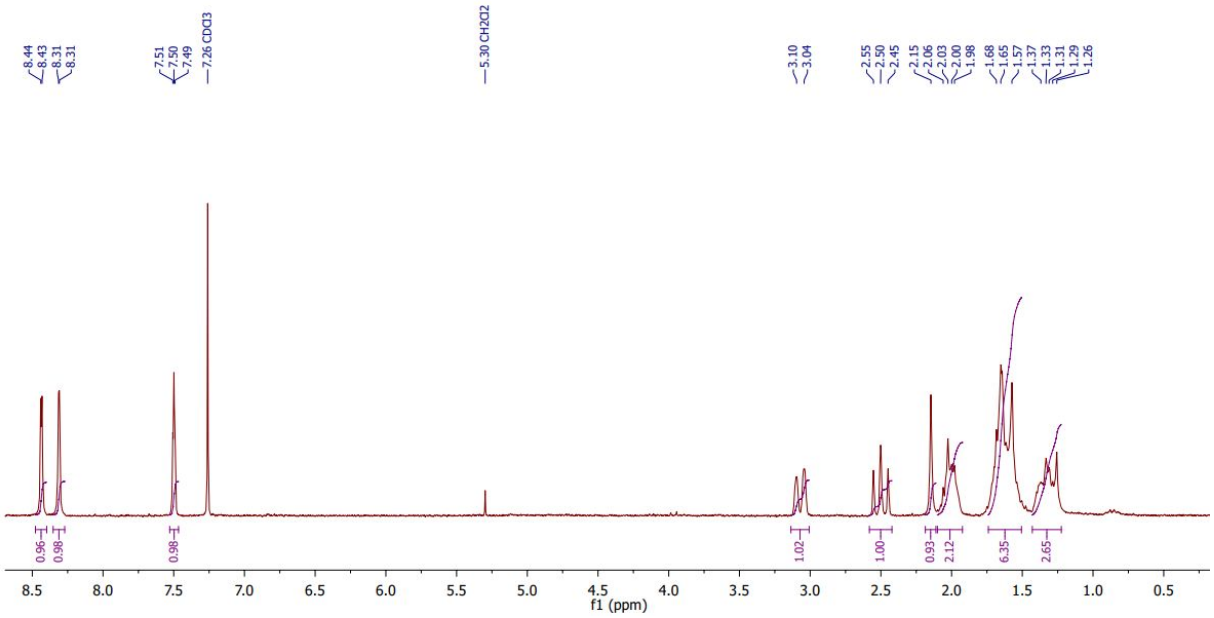


Figure S21. ^{1}H-NMR spectrum of compound 27f

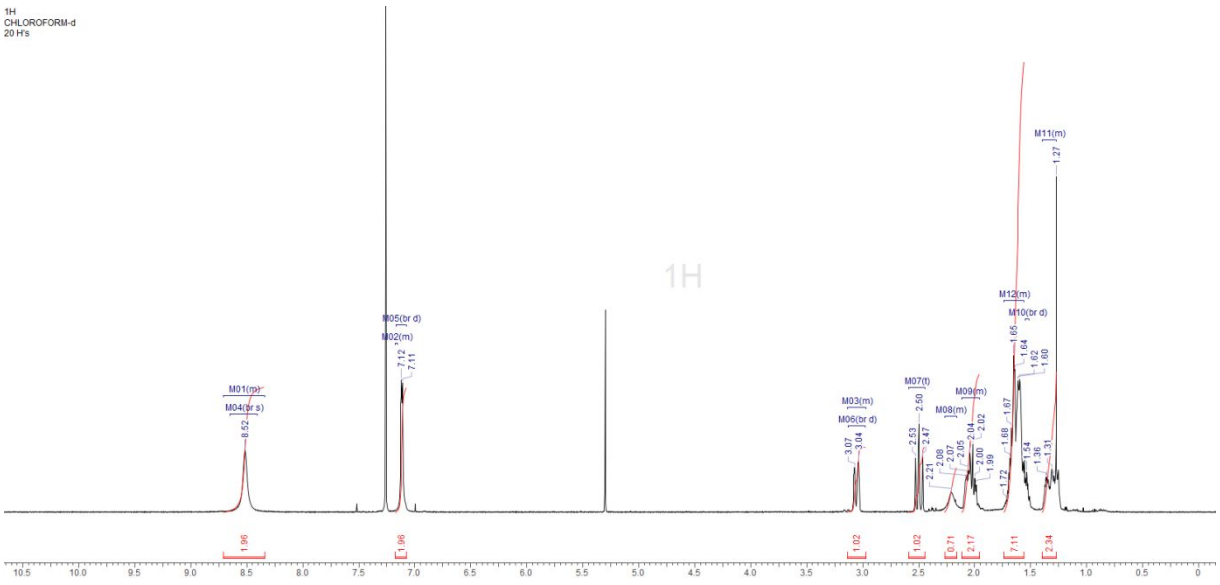


Figure S22. ^{1}H-NMR spectrum of compound 27g

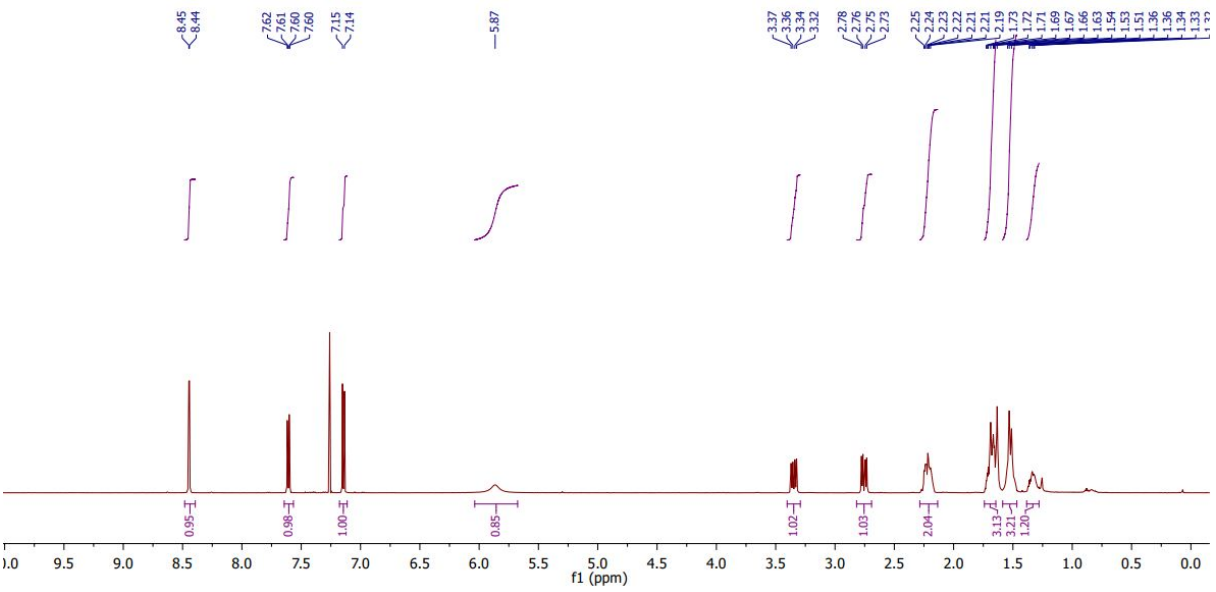


Figure S23. ^{1}H-NMR spectrum of compound 27h

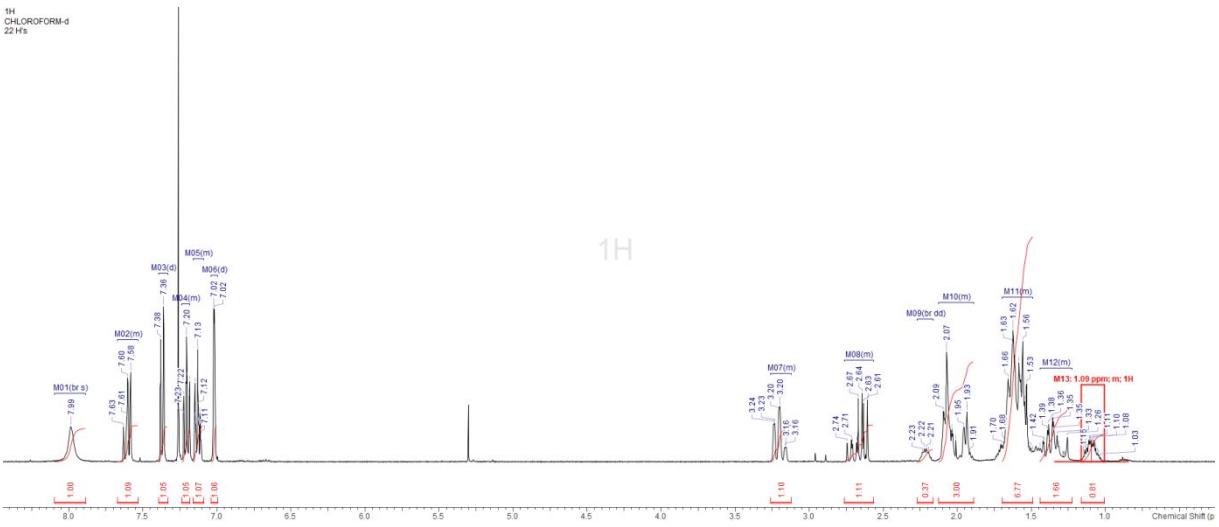


Figure S24. ^{1}H-NMR spectrum of compound 31

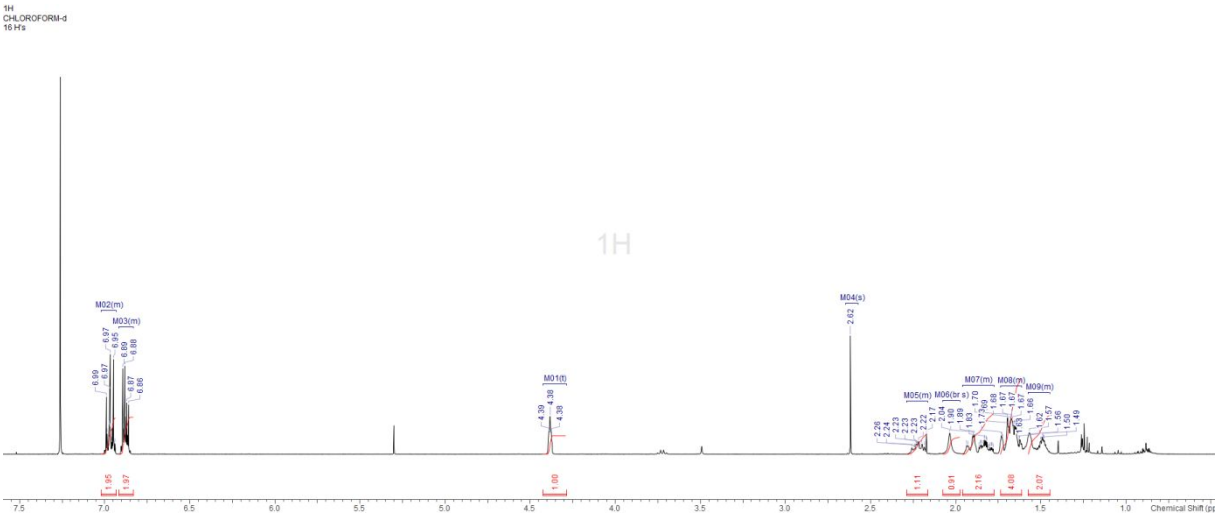


Figure S25. ^{1}H-NMR spectrum of compound 32a

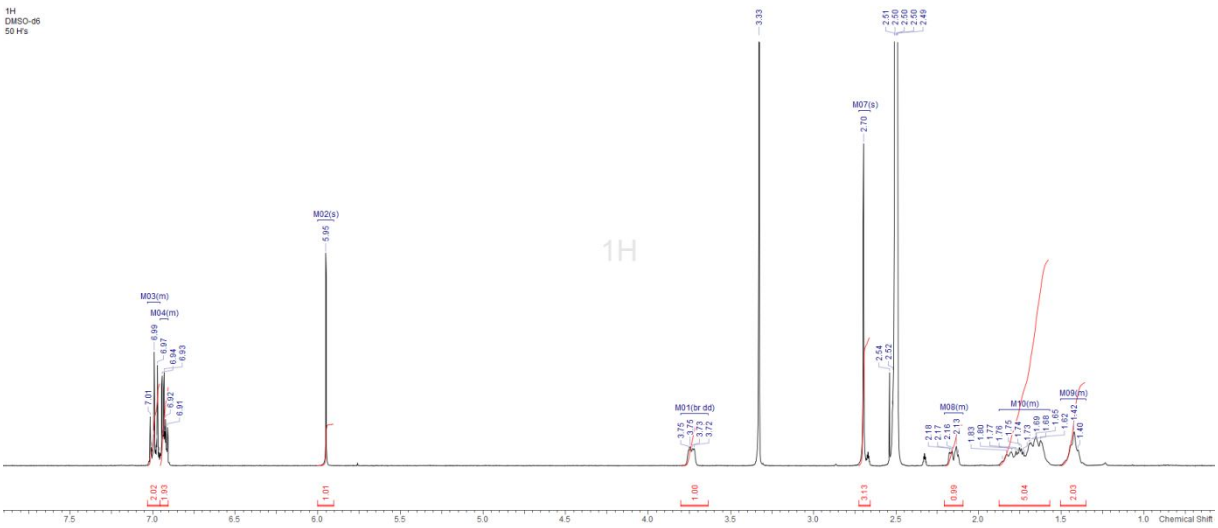


Figure S26. ^{1}H-NMR spectrum of compound 32b

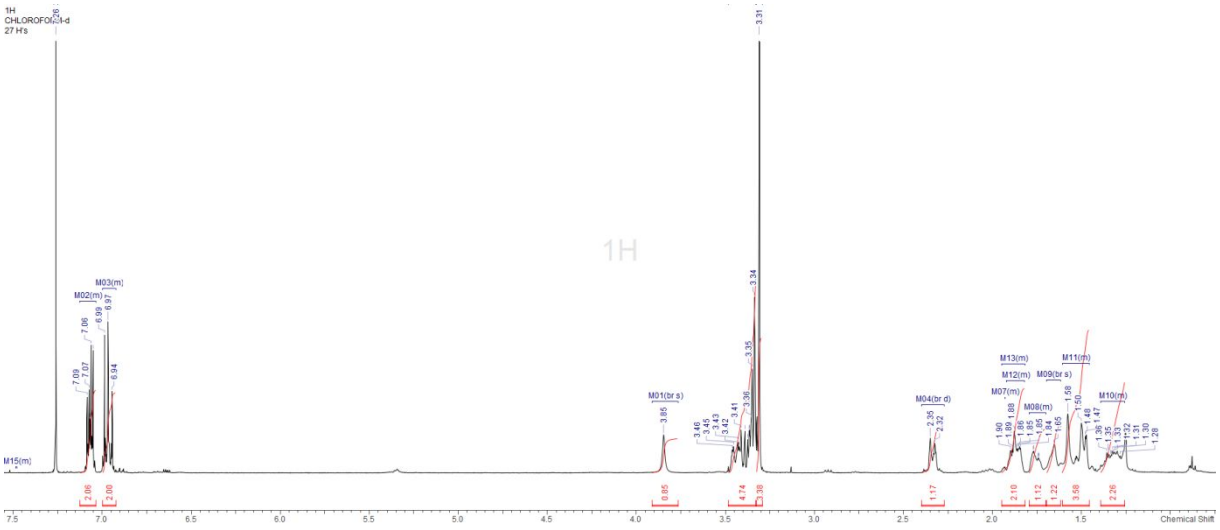


Figure S27. ^{1}H-NMR spectrum of compound 32c

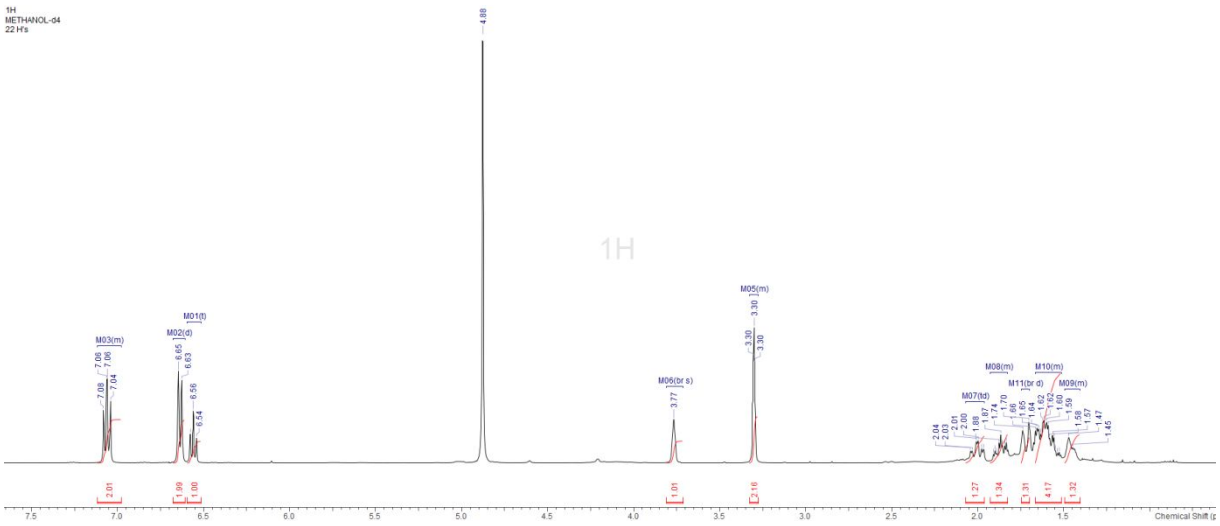


Figure S28. ^{1}H-NMR spectrum of compound 32d

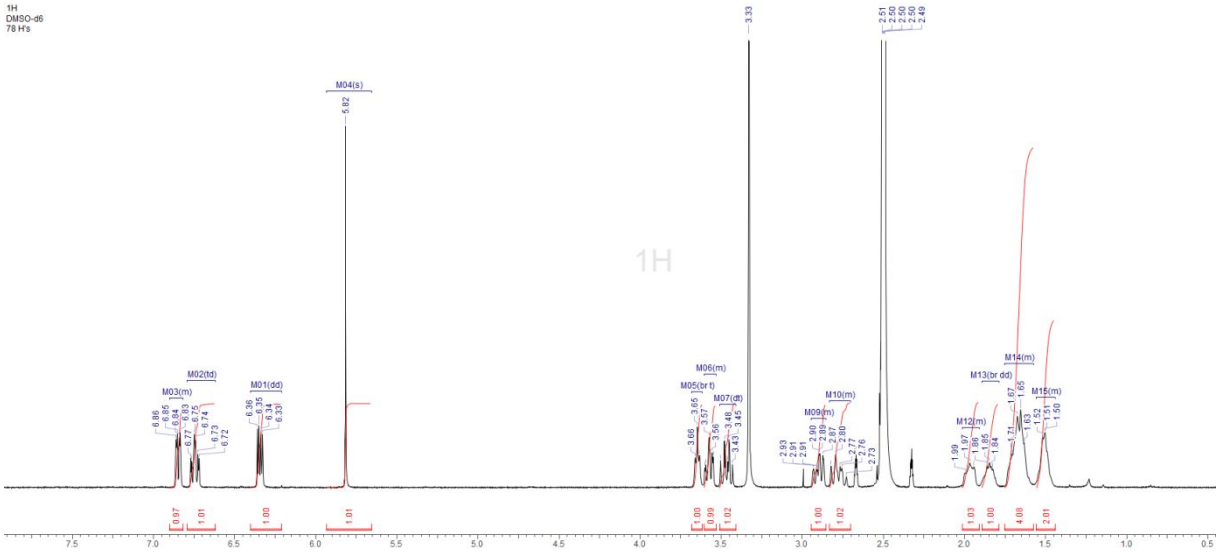
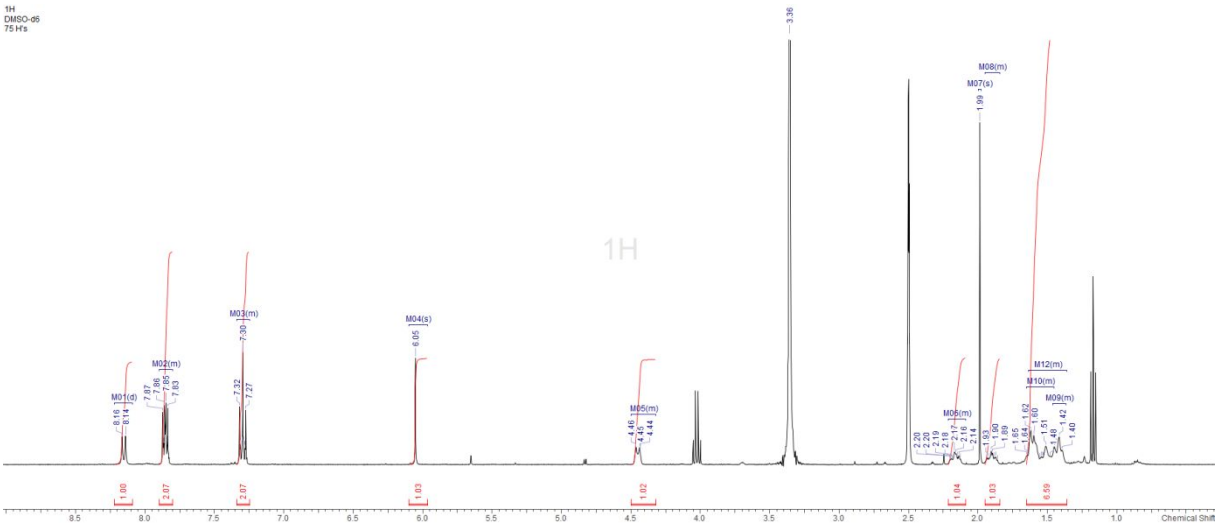


Figure S29. ^{1}H-NMR spectrum of compound 32e



contains residual EtOAc

Figure S30. ^{1}H-NMR spectrum of compound 32f

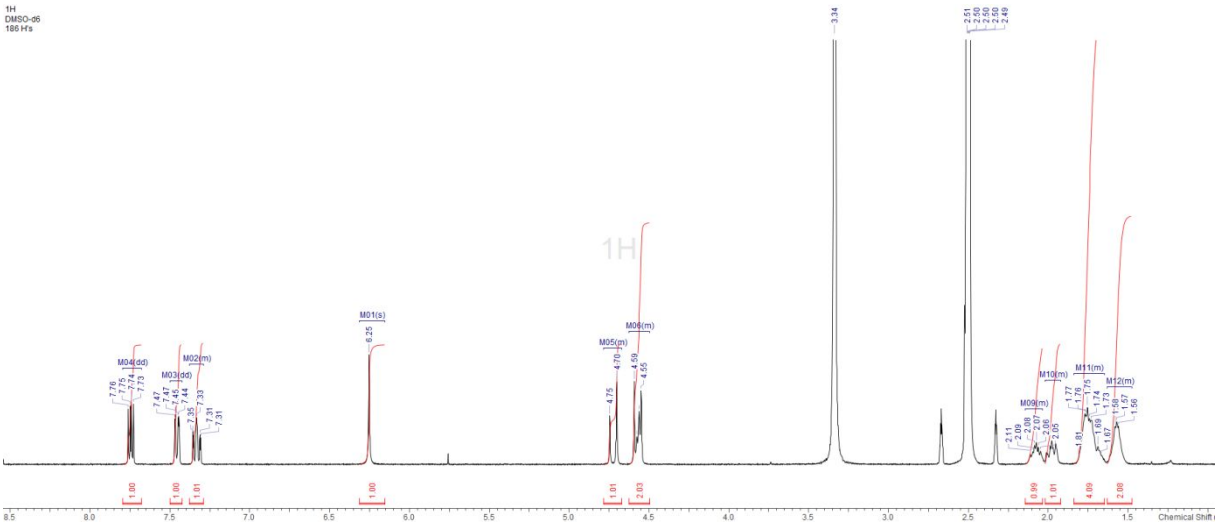


Figure S31. ^{1}H-NMR spectrum of compound 33

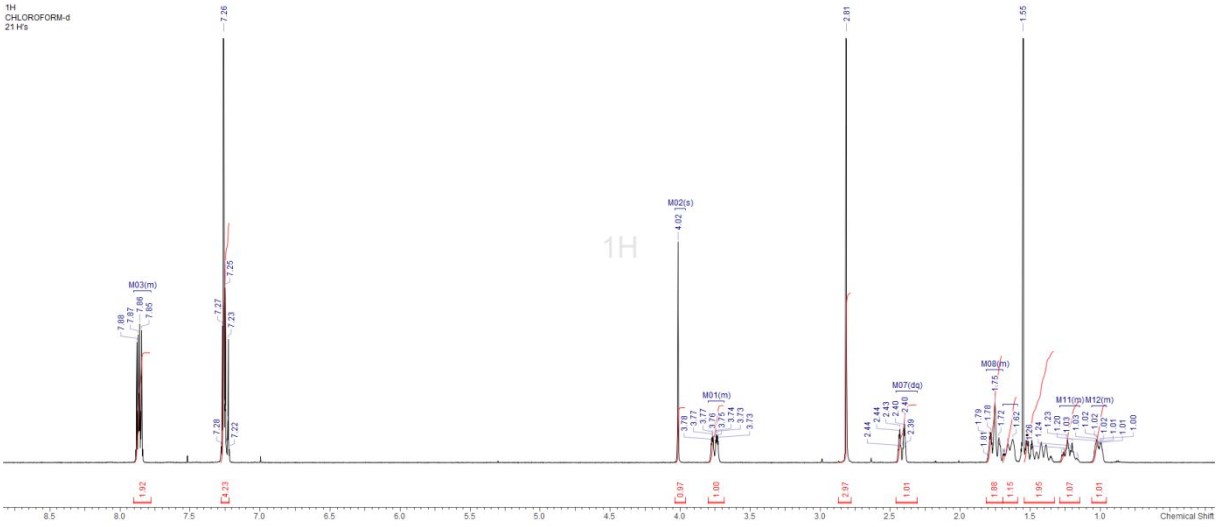


Figure S32. ^{1}H-NMR spectrum of compound 34a

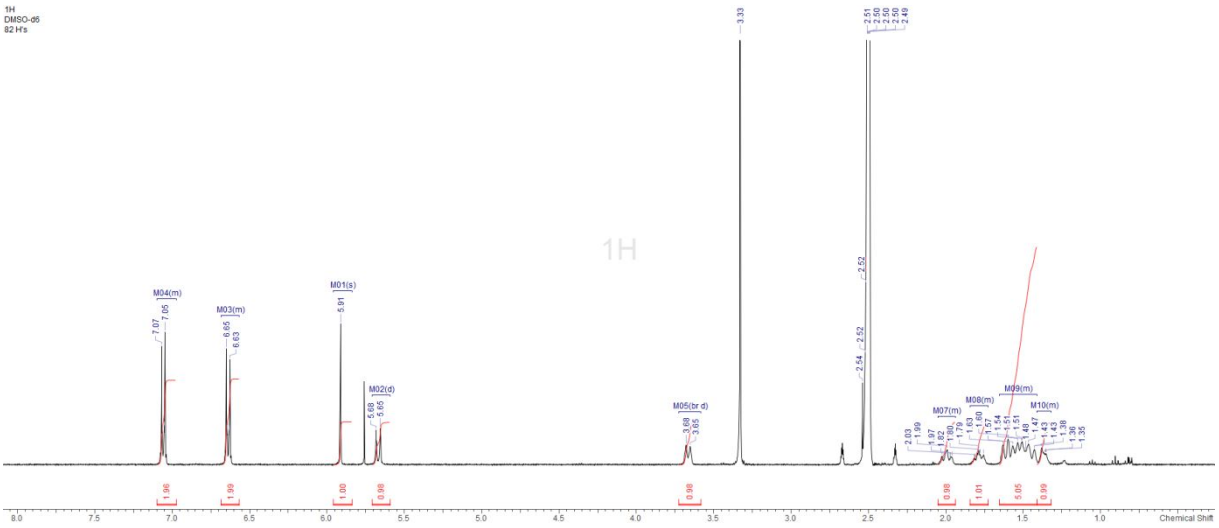


Figure S33. ^{1}H-NMR spectrum of compound 34b

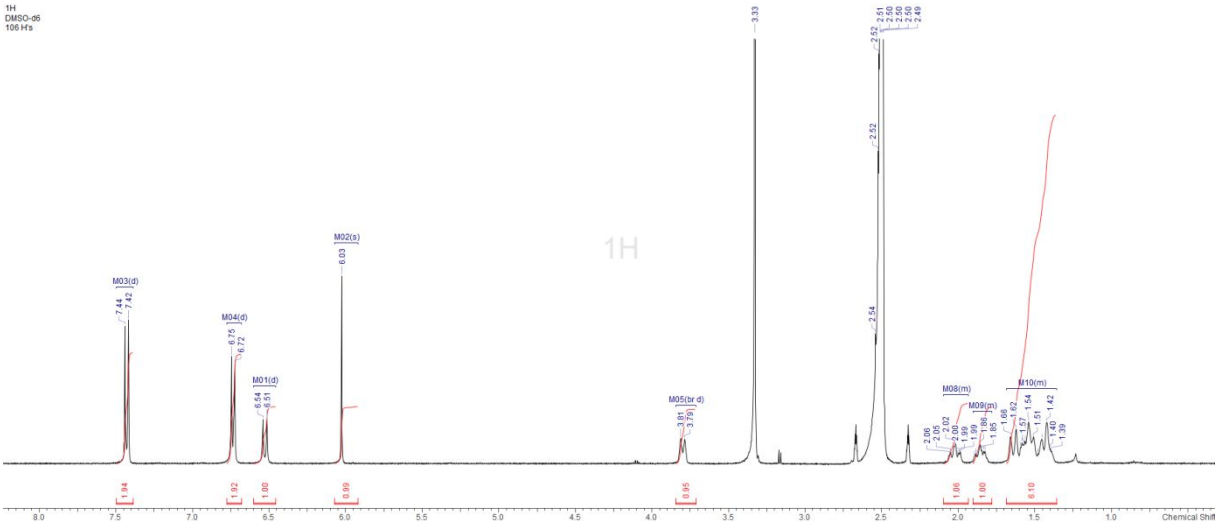
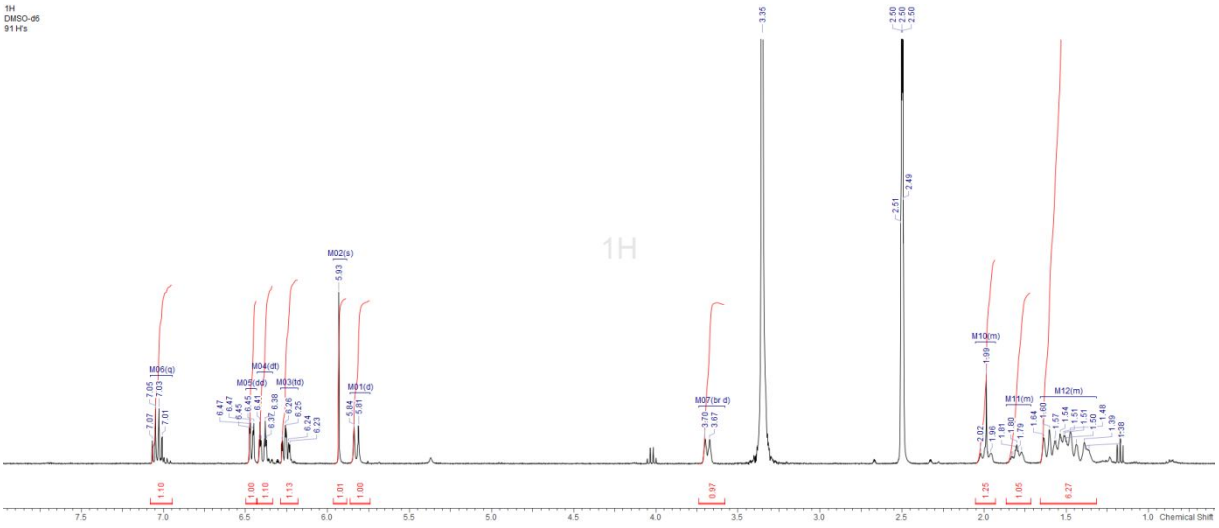


Figure S34. ^{1}H-NMR spectrum of compound 34c



contains residual EtOAc

Figure S35. ^{1}H-NMR spectrum of compound 34d

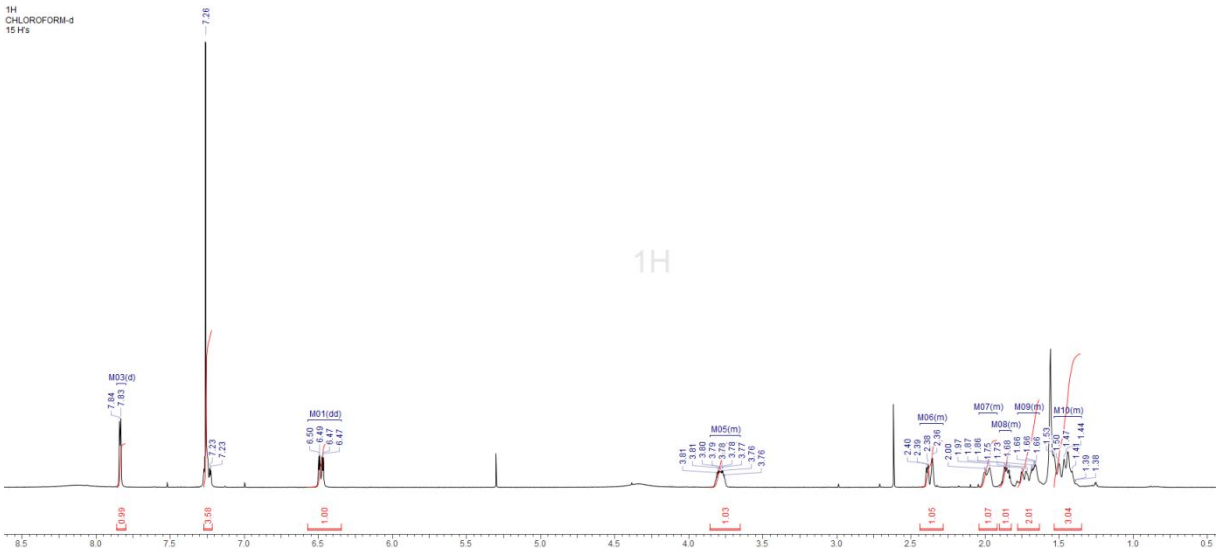


Figure S36. ^{1}H-NMR spectrum of compound 34e

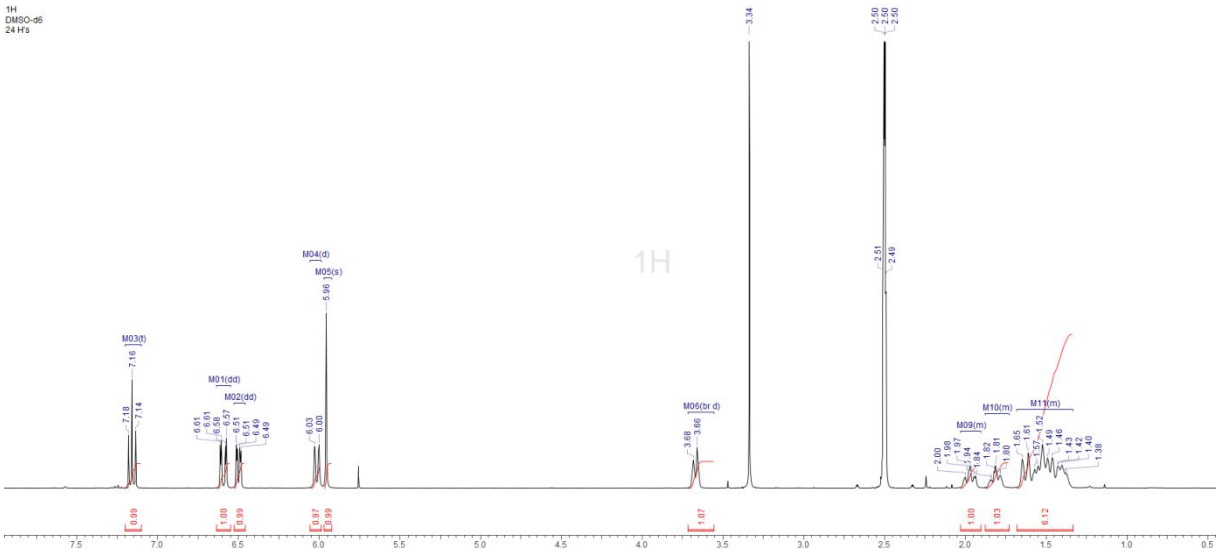


Figure S37. ^{1}H-NMR spectrum of compound 34f

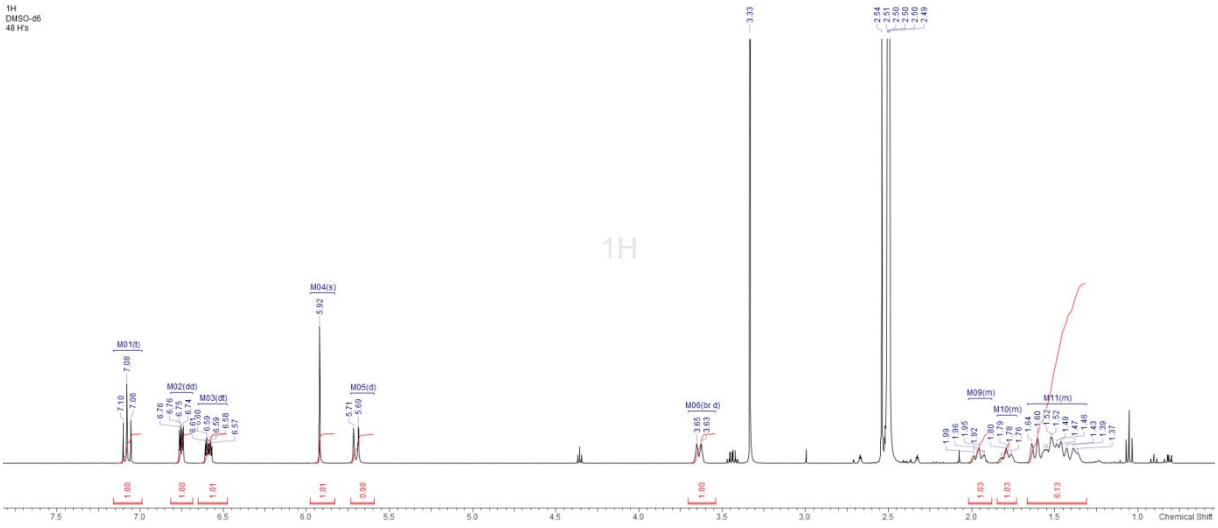
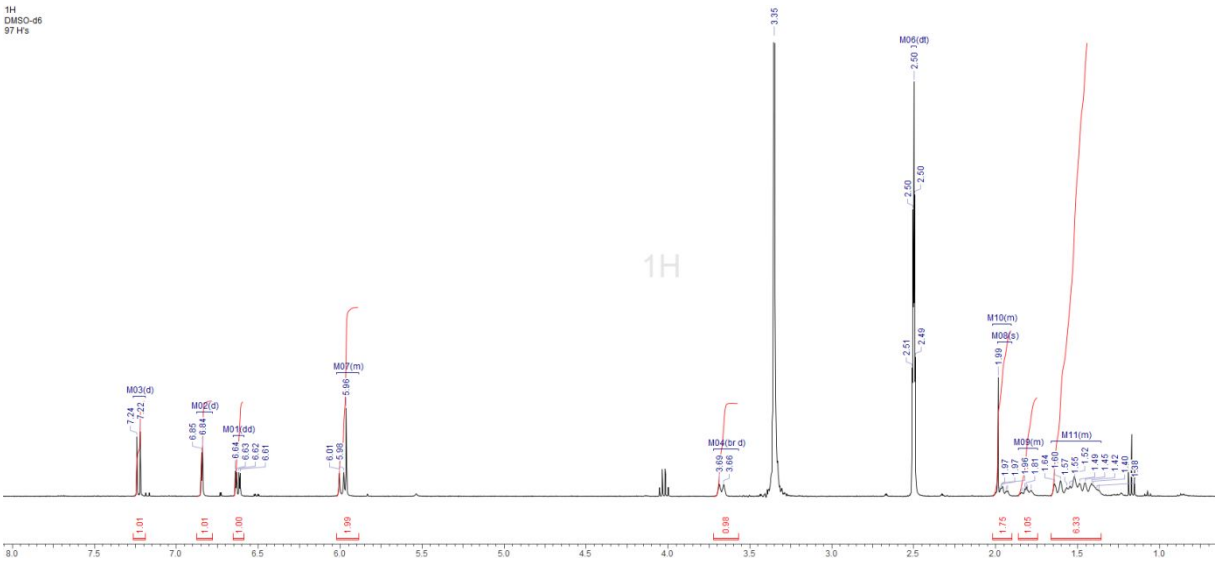


Figure S38. ^{1}H-NMR spectrum of compound 34g



contains residual EtOAc

Figure S39. ^{1}H-NMR spectrum of compound 34h

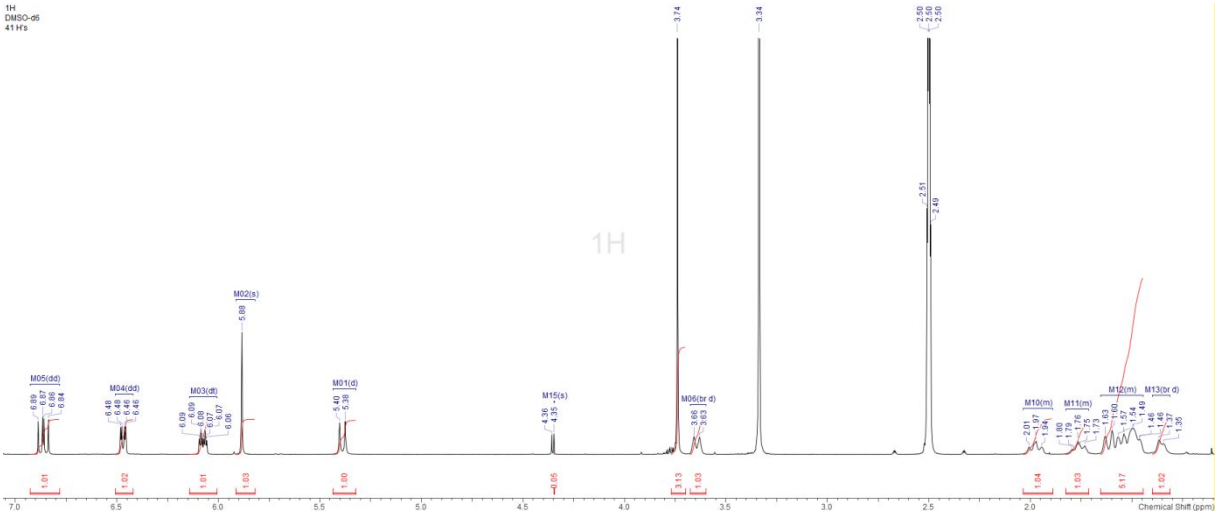
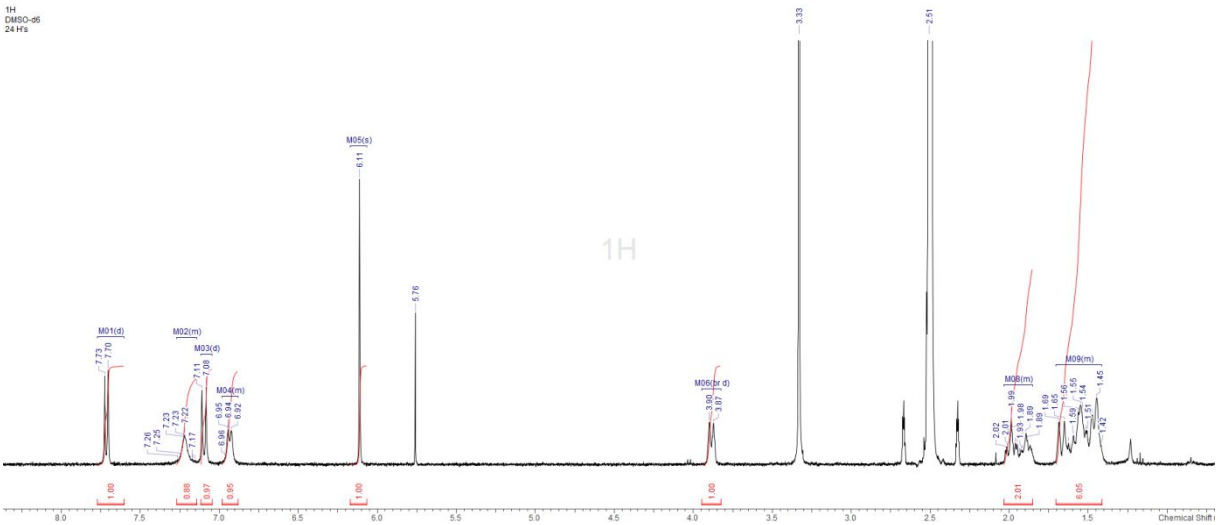


Figure S40. ^{1}H-NMR spectrum of compound 34i



Vibrational circular dichroism measurements of compound 18

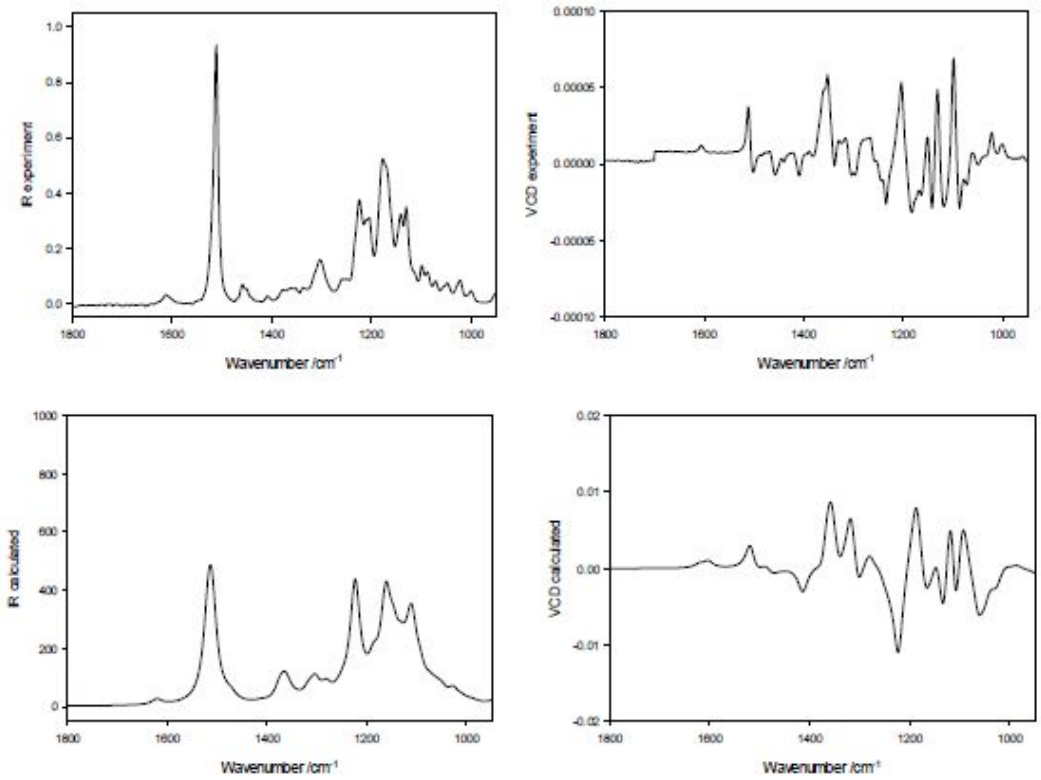
The measurements were performed by BioTools (Antwerp, Belgium).

MEASUREMENT PARAMETERS	
Concentration	4.1 mg / 100 µL
Solvent	CDCl3
Resolution	4 cm–1
PEM setting	1400 cm–1
Number of scans/Measurement time	75.000 scans / 24.0 hours
Sample cell	BaF2
Path length	100 µm
CALCULATION DETAILS	
Force fields used in MolMec conformational analyses	MMFF94S, MMFF, SYBYL
Number of conformations generated	38
Methodology and basis set for DFT calculations	SCRF-B3LYP/6-31G(d) SCRF-B3PW91/6-31G(d)
Enantiomer used for calculation	(R,R)
Number of conformations used in calculated spectrum	11
Number of low-energy conformations shown in report	2

Remarks:
VCD spectra were recorded using $CDCl_3$ as a solvent. Baseline corrections were introduced by using spectra of both enantiomers.
Calculations were performed using B3LYP and B3PW91 functionals, to check for consistency.

Figure S41. Calculated and experimental IR and VCD spectra for the (R,R) enantiomer of compound 18

Inspection of the calculated and experimental spectra shows that the B3LYP IR and VCD spectra for the (*R,R*) enantiomer reproduce the experimental IR and VCD spectra of **S17120**.



The assignment of the AC of S17120 to (*R,R*) is confirmed by the neighborhood similarities and confidence levels calculated.

Off-target selectivity data (Eurofins-Panlabs radioligand binding assay) of compound 18

Table S1. Eurofins-Panlabs radioligand binding assays on selected targets using compound 18 – Part I

Experimental Results

Cat #	Assay Name	Batch*	Spec.	Rep.	Conc.	% Inh.
Compound: CHH039-2017, PT #: 1207556						
107000	Aldose Reductase	401131	rat	2	10 µM	3
107710	ATPase, Na^+/K^+, Heart, Pig	401160	pig	2	10 µM	3
112020	Carbonic Anhydrase II	401132	hum	2	10 µM	7
104010	Cholinesterase, Acetyl, ACES	401128	hum	2	10 µM	-2
116020	Cyclooxygenase COX-1	401157	hum	2	10 µM	8
118010	Cyclooxygenase COX-2	401158	hum	2	10 µM	23
124010	HMG-CoA Reductase	401134	hum	2	10 µM	8
132000	Leukotriene LTC_4 Synthase	401133	gp	2	10 µM	-15
199017	Lipoxygenase 15-LO	401198	hum	2	10 µM	23
140010	Monoamine Oxidase MAO-A	401130	hum	2	10 µM	3
140120	Monoamine Oxidase MAO-B	401159	hum	2	10 µM	7
142000	Nitric Oxide Synthase, Neuronal (nNOS)	401135	rat	2	10 µM	-3
199010	Nitric Oxide Synthetase, Inducible (iNOS)	401197	mouse	2	10 µM	5
107300	Peptidase, Angiotensin Converting Enzyme	401129	rabbit	2	10 µM	3
152000	Phosphodiesterase PDE3	401193	hum	2	10 µM	3
154000	Phosphodiesterase PDE4	401194	hum	2	10 µM	-11
156000	Phosphodiesterase PDE5	401393	hum	2	10 µM	7
194020	Thromboxane Synthase	401136	hum	2	10 µM	6
200510	Adenosine A_1	401253	hum	2	10 µM	18
200610	Adenosine A_{2A}	401253	hum	2	10 µM	3
200720	Adenosine A_3	401176	hum	2	10 µM	4
203100	Adrenergic α_{1A}	401240	rat	2	10 µM	18
203630	Adrenergic α_{2A}	401239	hum	2	10 µM	7
203710	Adrenergic α_{2B}	401142	hum	2	10 µM	2
203810	Adrenergic α_{2C}	401211	hum	2	10 µM	-1
204010	Adrenergic β_1	401230	hum	2	10 µM	3
204110	Adrenergic β_2	401239	hum	2	10 µM	11
204200	Adrenergic β_3	401359	hum	2	10 µM	1
206000	Androgen (Testosterone)	401322	hum	2	10 µM	47
210030	Angiotensin AT_1	401212	hum	2	10 µM	4
210120	Angiotensin AT_2	401213	hum	2	10 µM	1
212510	Bradykinin B_1	401177	hum	2	10 µM	9
212620	Bradykinin B_2	401170	hum	2	10 µM	4
217030	Cannabinoid CB_1	401143	hum	2	10 µM	-15

Note: Items meeting criteria for significance (≥50% stimulation or inhibition) are highlighted.
* Batch: Represents compounds tested concurrently in the same assay(s).
gp=Guinea pig; hum=Human

Table S2. Eurofins-Panlabs radioligand binding assays on selected targets using compound 18 – Part II

Experimental Results

Cat #	Assay Name	Batch*	Spec.	Rep.	Conc.	% Inh.
217100	Cannabinoid CB_2	401141	hum	2	10 µM	21
219500	Dopamine D_1	401243	hum	2	10 µM	-14
219600	Dopamine D_{2L}	401370	hum	2	10 µM	12
219700	Dopamine D_{2S}	401241	hum	2	10 µM	13
219800	Dopamine D_3	401243	hum	2	10 µM	9
224010	Endothelin ET_A	401229	hum	2	10 µM	7
224110	Endothelin ET_B	401366	hum	2	10 µM	-4
226010	Estrogen ERα	401171	hum	2	10 µM	75
226810	$GABA_A$, Chloride Channel, TBOB	401172	rat	2	10 µM	-8
226600	$GABA_A$, Flunitrazepam, Central	401246	rat	2	10 µM	-17
228510	$GABA_B$, Non-Selective	401185	rat	2	10 µM	3
232030	Glucocorticoid	401208	hum	2	10 µM	4
232600	Glutamate, AMPA	401173	rat	2	10 µM	8
232700	Glutamate, Kainate	401296	rat	2	10 µM	-3
232810	Glutamate, NMDA, Agonism	401224	rat	2	10 µM	5
232910	Glutamate, NMDA, Glycine	401224	rat	2	10 µM	9
239300	Growth Hormone Secretagogue (GHS, Ghrelin)	401154	hum	2	10 µM	3
239610	Histamine H_1	401249	hum	2	10 µM	6
239710	Histamine H_2	401256	hum	2	10 µM	-8
239820	Histamine H_3	401166	hum	2	10 µM	-4
243000	Insulin	401420	rat	2	10 µM	8
252200	Motilin	401156	hum	2	10 µM	13
252610	Muscarinic M_1	401216	hum	2	10 µM	-1
252710	Muscarinic M_2	401231	hum	2	10 µM	-2
252810	Muscarinic M_3	401231	hum	2	10 µM	21
252910	Muscarinic M_4	401217	hum	2	10 µM	14
258590	Nicotinic Acetylcholine	401227	hum	2	10 µM	-7
260130	Opiate δ_1 (OP1, DOP)	401150	hum	2	10 µM	-6
260210	Opiate κ(OP2, KOP)	401150	hum	2	10 µM	7
260410	Opiate µ(OP3, MOP)	401232	hum	2	10 µM	5
299005	Progesterone PR-B	401321	hum	2	10 µM	65
268700	Purinergic P2X	401151	rabbit	2	10 µM	3
268810	Purinergic P2Y	401152	rat	2	10 µM	-6
271110	Serotonin (5-Hydroxytryptamine) 5-HT_{1A}	401182	hum	2	10 µM	10
271650	Serotonin (5-Hydroxytryptamine) 5-HT_{2A}	401181	hum	2	10 µM	-2

Note: Items meeting criteria for significance (≥50% stimulation or inhibition) are highlighted.
* Batch: Represents compounds tested concurrently in the same assay(s).
gp=Guinea pig; hum=Human

Table S3. Eurofins-Panlabs radioligand binding assays on selected targets using compound 18 – Part III

Experimental Results

Cat #	Assay Name	Batch*	Spec.	Rep.	Conc.	% Inh.
271700	Serotonin (5-Hydroxytryptamine) 5-HT$_{2B}$	401168	hum	2	10 µM	-4
271800	Serotonin (5-Hydroxytryptamine) 5-HT$_{2C}$	401180	hum	2	10 µM	3
202000	Transporter, Adenosine	401137	gp	2	10 µM	-2
220320	Transporter, Dopamine (DAT)	401164	hum	2	10 µM	84
226400	Transporter, GABA	401186	rat	2	10 µM	-5
204410	Transporter, Norepinephrine (NET)	401164	hum	2	10 µM	16
274030	Transporter, Serotonin (5-Hydroxytryptamine) (SERT)	401182	hum	2	10 µM	3
287530	Vasopressin V$_{1A}$	401219	hum	2	10 µM	-7

Estrogen ERalpha

IC50 [µM]=5 Ki [µM]=0.72

Progesterone PR-B

IC50 [µM]=5 Ki [µM]= 4

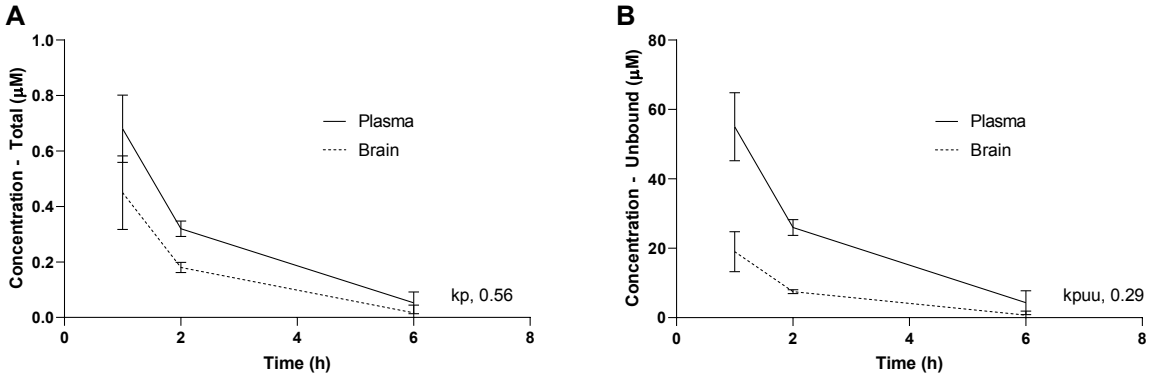
Transporter, Dopamine (DAT)

IC50 [µM]=1.2 Ki [µM]=0.94

Determination of brain/plasma ratio and measured plasma exposure levels in rat using compound 18 (BAY-390)

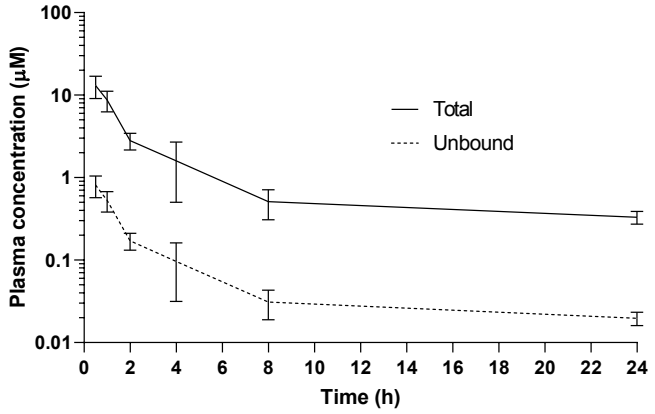
Brain/Plasma ratio of BAY-390 was assessed in female CD mice following i.v. administration. Total brain and plasma concentrations (A) were measured, and unbound level (B) were estimated using fraction unbound value of 8.1% for mouse plasma and 4.3% for brain tissues.

Figure S42. (A) total brain and plasma concentration, (B) estimated unbound brain and plasma levels using measured fraction unbound values of BAY-390



BAY-390 plasma concentration in Rat Sprague Dawley male following p.o. administration of a 50mg/kg solution in DMSO/PEG400/Water (20/20/60, vol/vol). Unbound level were estimated using fraction unbound value of 6.0% for rat plasma.

Figure S43. total and unbound plasma concentration of BAY-390 in rat after p.o administration of a 50 mg/kg in DMSO/PEG400/Water (20/20/60, vol/vol)



Method: Estimation of Plasma Protein Binding by Equilibrium Dialysis

Binding of test compounds to plasma proteins is measured by equilibrium dialysis in a 96-well format using ht-dialysis equipment made of Teflon and a semipermeable membrane (regenerated cellulose, MWCO 12-14K). The membrane separates the plasma and buffer side (50 mM phosphate buffer) filled with 150 µl each. The test compound is added in a concentration of 3 µM to the plasma side and binds to plasma proteins. The unbound fraction of the test compound passes the membrane and distributes on both sides until equilibrium is reached, which is usually the case after 6-8h at 37˚C. Compound concentration of plasma and buffer side is measured by LC-MSMS analytics. For this both sides are diluted with buffer and plasma to achieve the same matrix (10% plasma) and subsequently are precipitated with methanol. From the quotient of buffer and plasma concentration the free (unbound) fraction (fu) is calculated. Stability and recovery controls are included. Additionally, the test compound is dialyzed in buffer against buffer in order to estimate non-specific binding to equipment and/or membrane and to investigate in the establishment of the equilibrium. Due to

the osmotic pressure of the plasma proteins a dilution of the plasma takes place during the incubation (volume shift). The potential imprecision is addressed by inclusion of an empirical factor in the calculation of the fu. Establishment of equilibrium and stability in plasma should be at least 80% and the recovery in plasma should at least be 30%. A free fraction of <1% is designated as high, between 1 and 10% as moderate and of >10% as low plasma protein binding.

Method: Brain/Plasma ratio determination

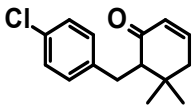
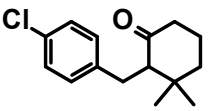
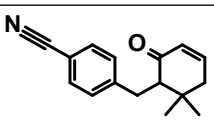
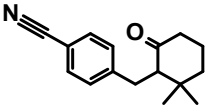
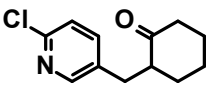
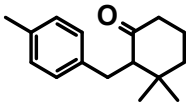
Penetration of test compounds into the brain was assessed in female CD mice after intravenous administration. Test compounds were administered i.v., at a dose of 10 mg/kg as solution using solubilizers such as PEG400 in well-tolerated amounts. Separate groups of animals (3 animals per group) were sacrificed at 1, 2, and 6h after dosing and blood and brain were sampled. Blood was collected into Lithium-Heparintubes (Monovetten® , Sarstedt) and centrifuged for 15 min at 3000 rpm. An aliquot of 100 µL from the supernatant (Plasma) was taken and precipitated by addition of 400 µL cold acetonitril and frozen at -20 ˚C over night. Brain samples were homogenized with 50 mM Tris-HCl buffer, pH7.5 (1:5 w/v), precipitated with acetonitril (1:5, v/v) and frozen at -20 ˚C over night. Plasma and brain samples were subsequently thawed and centrifuged at 3000 rpm, 4˚C for 20 minutes. Aliquots of the supernatants were taken for analytical testing using an Agilent 1200 HPLC-system with LCMS/MS detection.

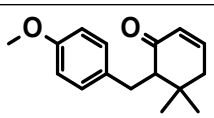
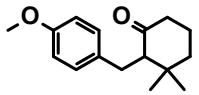
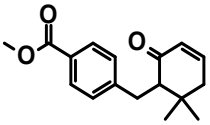
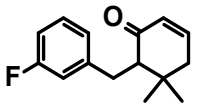
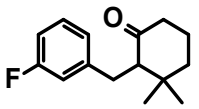
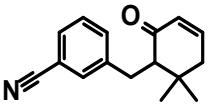
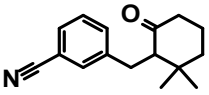
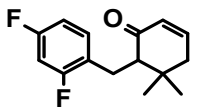
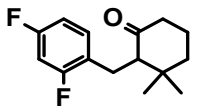
From the concentration-time profiles the AUC (area under the concentration-time curve) in plasma and brain were calculated and the ratio AUCbrain/AUCplasma was reported as the

brain-plasma ratio. Due to residual blood in the non-perfused brain tissue the lower limit for the brain-plasma ratio by this method approximates 1-2%.

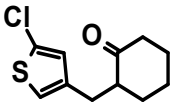
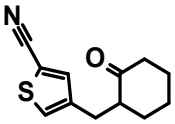
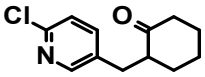
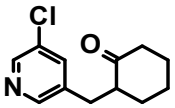
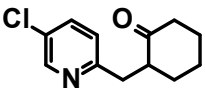
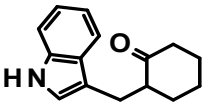
Characterization and synthesis of intermediates 22b-i, 23b-i and 26b-i

Table S4. Analytical data of intermediates 22b-i, 23b-i and 26b-i

Name (yield; purity)	Structure	Analytics
6-[(4-Chlorophenyl)methyl]-5,5-dimethyl-cyclohex-2-en-1-one (**22b**; yield: 27%, purity 93%).		MS (ESIpos): m/z = 248.95 / 250.95 $(M+H)^+$; ^{1}H-NMR (500 MHz, $CDCl_3$) δ [ppm]: 1.01 (s, 3H), 1.12 (s, 3H), 2.28 – 2.32 (m, 2H), 2.42 (dd, 1H), 2.73 (dd, 1H), 2.95 (dd, 1H), 5.98 (dt, 1H), 6.77 (dt, 1H), 7.13 (d, 2H), 7.20 – 7.23 (d, 2H)
2-[(4-Chlorophenyl)methyl]-3,3-dimethyl-cyclohexanone (**23b;** yield: 91%, purity 93%).		^{1}H-NMR (500 MHz, $CDCl_3$) δ [ppm]: 0.84 (s, 3H), 1.19 (s, 3H), 1.62 (dtd, 1H), 1.72 (td, 1H), 1.77 – 1.87 (m, 1H), 1.93 (m, 1H), 2.18 – 2.25 (dtd, 1H), 2.29 – 2.35 (m, 1H), 2.42 – 2.46 (dd, 1H), 2.52 (dd, 1H), 3.05 (dd, 1H), 7.13 (d, 2H), 7.17 – 7.21 (d, 2H)
4-[(6,6-Dimethyl-2-oxo-cyclohex-3-en-1-yl)methyl]benzonitrile (**22c**; yield: 14%, purity 85%).		MS (ESIpos): m/z = 239.95 $(M+H)^+$; ^{1}H-NMR (500 MHz, $CDCl_3$) δ [ppm]: 1.01 (s, 3H), 1.15 (s, 3H), 2.28 (ddd, 1H), 2.37 (dt, 1H), 2.47 (dd, 1H), 2.79 (dd, 1H), 3.05 (dd, 1H), 5.99 (dt, 1H), 6.79 (ddd, 1H), 7.32 (d, 2H), 7.52 – 7.55 (d, 2H)
4-[(2,2-Dimethyl-6-oxo-cyclohexyl)methyl]benzonitrile (**23c**; yield: 96%, purity 90%).		^{1}H-NMR (500 MHz, $CDCl_3$) δ [ppm]: 0.83 (s, 3H), 1.19 (s, 3H), 1.59 – 1.65 (m, 1H), 1.70 – 1.86 (m, 2H), 1.89 – 1.96 (m, 1H), 2.17 – 2.24 (m, 1H), 2.30 (ddt, 1H), 2.44 – 2.50 (m, 1H), 2.59 (dd, 1H), 3.11 (dd, 1H), 7.30 (d, 2H), 7.47 – 7.51 (m, 2H)
5,5-Dimethyl-6-(p-tolylmethyl)cyclohex-2-en-1-one (**22d**; yield: 12%, purity 95%).		LCMS (ESIpos): m/z = 229 $(M+H)^+$; ^{1}H-NMR (500 MHz, $CDCl_3$) δ [ppm]: 7.27 (s, 4H), 7.03 – 6.87 (m, 1H), 6.24 – 6.11 (m, 1H), 3.13 (dd, 1H), 2.95 (dd, 1H), 2.63 (dd, 1H), 2.50 (s, 5H), 1.31 (s, 3H), 1.23 (s, 3H)
3,3-Dimethyl-2-(p-tolylmethyl)cyclohexanone (**23d**; yield: 83%, purity 95%).		^{1}H-NMR (500 MHz, $CDCl_3$) δ [ppm]: 7.02 (d, 2H), 6.97 (d, 2H), 2.98 (dd1H), 2.49 – 2.44 (m, 1H), 2.42 – 2.39 (m, 1H), 2.28 – 2.23 (m, 1H), 2.21 (s, 3H), 2.18 – 2.10 (m, 1H), 1.88 – 1.70 (m, 2H), 1.63 (td, 1H), 1.55 (dtd, 1H), 1.11 (s, 3H), 0.77 (s, 3H)

6-[(4-Methoxyphenyl)methyl]-5,5-dimethyl-cyclohex-2-en-1-one (**22e**; yield: 11%, purity 95%).		LCMS (ESIpos): m/z = 245 $(M+H)^+$; ^{1}H-NMR (500 MHz, $CDCl_3$) δ [ppm]: 7.15 – 7.08 (m, 2H), 6.83 – 6.79 (m, 2H), 6.78 – 6.74 (m, 1H), 5.98 (dt, 1H), 3.78 (s, 3H), 2.95 – 2.85 (m, 1H), 2.75 (dd, 1H), 2.41 (dd, 1H), 2.35 – 2.24 (m, 2H), 1.12 (s, 3H), 1.03 (s, 3H)
2-[(4-Methoxyphenyl)methyl]-3,3-dimethyl-cyclohexanone (**23e**; yield: 73%, purity 95%).		^{1}H-NMR (500 MHz, $CDCl_3$) δ [ppm]: 7.16 – 7.11 (m, 2H), 6.83 – 6.77 (m, 2H), 3.78 (s, 3H), 3.05 (dd, 1H), 2.55 (dd, 1H), 2.51 – 2.45 (m, 1H), 2.39 – 2.31 (m, 1H), 2.29 – 2.19 (m,1H), 1.99 – 1.79 (m, 2H), 1.73 (td, 1H), 1.67 – 1.63 (m, 1H), 1.20 (s, 3H), 0.87 (s, 3H)
Methyl 4-[(6,6-dimethyl-2-oxo-cyclohex-3-en-1-yl)methyl]benzoate (**22f**; yield: 19%, purity 88%).		LC-MS (ESIpos) m/z =273.2 $(M+H)^+$
6-[(3-Fluorophenyl)methyl]-5,5-dimethyl-cyclohex-2-en-1-one (**22g**; yield: 23%, purity 72%).		LCMS (ESIpos) m/z = 233.0 $(M+H)^+$; ^{1}H-NMR (500 MHz, $CDCl_3$) δ [ppm]: 1.01 (s, 3H), 1.13 (s, 3H), 2.30 (dd, 1H), 2.31 (td, 1H), 2.46 (dd, 1H), 2.74 (dd, 1H), 3.00 (dd, 1H), 5.99 (dt, 1H), 6.78 (dt, 1H), 6.85 (td, 1H), 6.92 (dt, 1H), 6.98 (d, 1H), 7.20 (td, 1H)
2-[(3-Fluorophenyl)methyl]-3,3-dimethyl-cyclohexanone (**23g**; yield: 37%, purity 99%).		^{1}H-NMR (500 MHz, $CDCl_3$) δ [ppm]: 0.77 (s, 3H), 1.10 (s, 3H), 1.49 – 1.62 (m, 1H), 1.64 – 1.90 (m, 3H), 2.10 – 2.25 (m, 1H), 2.25 – 2.37 (m, 1H), 2.54 – 2.68 (m, 2H), 2.94 (td, 1H), 6.86 – 7.05 (m, 3H), 7.19 – 7.32 (m, 1H)
3-[(6,6-Dimethyl-2-oxo-cyclohex-3-en-1-yl)methyl]benzonitrile (**22h**; yield: 15%, purity 95%).		LCMS (ESIPos): m/z = 240 $(M+H)^+$; ^{1}H-NMR (500 MHz, $CDCl_3$) δ [ppm]: 7.55 – 7.44 (m, 3H), 7.37 (t, 1H), 6.82 (ddd, 1H), 6.01 (dt, 1H), 3.04 (dd, 1H), 2.79 (dd, 1H), 2.47 (dd, 1H), 2.42 – 2.26 (m, 2H), 1.18 (s, 3H), 1.03 (s, 3H)
3-[(2,2-Dimethyl-6-oxo-cyclohexyl)methyl]benzonitrile (**23h**; yield: 92%, purity 95%).		^{1}H-NMR (500 MHz, $CDCl_3$) δ [ppm]: 7.53 – 7.43 (m, 3H), 7.35 (t, 1H), 3.12 (dd, 1H), 2.60 (dd, 1H), 2.48 (d, 1H), 2.40 – 2.32 (m, 1H), 2.29 – 2.19 (m, 1H), 1.97 (dtd, 1H), 1.91 – 1.74 (m, 2H), 1.69 – 1.63 (m, 1H), 1.61 (s, 1H), 1.24 (s, 3H), 0.86 (s, 3H)
6-[(2,4-Difluorophenyl)methyl]-5,5-dimethyl-cyclohex-2-en-1-one (**22i**; yield: 39%, purity 82%).		LCMS (ESIpos): m/z = 251.0 $(M+H)^+$; ^{1}H-NMR (500 MHz, $CDCl_3$) δ [ppm]: 1.04 (s, 3H), 1.13 (s, 3H), 2.31 (dd, 2H), 2.44 (dd, 1H), 2.81 (dd, 1H), 2.88 (dd, 1H), 5.96 (dt, 1H), 6.70 – 6.80 (m, 3H), 7.26 (s, 1H)
2-[(2,4-Difluorophenyl)methyl]-3,3-dimethyl-cyclohexanone (**23i**; yield: 85%, purity 74%).		LCMS (ESIpos): m/z = 253.0 $(M+H)^+$; ^{1}H-NMR (500 MHz, $CDCl_3$) δ [ppm]: 0.85 (s, 3H), 1.20 (s, 3H), 1.61 (dtd, 1H), 1.73 (td, 1H), 1.82 (ddt, 1H), 1.92 (ddt,

		1H), 2.22 (tdd, 1H), 2.31 (dtd, 1H), 2.49 (d, 1H), 2.69 (d, 1H), 2.92 (ddd, 1H), 6.68 – 6.76 (m, 2H), 7.30 (td, 1H)

Name (yield; purity)	Structure	Analytics
2-[(5-Chloro-3-thienyl)methyl]cyclohexanone (**26b**; yield: 43%, purity 99%).		^{1}H-NMR (500 MHz, $CDCl_3$) δ [ppm]: 1.29 - 1.42 (m, 1 H), 1.61 - 1.73 (m, 2 H), 1.79 - 1.94 (m, 1 H), 2.01 - 2.19 (m, 2 H), 2.25 - 2.36 (m, 1 H), 2.39 - 2.47 (m, 1 H), 2.47 - 2.58 (m, 1 H), 2.59 - 2.69 (m, 1 H), 3.14 - 3.29 (m, 1 H), 6.46 - 6.58 (m, 1 H), 6.69 (d, 1 H)
4-[(2-Oxocyclohexyl)methyl]thiophene -2-carbonitrile (**26c;** yield: 54%, purity 93%).		^{1}H-NMR (500 MHz, $CDCl_3$) δ [ppm]: 1.32 - 1.45 (m, 1 H), 1.60 - 1.74 (m, 2 H), 1.84 - 1.94 (m, 1 H), 2.04 - 2.19 (m, 2 H), 2.27 - 2.39 (m, 1 H), 2.54 - 2.65 (m, 1 H), 2.72 - 2.83 (m, 1 H), 3.17 - 3.39 (m, 1 H), 6.75 - 6.86 (m, 1 H), 7.43 (d, 1 H)
2-[(6-Chloro-3-pyridyl)methyl]cyclohexanone (**26e**; yield: 2%; purity 50%).		LCMS (ESIpos): m/z = 224.2 $(M+H)^+$
2-[(5-Chloro-3-pyridyl)methyl]cyclohexanone (**26f**; yield: 30%, purity 93%).		LCMS (ESIpos): m/z = 223.9, 225.9 $(M+H)^+$; ^{1}H-NMR (500 MHz, $CDCl_3$) δ [ppm]: 1.38 (qd, 1H), 1.57 – 1.73 (m, 2H), 1.83 – 1.93 (m, 1H), 2.01 – 2.14 (m, 2H), 2.28 – 2.37 (m, 1H), 2.40 – 2.49 (m, 2H), 2.51 – 2.60 (m, 1H), 3.17 (dd, 1H), 7.52 (t, 1H), 8.31 (d, 1H), 8.40 (d, 1H)
2-[(5-Chloro-2-pyridyl)methyl]cyclohexanone (**26h**; Yield: 14%, purity 60%).		1H NMR (250 MHz, $CDCl_3$) δ = 1.60 – 1.74 (m, 4H), 1.99 – 2.26 (m, 2H), 2.27 – 2.45 (m, 2H), 2.56 (dd, 1H), 2.89 – 3.05 (m, 1H), 3.28 (dd, J=14.2, 6.5, 1H), 7.17 (d, 1H), 7.54 (dd, 1H), 8.44 (d, 1H)
(**26i**; yield: 59%, purity 69%).		^{1}H-NMR (500 MHz, $CDCl_3$) δ [ppm]: 1.34 - 1.45 (m, 2H), 1.67 (s, 9H), 1.70 - 1.91 (m, 2H), 2.01 - 2.19 (m, 2H), 2.28 - 2.40 (m, 1H), 2.42 - 2.58 (m, 2H), 2.61 - 2.72 (m, 1H), 3.28 (ddd, 1H), 7.13 - 7.25 (m, 1H), 7.27 - 7.39 (m, 2H), 7.48 (d, 1H), 8.02 - 8.24 (m, 1H)

2-[(5-Chlorothiazol-2-yl)methyl]cyclohexanone (**26d**).

Step A: To vigorously stirred cyclohexanone (2.81 ml, 27.1 mmol) was added aq. NaOH (3.39 ml, 6.78 mmol, 2M) followed by 5-chloro-1,3-thiazole-2-carbaldehyde (1.00g, 6.78 mmol). After 1.5h, the reaction mixture was diluted with water (20 ml) and extracted with EtOAc (2 x 30 ml). The combined

organic layers were dried via a hydrophobic filter and concentrated to dryness under reduced pressure. The crude was purified by chromatography on silica gel (gradient hexane / EtOAc) to give 2-[(5-chlorothiazol-2-yl)-hydroxy-methyl]cyclohexanone (1.11g, 64% yield, 96% purity). LCMS (ESIpos): m/z = 245.9, 247.9 $[M+H]^+$.

Step B: To a stirred solution of 2-[(5-chlorothiazol-2-yl)-hydroxy-methyl]cyclohexanone (1.0 g, 4.07 mmol) in 1,4-dioxane (20 ml) was added burgess reagent (1.07 g, 4.48 mmol) and the reaction mixture was heated to reflux. After 1h, the mixture was concentrated under reduced pressure, diluted with aq. sodium carbonate solution (20 ml, 1M) and extracted with EtOAc (2 x 30 ml). The combined organic layers were dried over an hydrophobic filter and concentrated under reduced pressure. The crude was purified by chromatography on silica gel (gradient hexane / EtOAc) to give 2-[(5-chlorothiazol-2-yl)methylene]cyclohexanone (348 mg, 37% yield, 98% purity). LCMS (ESIpos): m/z = 227.9, 229.9 $[M+H]^+$.

Step C: Aforementioned 2-[(5-chlorothiazol-2-yl)methylene]cyclohexanone (300 mg, 1.32 mmol) in EtOAc (4 ml) and EtOH (4 ml) was added Pd/C (50 mg, 10%) and the flask was purged with hydrogen. The reaction was stirred for 24h, then filtered over celite and concentrated to dryness under reduced pressure to yield 2-[(5-chlorothiazol-2-yl)methyl]cyclohexanone (316 mg, 99% yield, 99% purity). LCMS (ESIpos): m/z = 229.9, 231.9 $[M+H]^+$.

2-(4-pyridylmethyl)cyclohexanone (**26g**)

The synthesis of intermediate **26g** was carried out following literature procedure. (J. Galambos *et al.* Bioorg. Med. Chem. Lett. 20 (2010), 4371 – 4375.)