

Supporting Information

Synthesis, Structure Activity Relationship, Radiofluorination and Biological Evaluation of [¹⁸F]RM365, a Novel Radioligand for Imaging the Human Cannabinoid Receptor Type 2 (CB2R) in the Brain with PET

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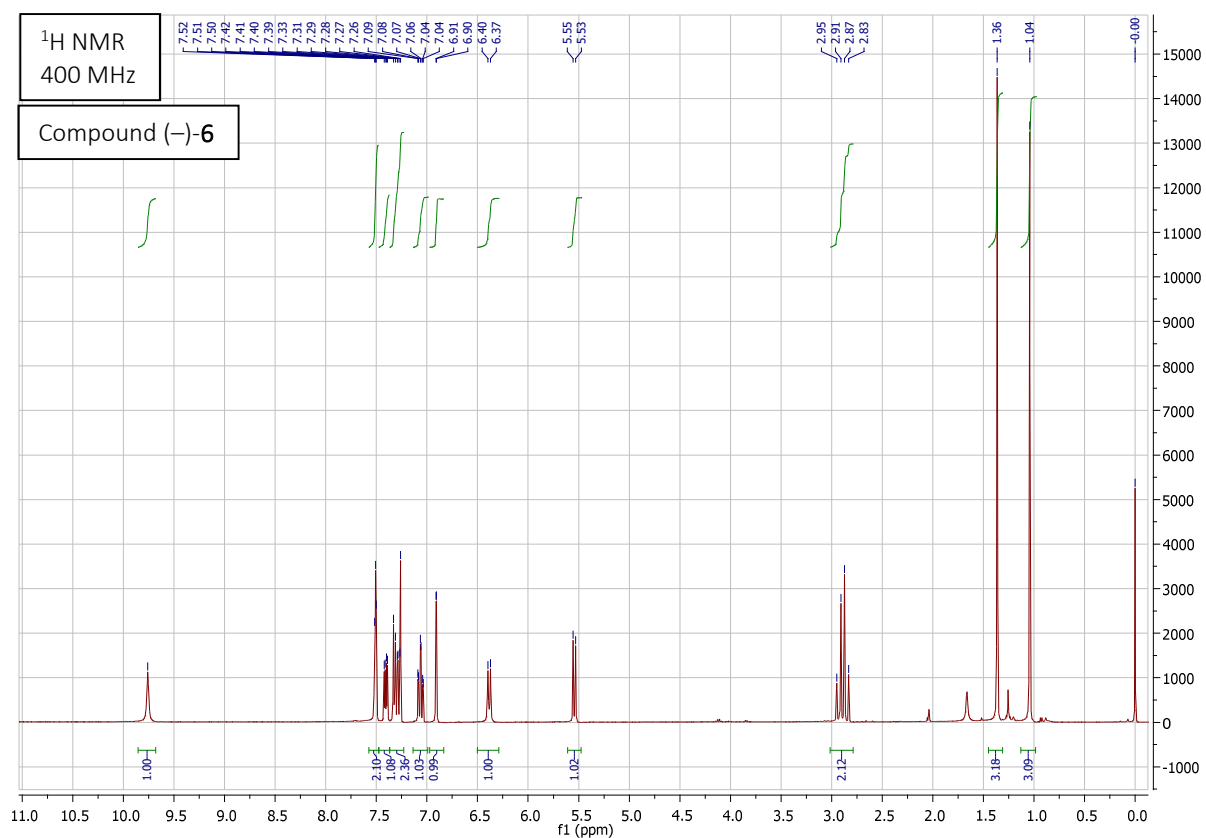
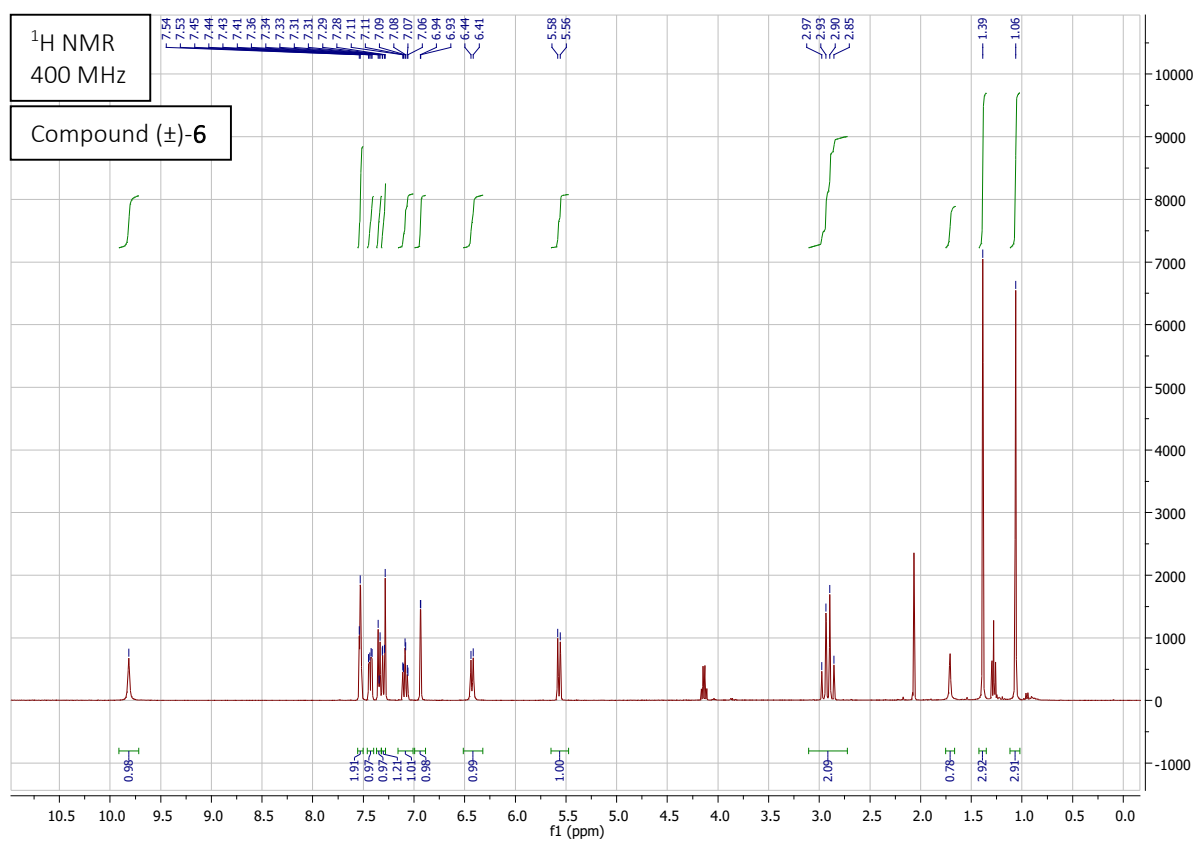
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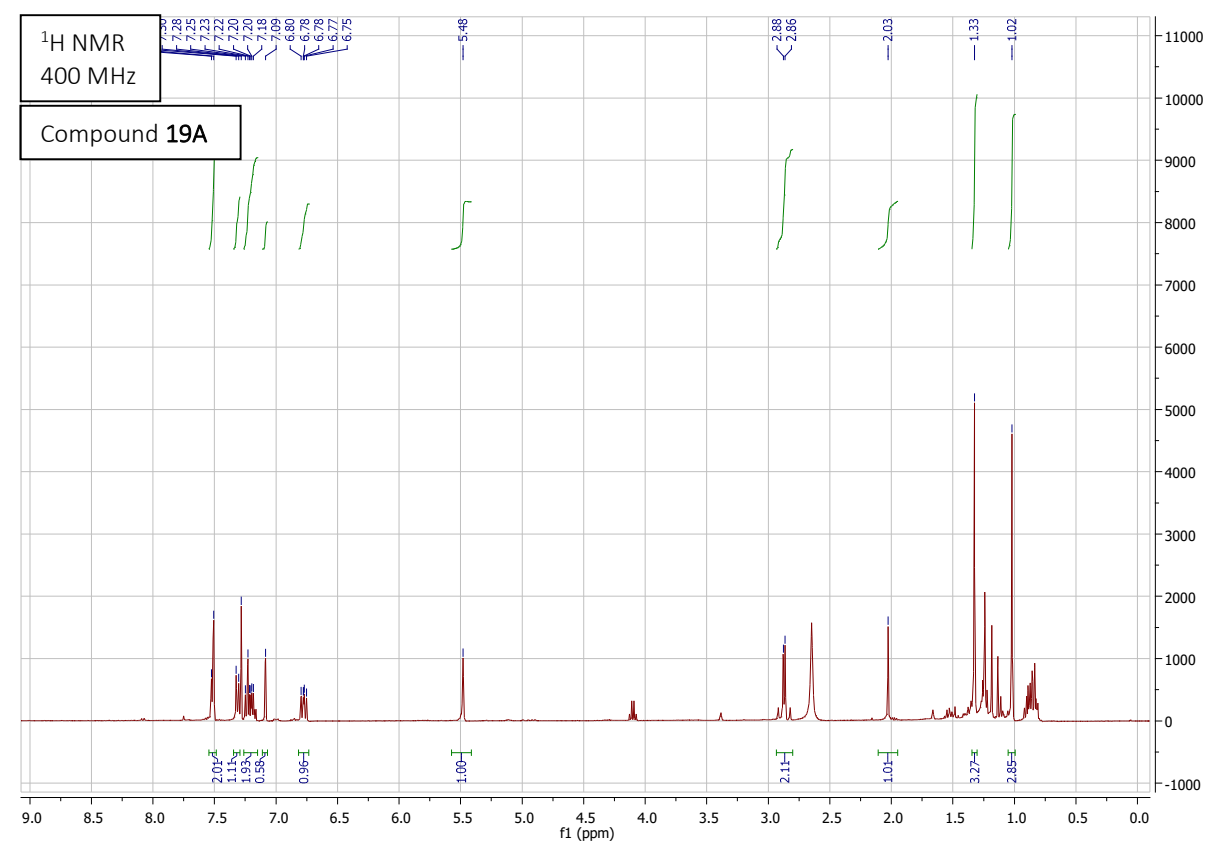
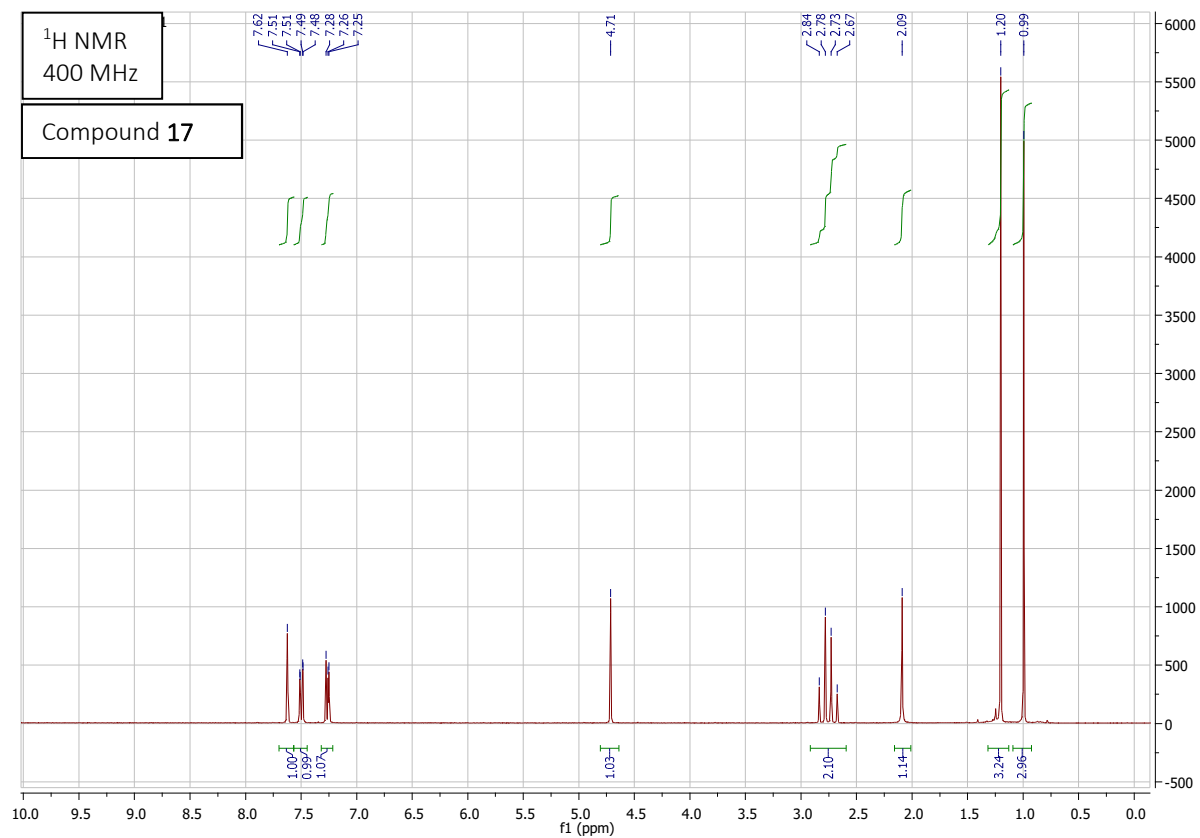
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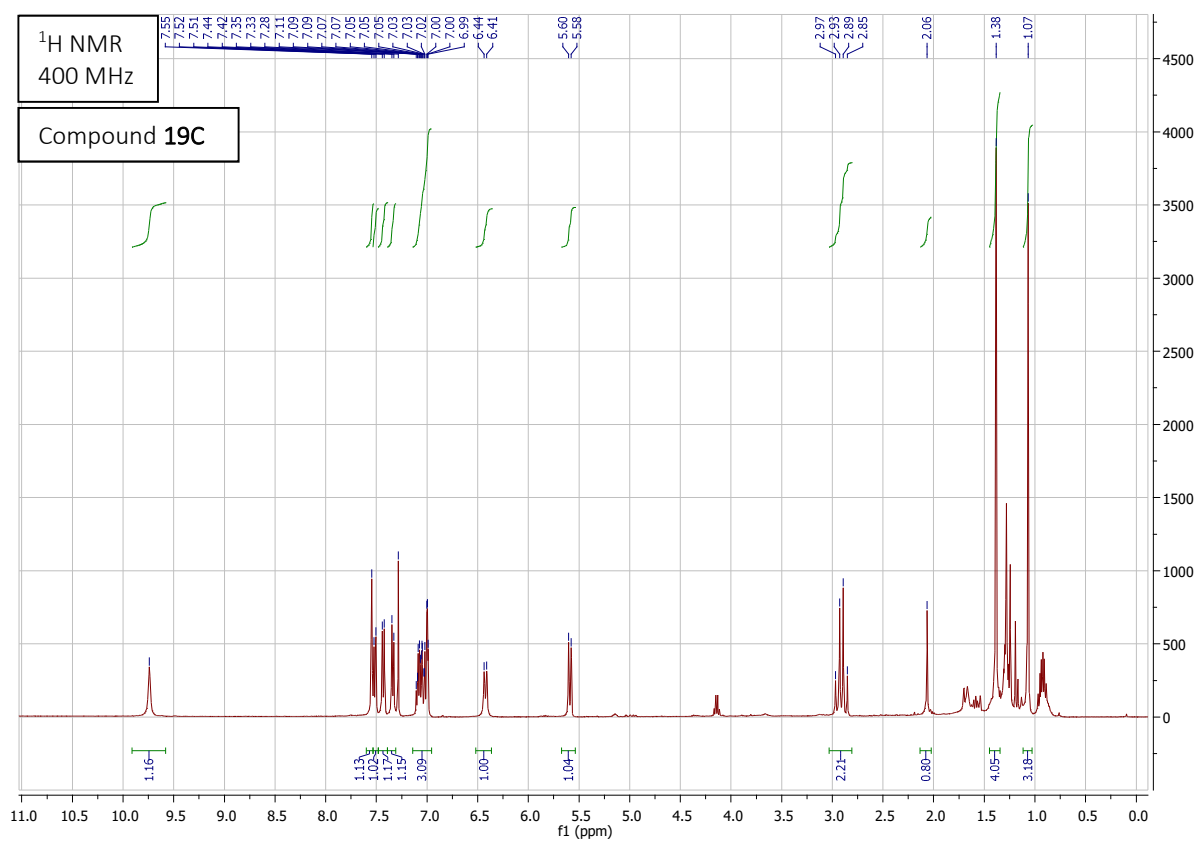
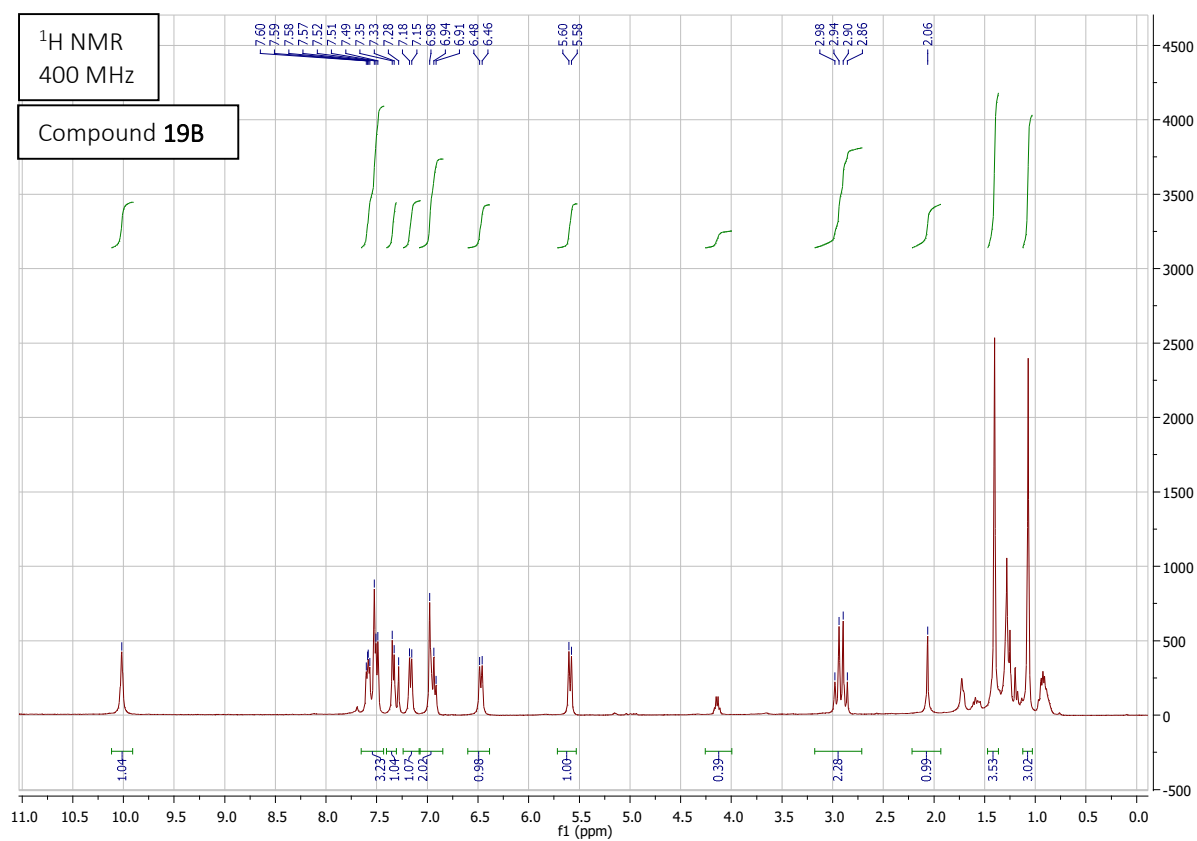
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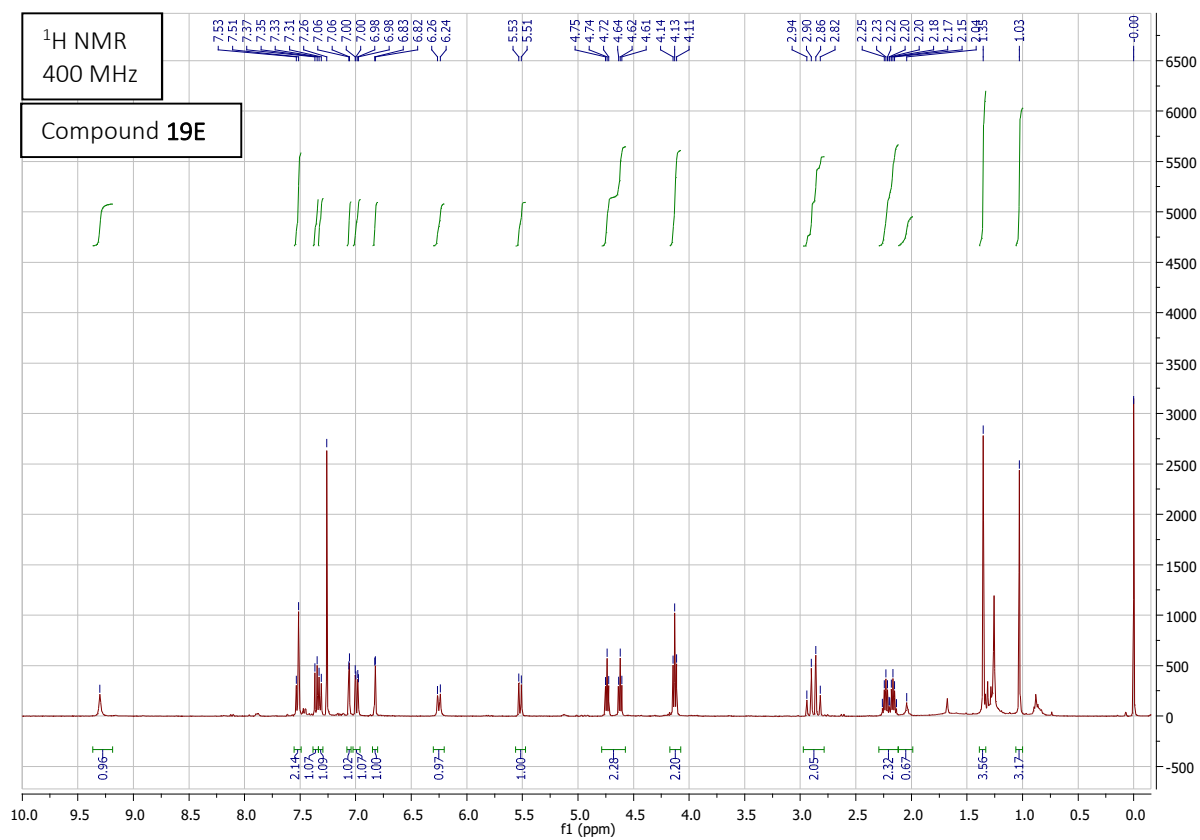
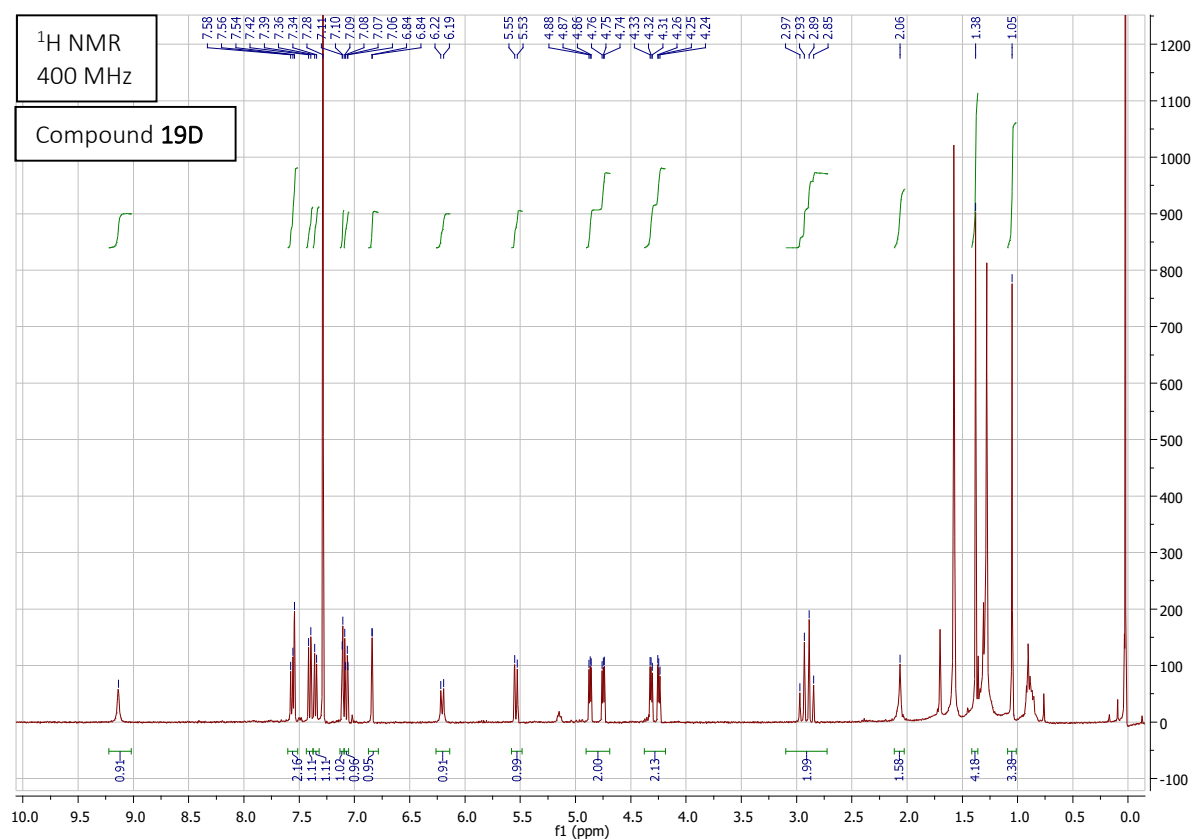
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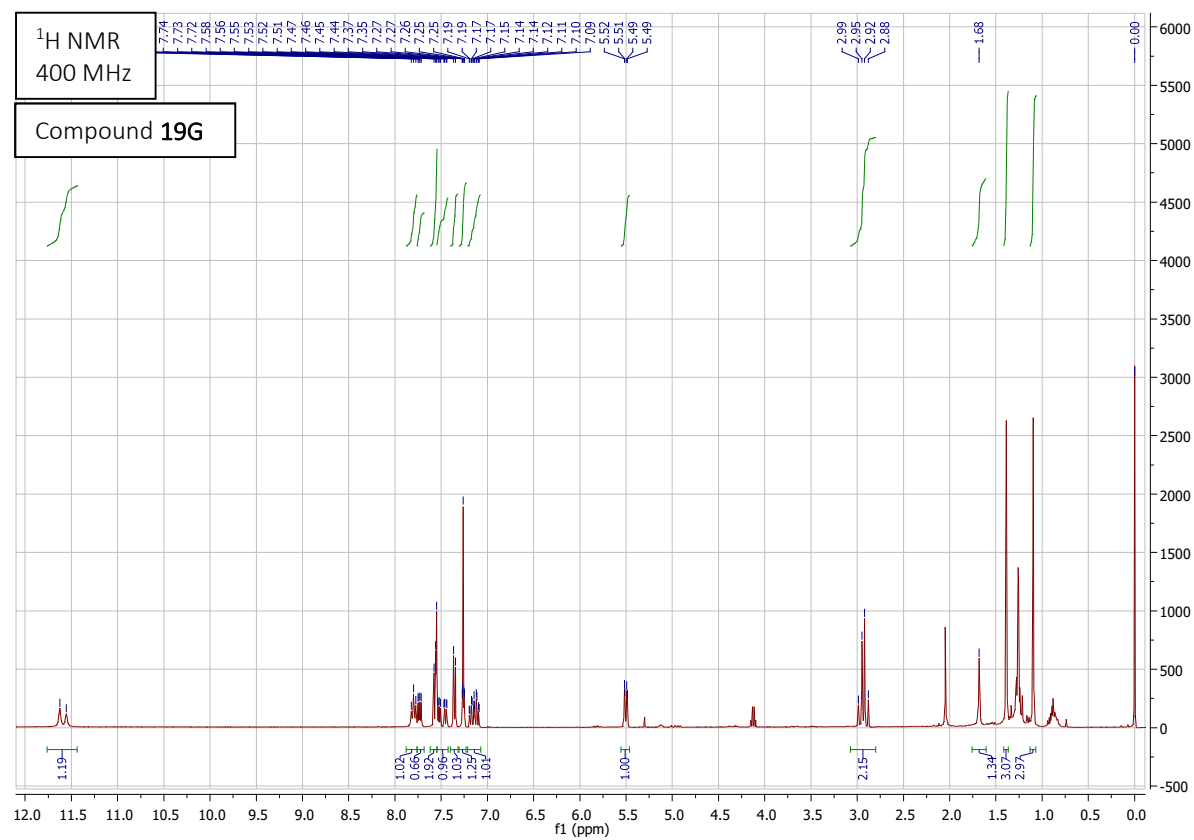
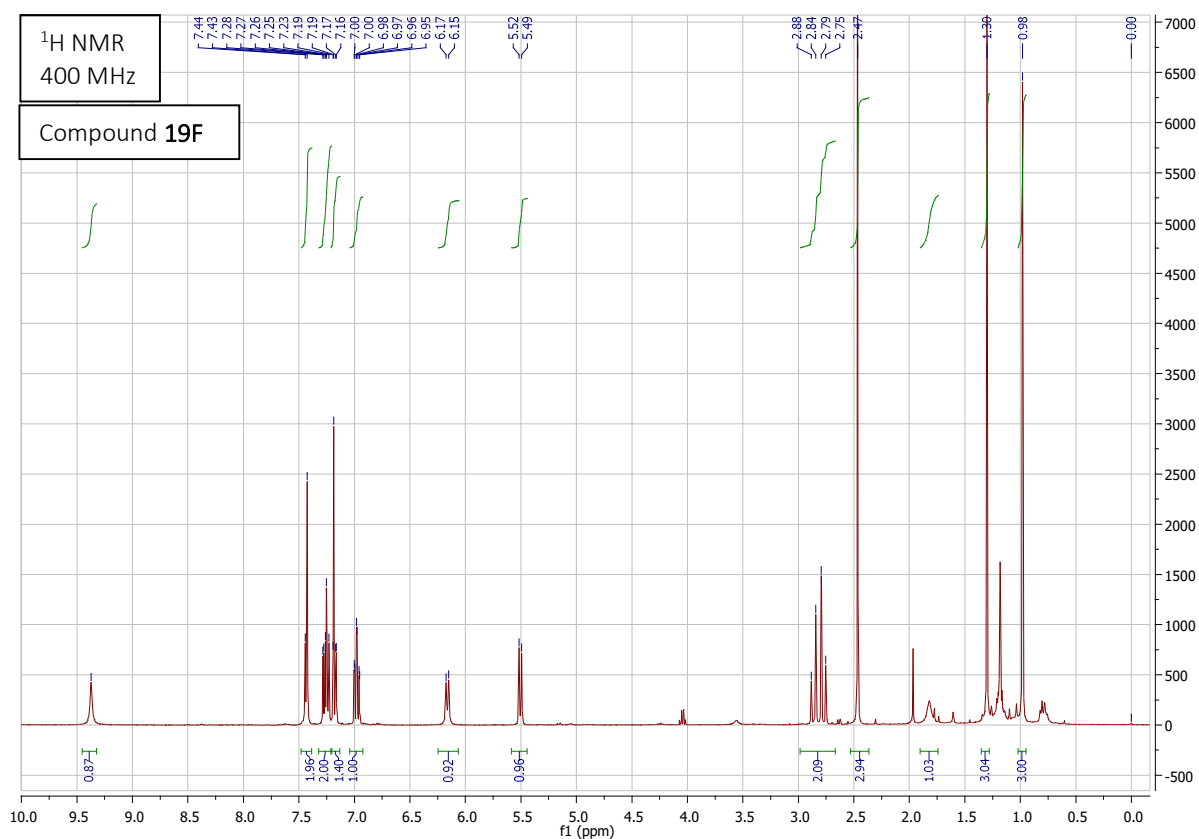
- NMR characterization of compounds **6**, **19A-H** and **21A-E**, **23** and **24**
- HPLC analysis of compounds **6**, **19A-H** and **21A-E**
- Analytical UV and OR chromatograms of (+)-**6** and (–)-**6** (Figure S1)
- In vitro autoradiography of [¹⁸F]RM365 (Figure S2)
- Computational Chemistry (Figure S3-S6)
- PET Imaging (Figure S7-S11)

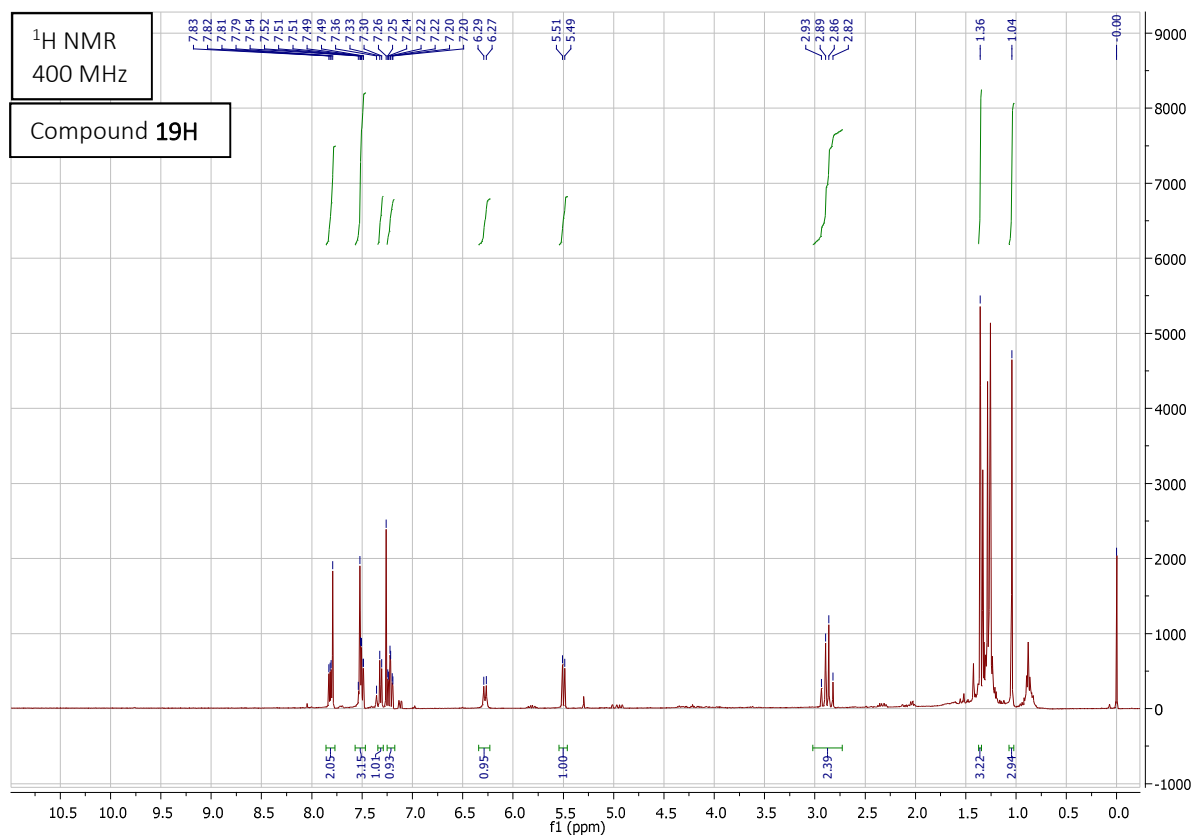


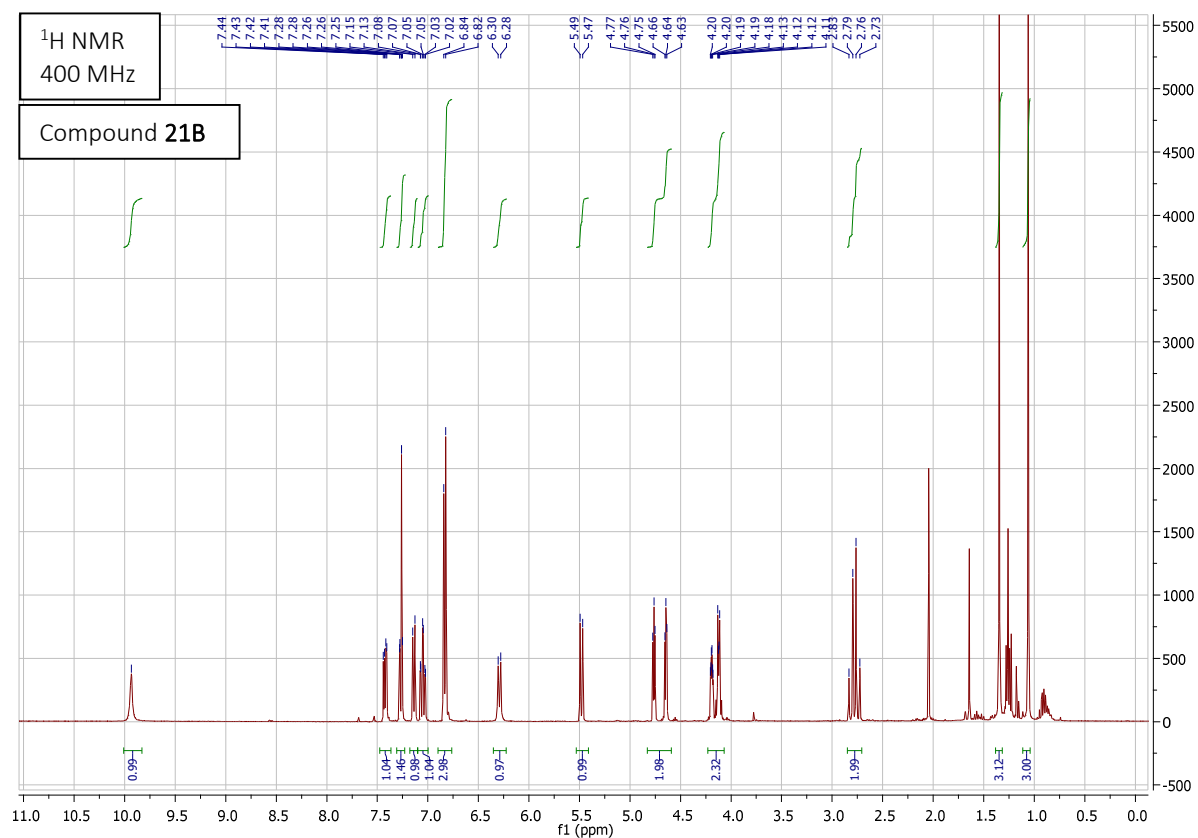
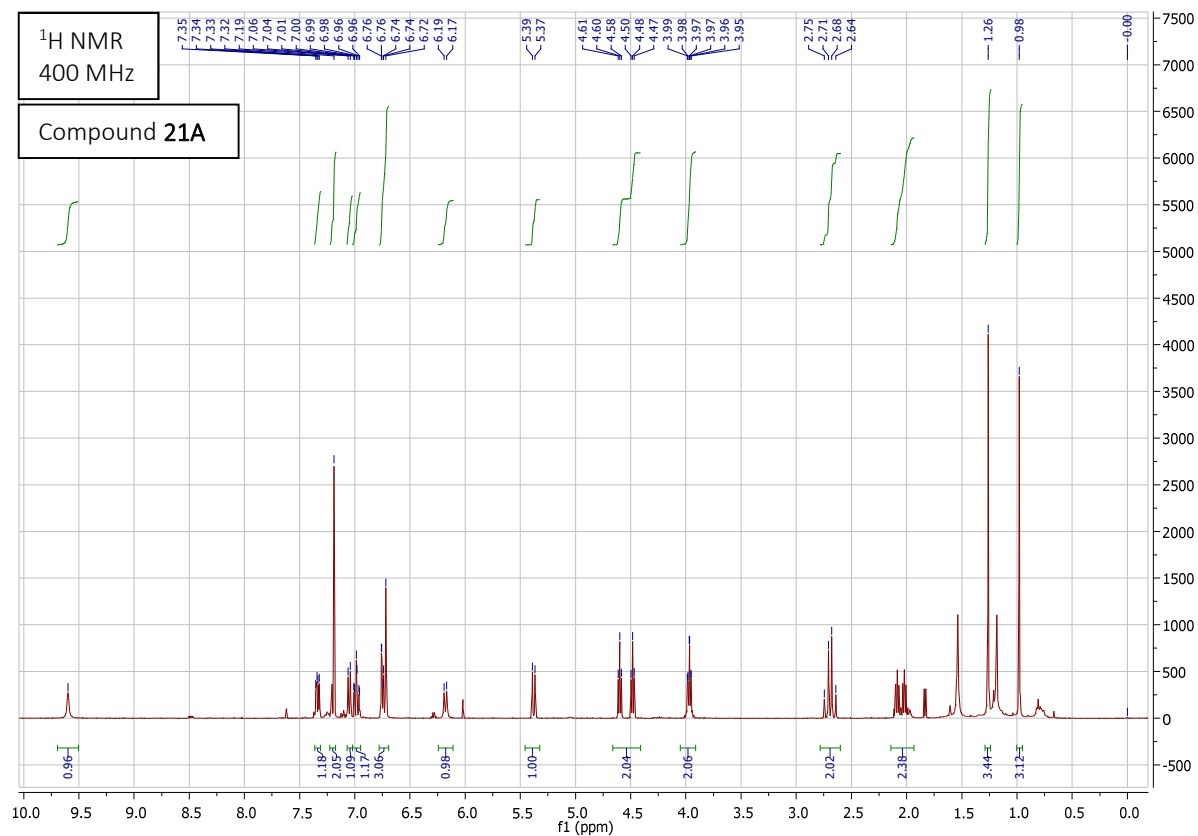


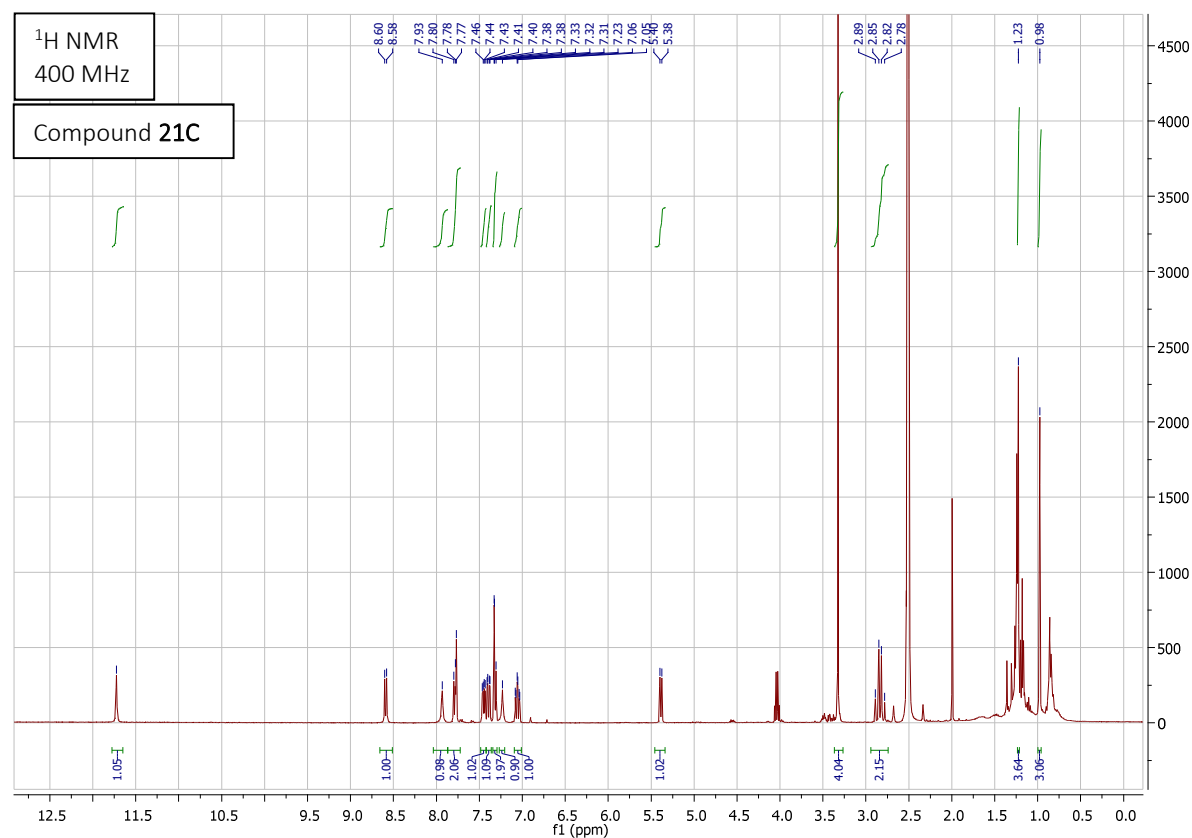
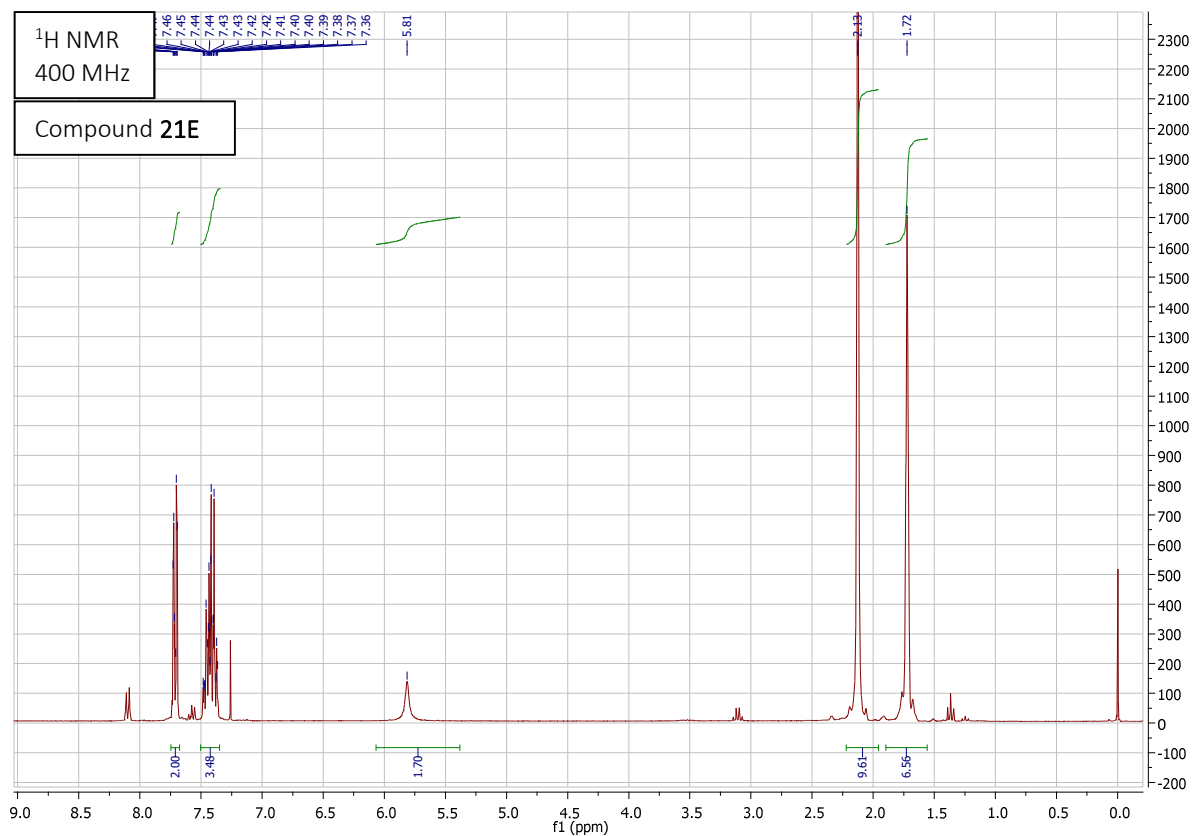


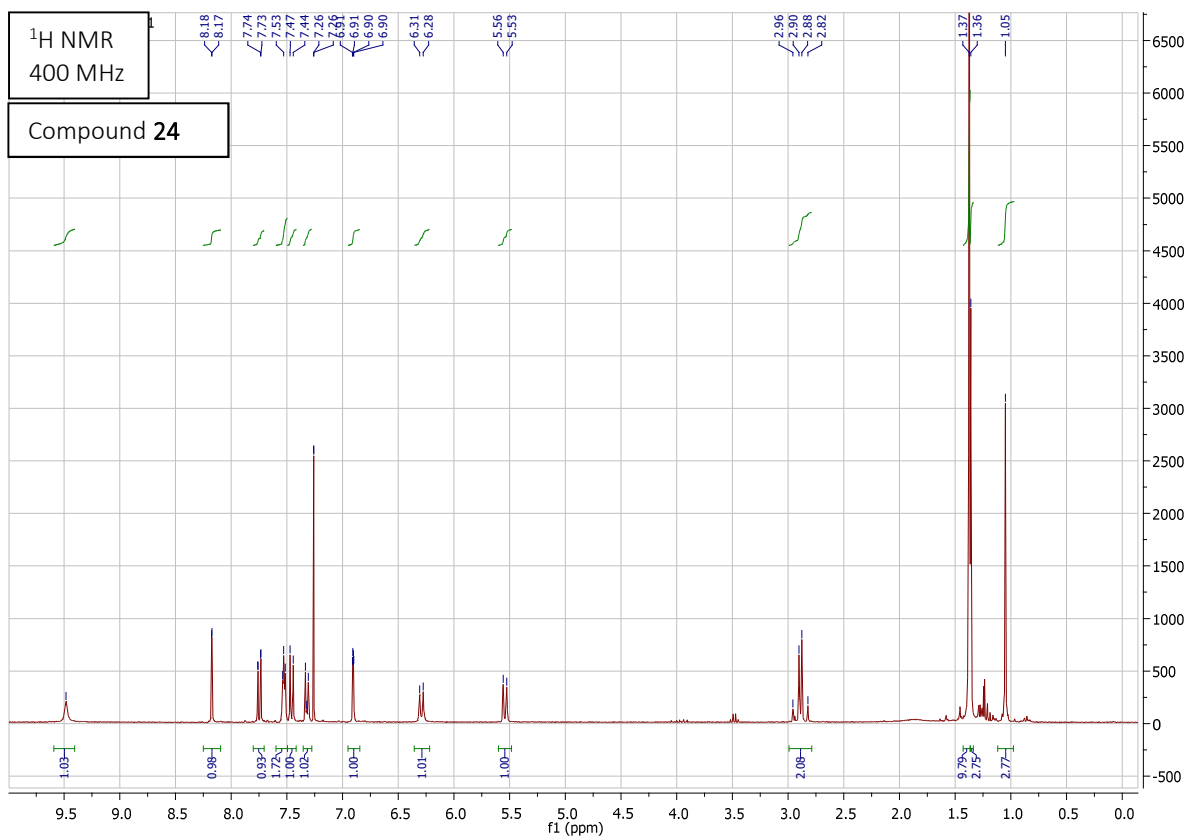
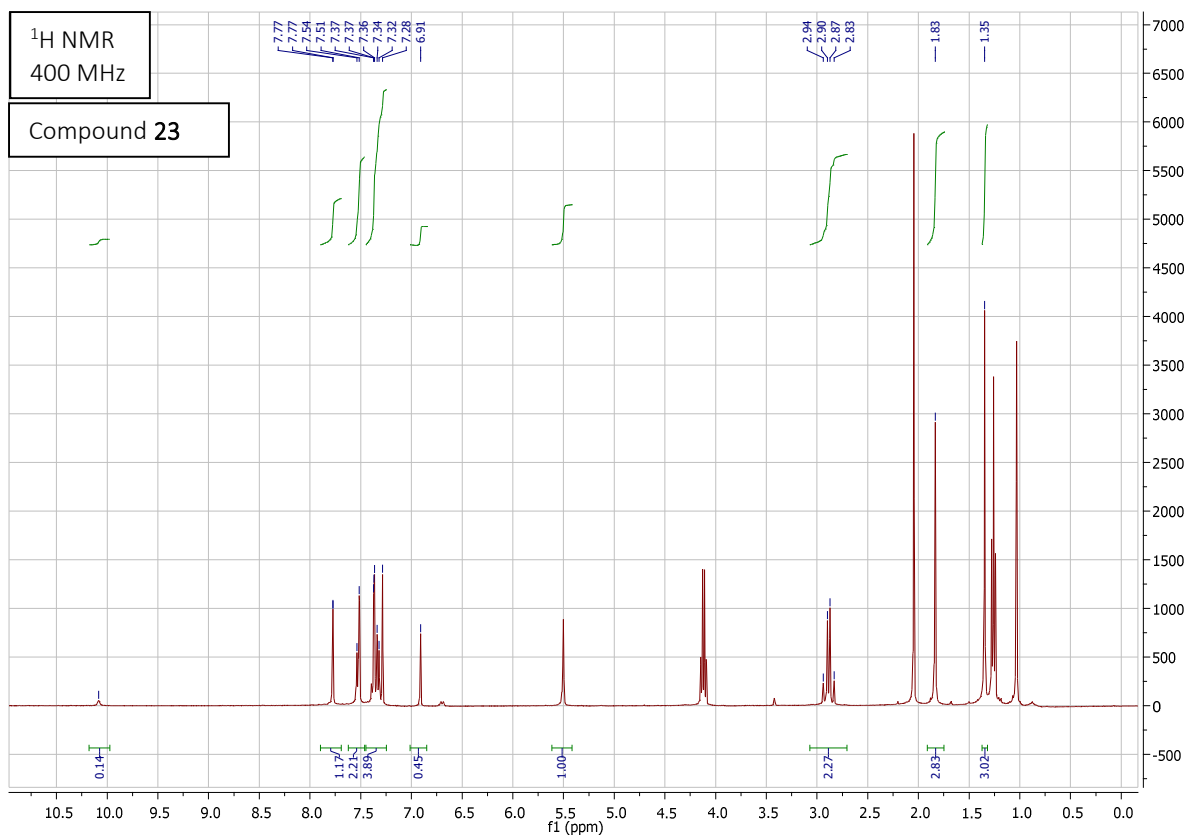


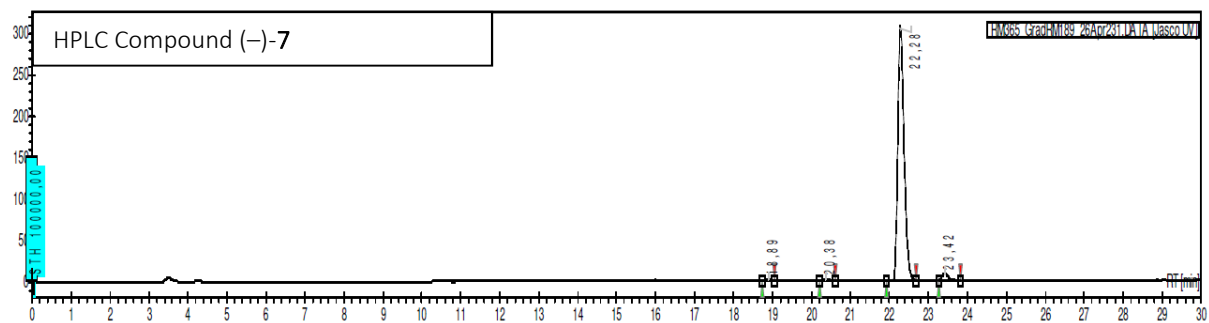




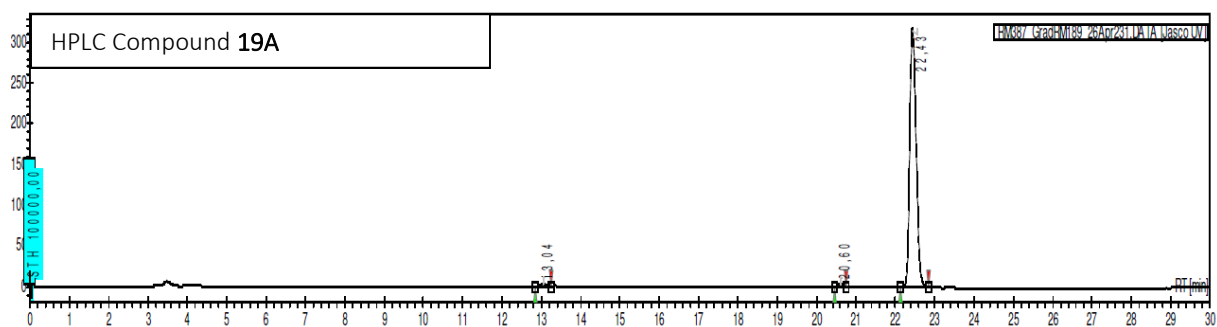




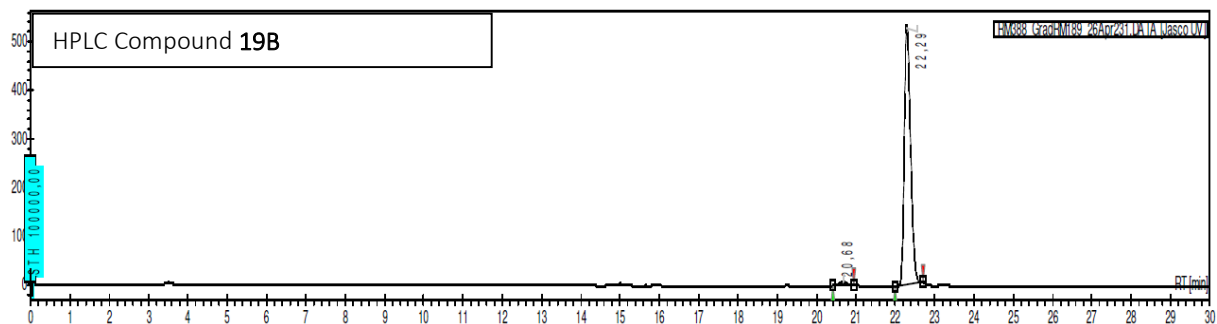




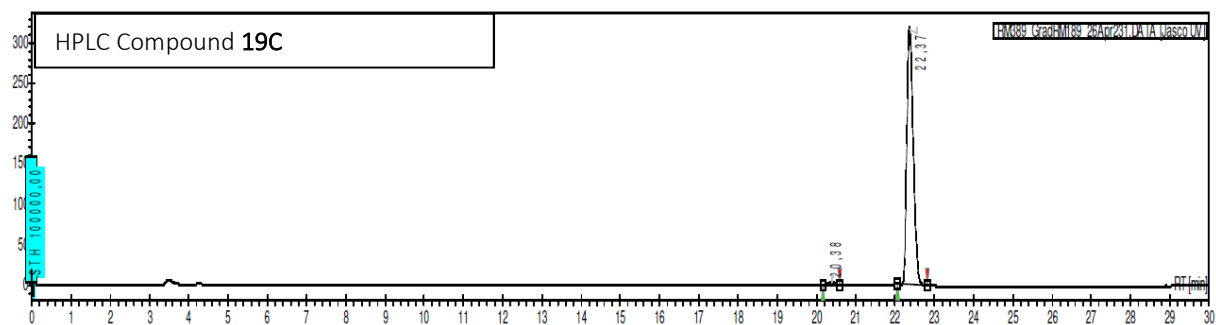
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2	UNKNOWN	20.38	0.58	1.9	0.4	0.578
4	UNKNOWN	22.28	96.91	308.8	59.2	96.905
1	UNKNOWN	23.42	2.39	7.7	1.5	2.388
Total			100.00	318.8	61.1	100.000



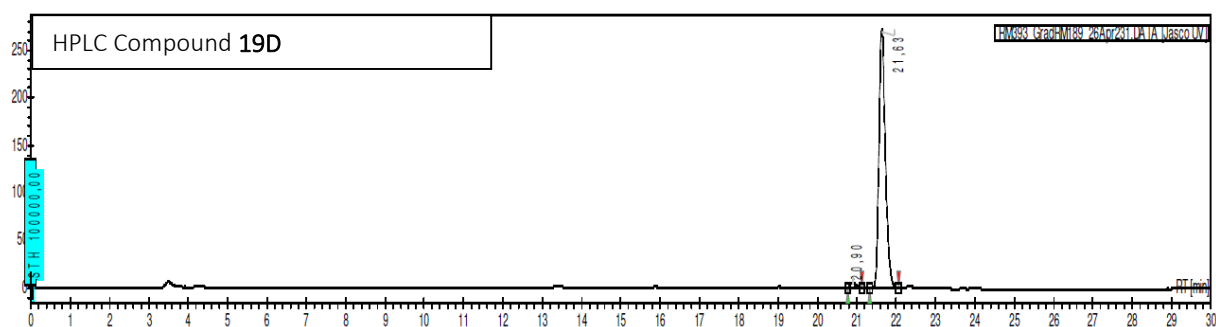
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2	UNKNOWN	20.60	1.00	3.9	0.6	1.000
1	UNKNOWN	22.43	98.10	318.9	58.4	98.100
Total			100.00	326.6	59.5	100.000



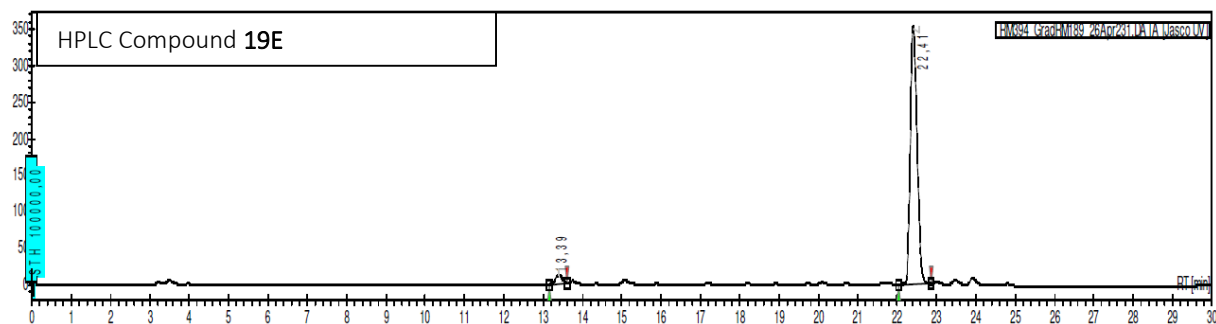
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2	UNKNOWN	20.68	1.45	7.2	1.5	1.452
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Total			100.00	539.3	101.7	100.000



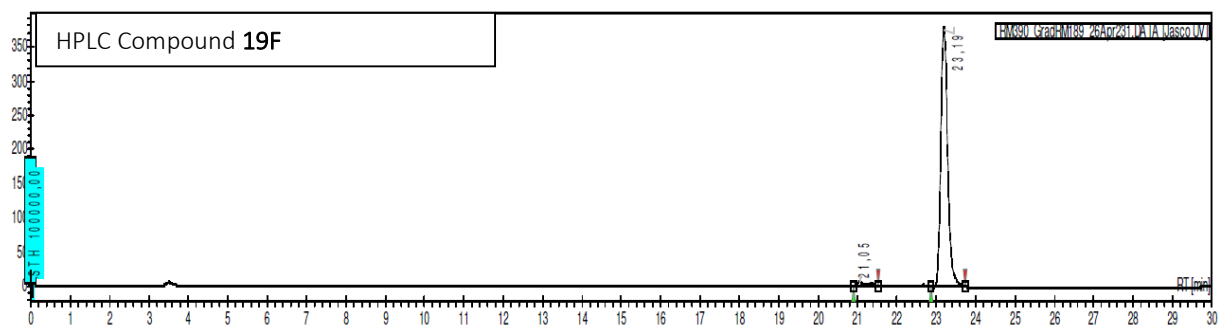
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2	UNKNOWN	20.38	1.18	3.7	0.7	1.175
1	UNKNOWN	22.37	98.82	320.3	59.0	98.825
Total			100.00	324.0	59.7	100.000



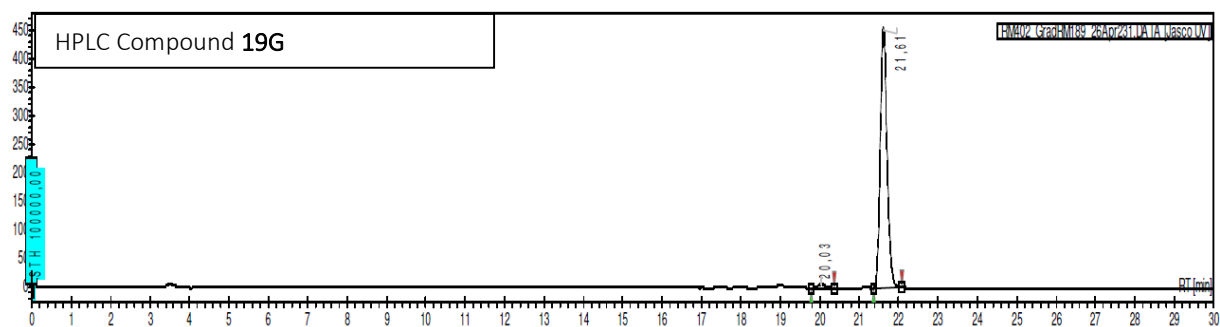
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2	UNKNOWN	20.90	1.70	5.3	0.9	1.696
1	UNKNOWN	21.63	98.30	273.8	52.6	98.304
Total			100.00	279.2	53.6	100.000



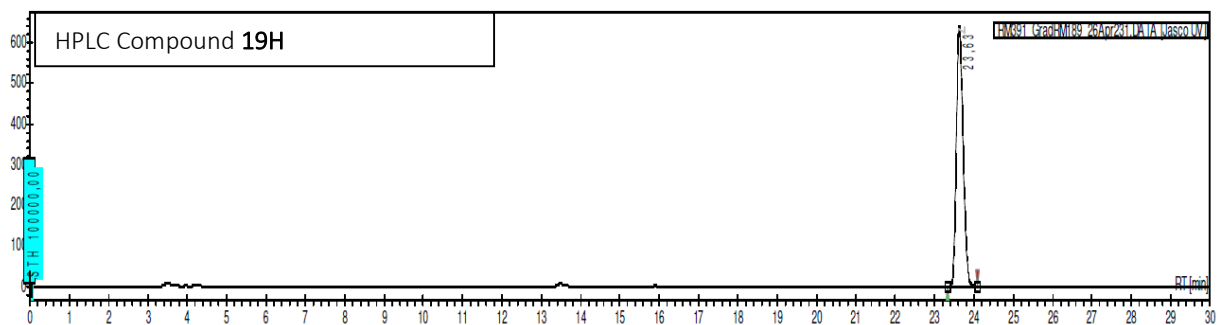
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2	UNKNOWN	13.39	3.56	13.1	2.5	3.556
1	UNKNOWN	22.41	96.44	353.8	68.6	96.444
Total			100.00	366.9	71.1	100.000



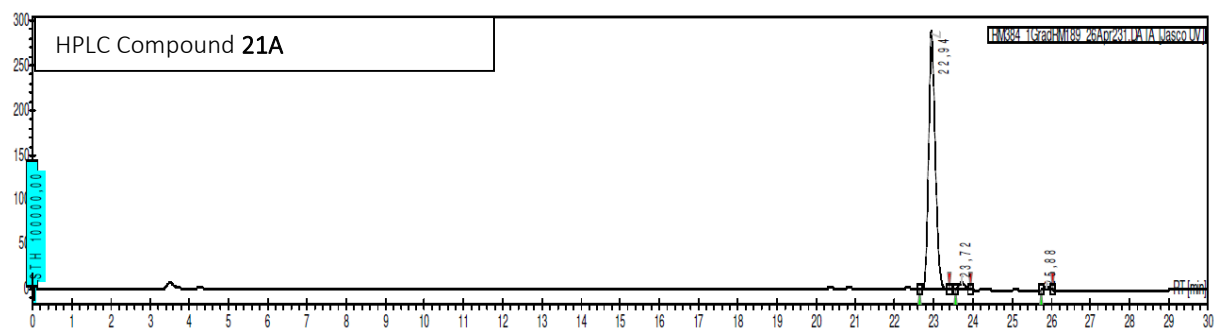
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2	UNKNOWN	21.05	2.60	7.1	2.1	2.599
1	UNKNOWN	23.19	97.40	380.8	77.0	97.401
Total			100.00	387.9	79.0	100.000



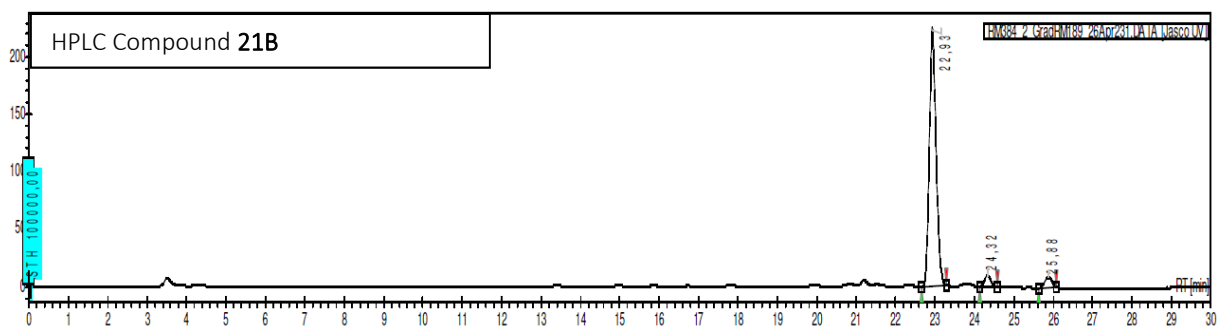
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2	UNKNOWN	20.03	1.44	6.7	1.3	1.440
1	UNKNOWN	21.61	98.56	457.3	91.6	98.560
Total			100.00	464.0	92.9	100.000



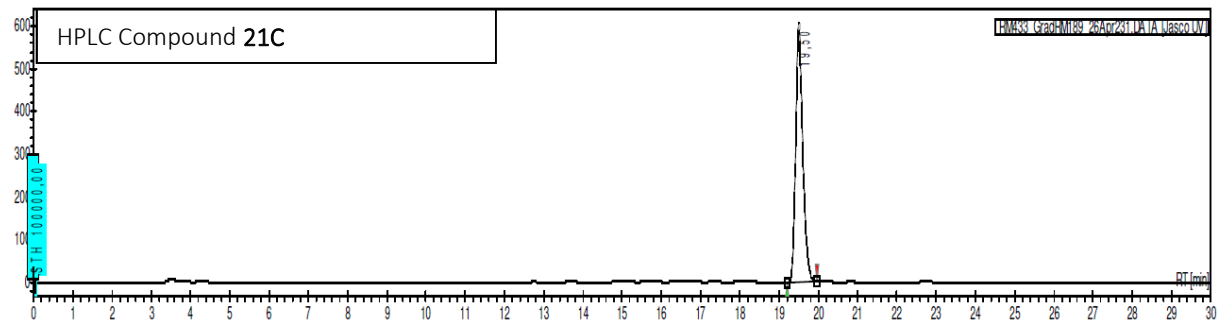
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1	UNKNOWN	23.63	100.00	644.2	130.5	100.000
Total			100.00	644.2	130.5	100.000



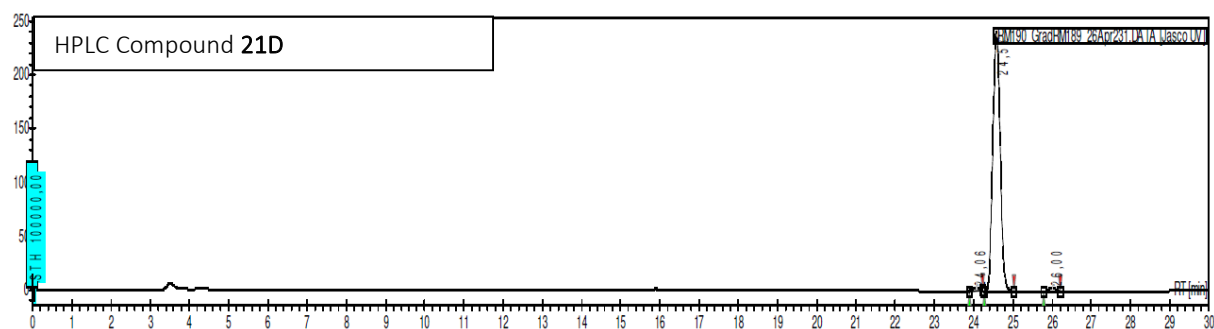
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1	UNKNOWN	22.94	96.96	290.4	57.9	96.964
2	UNKNOWN	23.72	2.20	7.2	1.3	2.198
3	UNKNOWN	25.88	0.84	3.0	0.5	0.837
Total			100.00	300.6	59.7	100.000



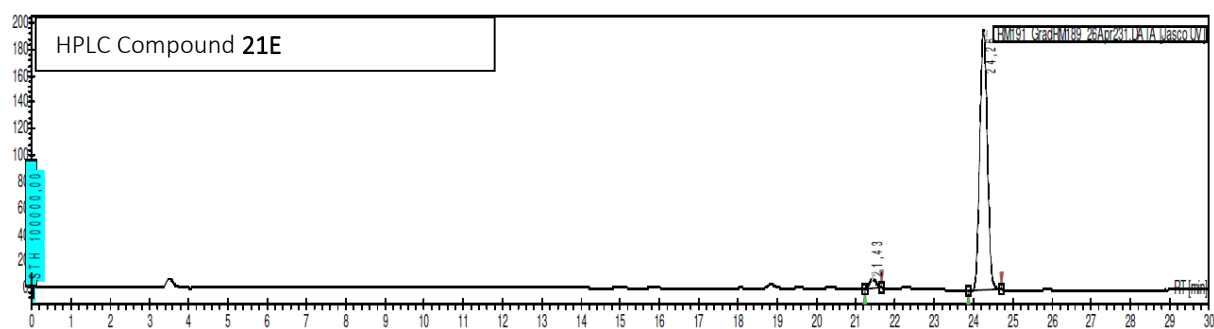
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1	UNKNOWN	22.93	92.51	226.6	45.5	92.513
2	UNKNOWN	24.32	3.82	10.1	1.9	3.821
3	UNKNOWN	25.88	3.67	8.6	1.8	3.666
Total			100.00	245.3	49.2	100.000



Index	Name	Time [Min]	Quantity [% Area]	Height [mV]	Area [mV.Min]	Area % [%]
1	UNKNOWN	19.50	100.00	609.2	129.3	100.000
Total			100.00	609.2	129.3	100.000



Index	Name	Time [Min]	Quantity [% Area]	Height [mV]	Area [mV.Min]	Area % [%]
2	UNKNOWN	24.06	0.96	3.0	0.5	0.958
1	UNKNOWN	24.58	97.63	243.1	53.0	97.634
3	UNKNOWN	26.00	1.41	3.6	0.8	1.408
Total			100.00	249.7	54.2	100.000



Index	Name	Time [Min]	Quantity [% Area]	Height [mV]	Area [mV.Min]	Area % [%]
2	UNKNOWN	21.43	3.20	7.0	1.4	3.205
1	UNKNOWN	24.26	96.80	196.6	43.5	96.795
Total			100.00	203.6	45.0	100.000

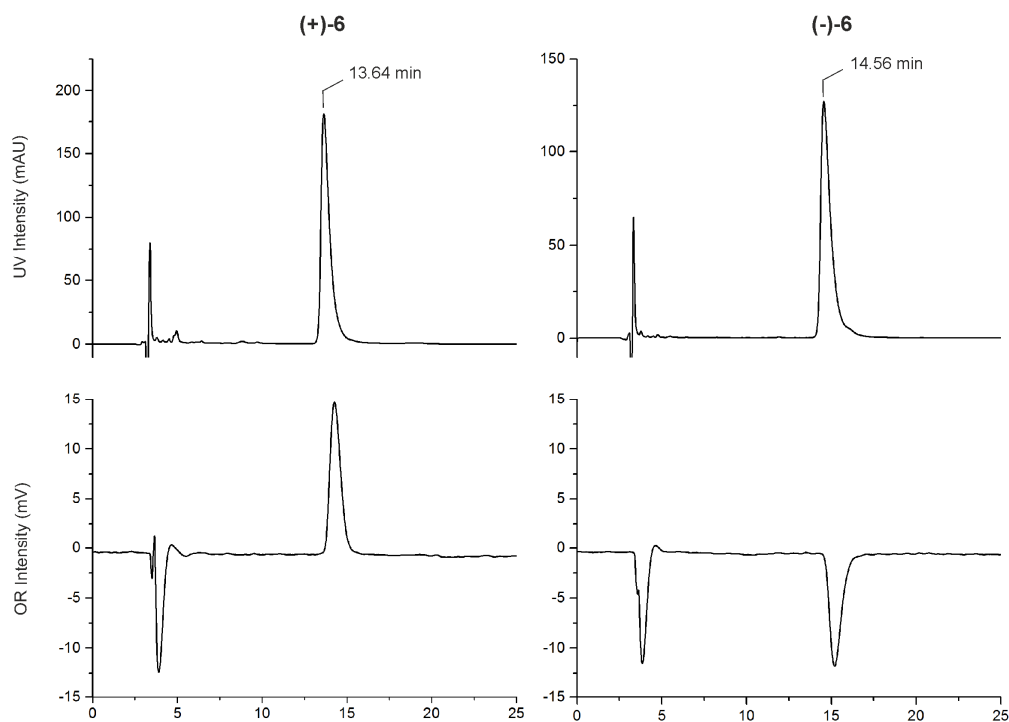
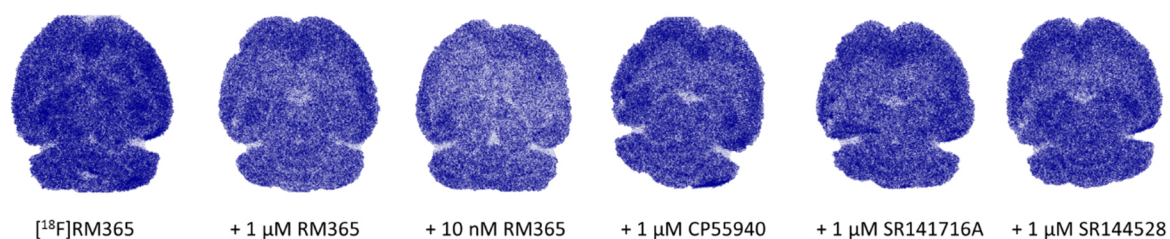


Figure S1. Analytical UV and OR chromatograms of (+)-**6** and (–)-**6**. Conditions: CHIRALPAK IA (250 × 4.6 mm), 58% ACN/ aq. 20mM NH₄OAc, flow 1.0

In vitro autoradiography

A



B

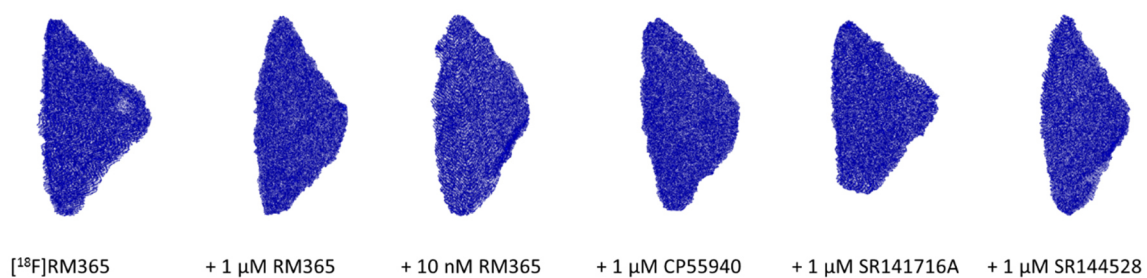


Figure S2. A) Mouse brain in vitro autoradiography of [¹⁸F]RM365. Cryosections of a brain of a female CD-1 mouse were incubated with [¹⁸F]RM365 alone (total binding) or

in the presence of RM365 at 1 μ M or 10 nM (self blocking), or of 1 μ M CP55940 (CB1R/CB2R non-selective agonist), SR141716A (CB1R-selective antagonist), or SR144528 (CB2R-selective antagonist). B) Rat spleen in vitro autoradiography of [18 F]RM365. Cryosections of spleens of two female SPRD rats were incubated with [18 F]RM365 alone (total binding) or in the presence of RM365 at 1 μ M or 10 nM (self blocking), or of 1 μ M CP55940 (CB1R/CB2R non-selective agonist), SR141716A (CB1R-selective antagonist), or SR144528 (CB2R-selective antagonist).

Computational Chemistry.

1. Geometry optimisation.

The geometry optimisation of R-(–)-**6** and S-(+)-**6** was done using density functional theory (DFT)¹ and performed with the ORCA 5.0 package². The functional B3LYP³ has been used. In order to improve the stability of the optimised geometry Becke-Johnson dispersion corrections of third order (D3BJ)⁴ were included in the functional. We applied the density fitting technique resolution-of-identity approximation (RI-J)⁵ and chain-of-sphere approximation (COSX)⁵ in the geometry optimisation to speed up the calculations. The optimised geometry was verified based on the frequency calculation, where no imaginary frequencies were observed.

Protein structure preparation

1. Human Cannabinoid Receptor type 2 (hCB2R).

The crystal structure of the hCB2R is available from the protein data bank (PDB) under the code 3ZTY⁶ and contains the synthetic ligand AM2051 in the binding pocket of the receptor. The ligand was eliminated from the binding pocket of the receptor and the initial structure of hCB2R was protonated using Reduce program⁷.

2. Rattus Cannabinoid Receptor type 2 (rCB2R).

2.1 Folding.

Since the crystallographic structure of rat CB2R is not available we carried out *de novo* modelling. One of the main goals of this modelling is the folding of the primary sequence (mentioned below).

```
MEECWVTEIANGSKDGLDSNPMKDYMLSGPQKTAVAVLCTLLGLLSALENVAVLYLILS  
SHQLRRKPSYLFIGSLAGADFLASVVFACSFVNFHVFHGVDSKAVFLLKIGSVTMTFTAS  
VGSLLLTAIDRYLCLRYPPSYKALLTRGRALVTLGIMWVLSALVSYLPLMGWTCCPRPCS  
ELFPLIPNDYLLSWLLFIAFLFSGIITYGHVWLWKAHQHVASLSGHQDRQVPGMARMRLD  
VRLAKTLGLVLAVLLICWFPVLALMAHSLATTLSDQVKKAFAFCSMLCLINSMVNPVIYA  
LRSGEIRSSAHHCCLAHWKCVRLGSEAKEEAPRSSVTETeadgKITPWPDSRDLDLSDC
```

For this purpose we applied I-TASSER⁸⁻⁹ software. I-TASSER identifies PDB templates with the similar primary sequences and constructs several models. Five models have been generated based on several PDB templates: 2HPY¹⁰, 2KS9¹¹, 2ZIY¹², 5TGZ¹³, 5UEN¹⁴, 5ZTY¹⁵, 7FEE¹⁶ and 7WC4¹⁷ (Figure S2). These five models differ from each other in their loop conformation and helix orientation. However for the further modelling we considered only the best model **1**. This model have been selected

based on the several scores: C-score, number of decoys and cluster density (Table S1).

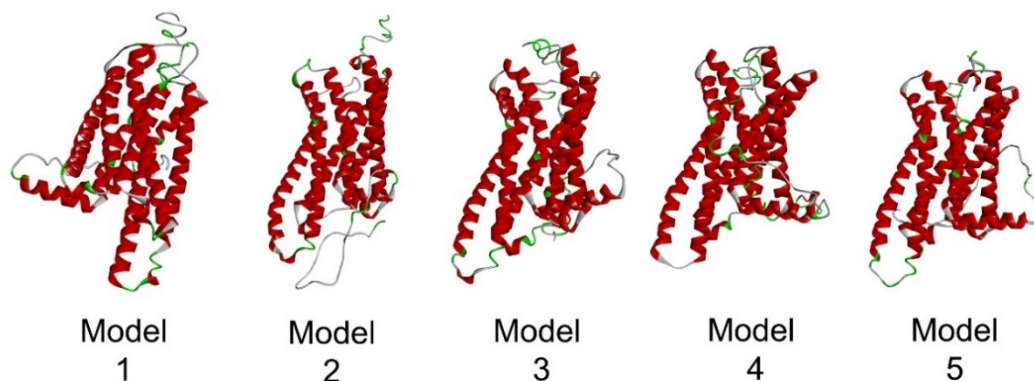


Figure S3. Five models of rat CB2R generated based on template modelling.

Table S1. Parameters estimating the quality of the generated models created via folding of the primary sequence.

Number of model	1	2	3	4	5
C-score	-1.51	-1.51	-2.54	-3.96	-2.80
Number of decoys	1165	889	481	105	269
Cluster density	0.0823	0.0821	0.0295	0.071	0.0226

C-score is a confident score allowing to estimate the quality of the predicted model. The better model has the higher C-score, which typically varies in the range [-5, 2]. Number of decoys (number of the low temperature replicas) are generated according to the algorithm and clustered by SPICKER. The cluster density is determined by the number of decoys at the unit space of the cluster. The higher number of decoys and cluster density indicate the model of higher quality. Thus, the model 1 based on these parameters is the best generated model. For further computations we considered only this model.

2.2 Molecular dynamic

In order to improve the generated model 1 we optimised its structure applying molecular dynamic in the water box. The force field CHARMM¹⁸ was assigned in NAMD¹⁹ for the atoms of the receptor. The molecular dynamic minimisation (Langevin dynamic)²⁰ has been applied in order to minimise the energy of the structure in the water. The dynamic was run for 500 ps with langevin thermostat at 310K. The integration timestep is 2 fs/step and the size of the water cell is 74 x 68 x 106 Å³ with the centre placed at 70.10, 71.40, 73.11 of x,y,z-coordinates. The rigid model was applied for the water molecules (TIP3P). For the long-range electrostatic interaction we used the particle-meshed Edwald method²¹ (PME).

Docking.

1. Human Cannabinoid receptor type 2.

The molecular docking was performed with the AutoDockTools4 software²² using the Lamarckian Genetic Algorithm²³. The water molecules were eliminated and the non-polar hydrogen atoms were merged. The docking area was limited by the constructed grid box of the size 40 x 52 x 48 centred at 8.21, -0.606, -52.343 of x,y,z-coordinates (based on the position of the ligand binding domain (LBD)). The following parameters were used in the docking: number of hybrid GA-LS runs: 500; population size: 150; maximum number of energy evaluations: 25,000,000, maximum number of top individuals to survive to next generation: 1; rate of gene mutation: 0.02; rate of crossover: 0.8; Mean of Cauchy distribution for gene mutation: 0.0; variance of Cauchy distribution for gene mutation: 1.0. The ligand binding domain (LBD) of hCB2R was selected based on the crystallographic data of the receptor.

2. Rat Cannabinoid receptor type 2.

After the energy of the model **1** was minimised, we took optimised coordinates of the receptor in order to carry out the docking. In order to establish the possible binding pockets of rCB2R, we determined the hydrophobic sites of the receptor model and compared them to the binding sites of the known protein templates. Based on this comparison we have selected three possible sites of the receptor (Fig. S3) and compared the binding energies of the best ranked poses of R-(–)-**6** inside of the each site. The molecular docking was performed with the similar algorithm and software as for hCB2R.

The following parameters were used for all three binding positions: number of hybrid GA-LS runs: 500; population size: 150; maximum number of energy evaluations: 25,000,000, maximum number of top individuals to survive to next generation: 1; rate of gene mutation: 0.02; rate of crossover: 0.8; Mean of Cauchy distribution for gene mutation: 0.0; variance of Cauchy distribution for gene mutation: 1.0. The constructed grid box limiting the docking area had the same size for A, B and C: 46, 40, 44 centered at 84.237, 62.779, 83.571 for A, at 72.544, 73.01, 60.599 for B and 61.498, 83.45, 76.266 for C of x,y,z-coordinates.

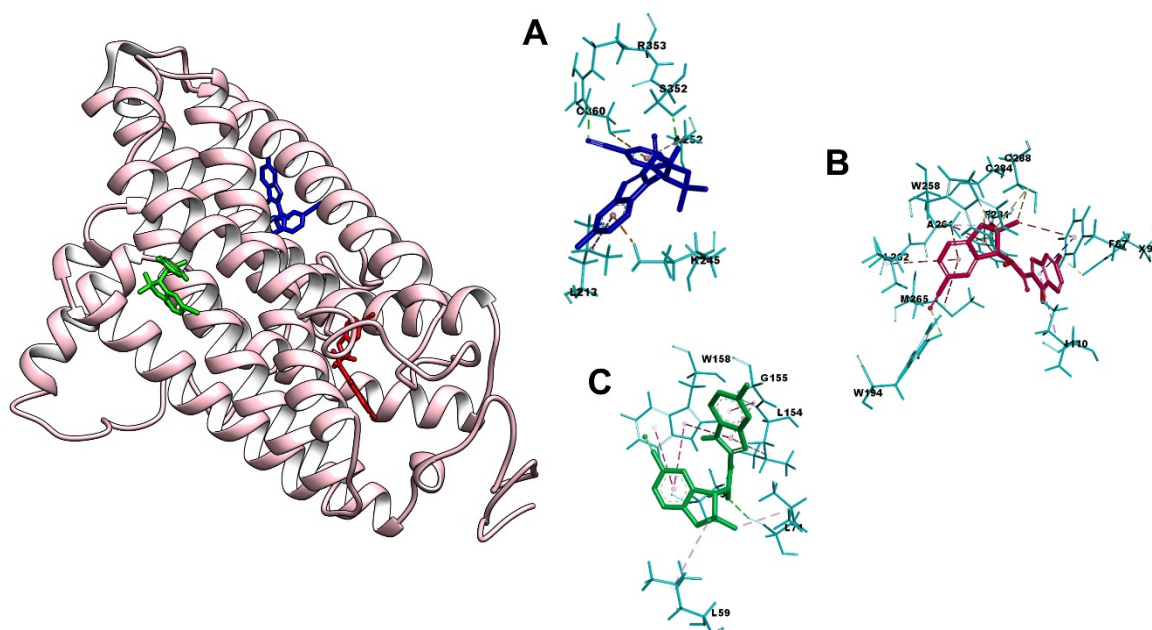


Figure S4. *In silico* investigation of the binding modes of compound **6** based on docking in the three binding sites of rCB2R (left). The highest ranked docked positions of R-(–)-**6** are shown as the pose A (blue) interacting with A252, R353, S352, C360, K245 and L213; the pose B (red) interacting with A261, W258, C284, C288, L252, M265, W194, I110, F87 and X95; the pose C (green) interacting with W158, G155, L154, L59 and L71.

The binding energies of the best three ranked poses made up -6.04, -6.64 and -6.79 kcal mol⁻¹ for A, B and C accordingly. According to the binding energy score the most stable poses are B and C. The pose C exhibit slightly higher stability than B; however, these poses are almost equally stable. The pose B is involved in the interactions with the similar amino acid residues in rCB2R as R-(–)-**6** in hCB2R. For further comparison with hCB2R we considered only the pose B in the model of rCB2R.

3. Docking study of S-(+)-**6**.

In order to investigate the binding abilities of S-(+)-**6** in hCB2R and rCB2R we carried out docking of the optimised geometry of S-(+)-**6** in the ligand binding pockets of the receptors. The same docking parameters, algorithm and software were applied as described above for R-(–)-**6**. In spite of docking was not able to reproduce the affinity for R and S isomers precisely²⁴, it shows the correlation with the experimental results. First of all, the best ranked pose of S-(+)-**6** exhibited the less stable binding with the binding energy -9.71 kcal mol⁻¹ in the LBD of hCB2R compare to R-(–)-**6** with the energy -10.45 kcal mol⁻¹. Interesting that the best binding pose of S-isomer is moved closer to the loop of the receptors, where the dominating pi-alkyl interactions have weaker stabilising factor on S-isomer than the pi-pi-T shaped interactions on R-(–)-**6** (Figure S5).

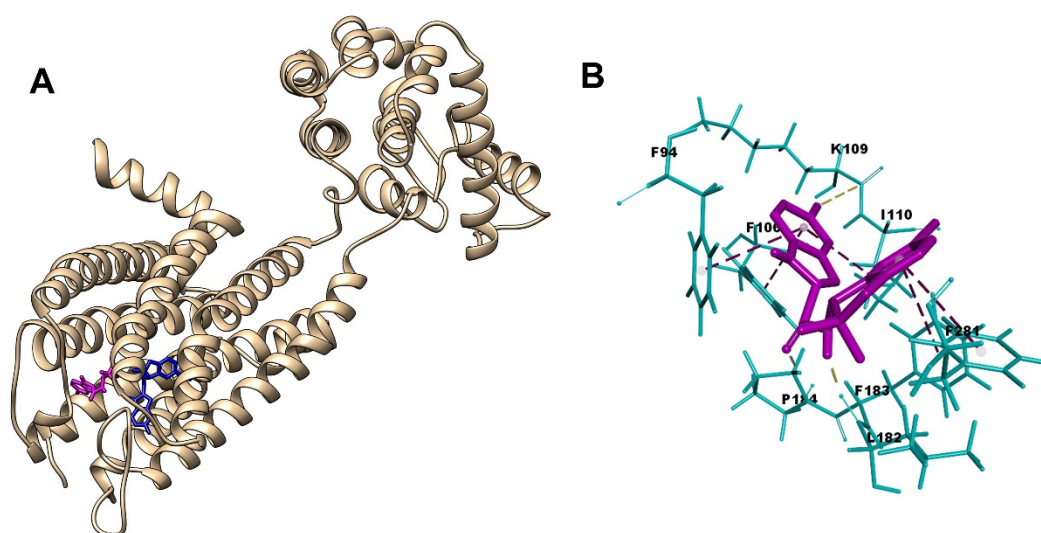


Figure S5. *In silico* investigation of the binding modes of compound **6** based on docking in hCB2R. The highest ranked docked positions of the compounds R-(-)-**6** (blue) and S-(+)-**6** (purple) are shown in the ligand binding domain (A). S-(+)-**6** interacts with K109, I110, F94, F281, F183, P184, L182 and F106 (B).

The weaker binding was observed also when S-(+)-**6** was docked in rCB2R. The binding energy for the best docked pose of S-isomer made up -6.15 kcal mol⁻¹ compare to -6.64 kcal mol⁻¹ of R-(-)-**6** (Figure S5).

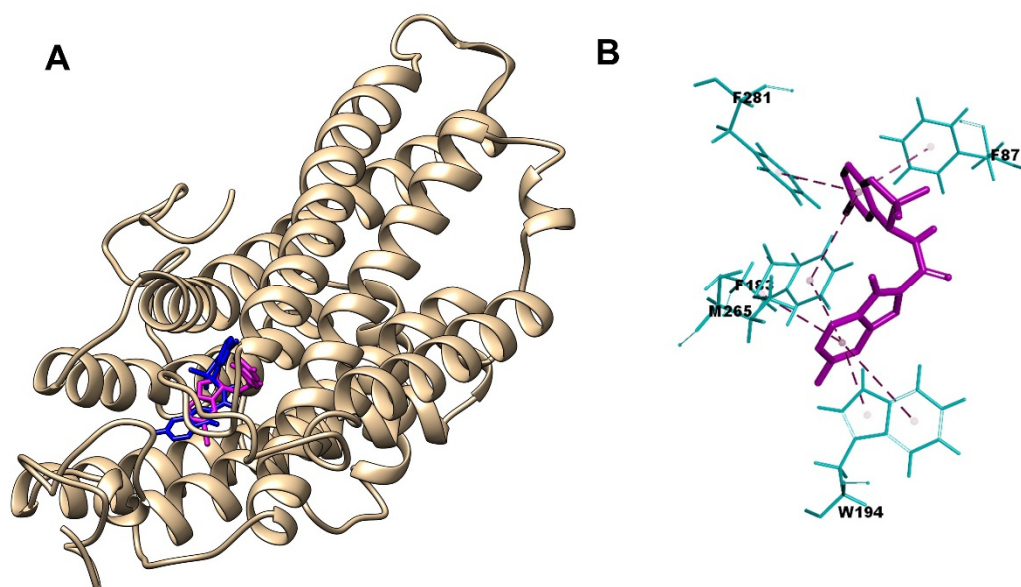
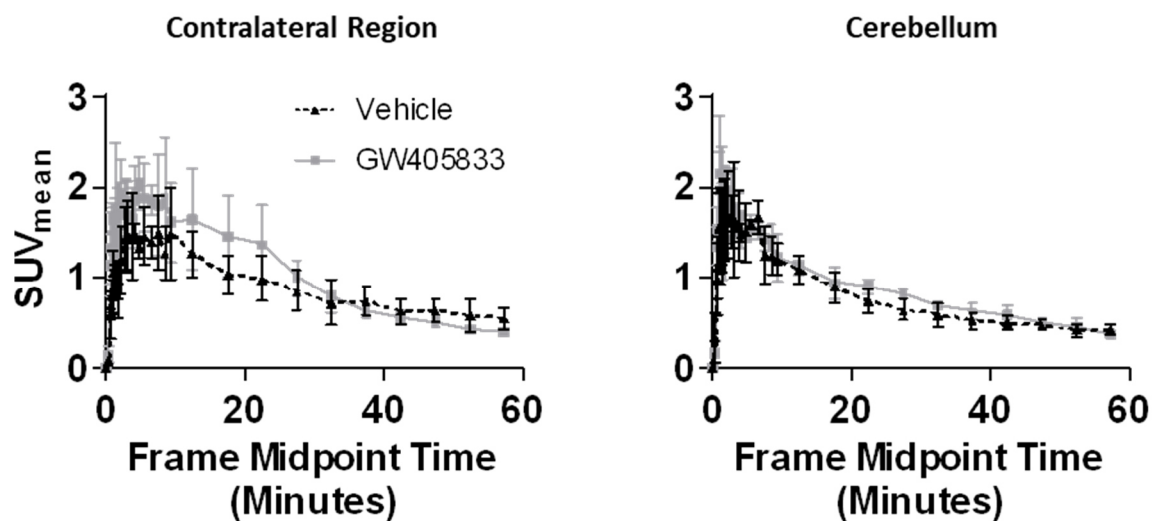


Figure S6. *In silico* investigation of the binding modes of compound **6** based on docking in rCB2R. The highest ranked docked positions of the compound R-(-)-**6** (blue) and S-(+)-**6** (purple) are shown (A). S-(+)-**6** interacts with W194, F87, F281, F183 and M265 (B).

PET imaging



TAC Parameter	Contralateral			Cerebellum		
	Vehicle	GW405833	p-Value	Vehicle	GW405833	p-Value
AUC ₀₋₁₀ (SUV · min)	12 (9 to 15)	14 (2 to 34)	0.077	13 (2 to 28)	14 (2 to 30)	0.293
AUC ₁₀₋₂₀ (SUV · min)	10 (7 to 12)	11 (2 to 27)	0.176	8 (1 to 18)	9 (1 to 19)	0.368
AUC ₂₀₋₄₀ (SUV · min)	17 (12 to 23)	20 (3 to 45)	0.205	13 (2 to 29)	16 (2 to 34)	0.109
AUC ₄₀₋₆₀ (SUV · min)	12 (9 to 16)	10 (1 to 21)	0.138	9 (1 to 20)	10 (1 to 22)	0.251

Figure S7. Time activity curves of the contralateral site and the cerebellum, with and without (Vehicle) GW405833 (5 mg/Kg, n = 3) administered at 20 min post injection of [¹⁸F]LU13 and the corresponding AUCs (n = 3; Mean ± SD).

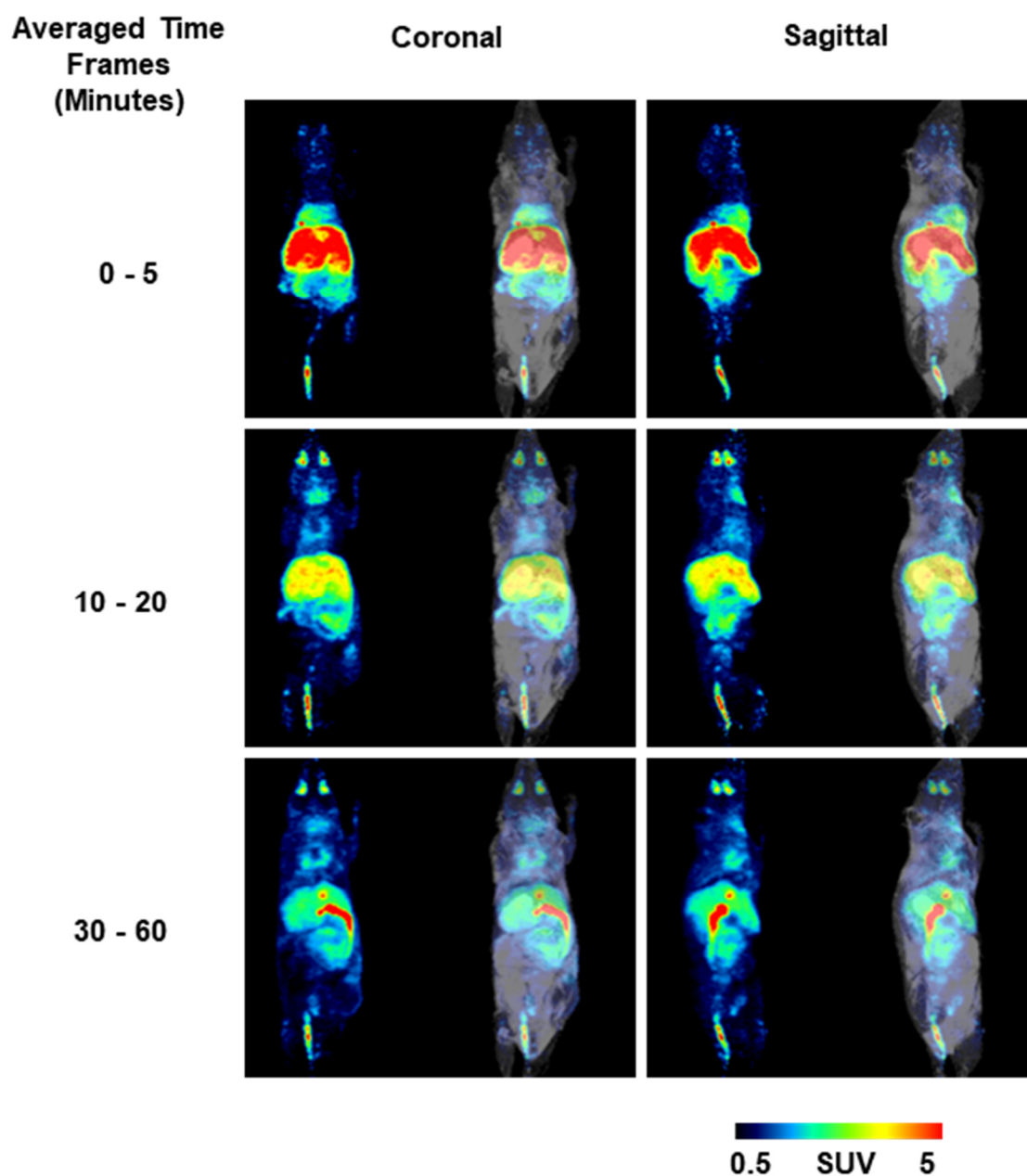


Figure S8. In vivo biodistribution of [^{18}F]RM365 after intravenous administration in an exemplary CD-1 mouse. Shown are maximal intensity projections (MIP) of the time averaged reconstructed PET recordings in coronal and sagittal view. MIPs of PET merged with T1w MR image (right).

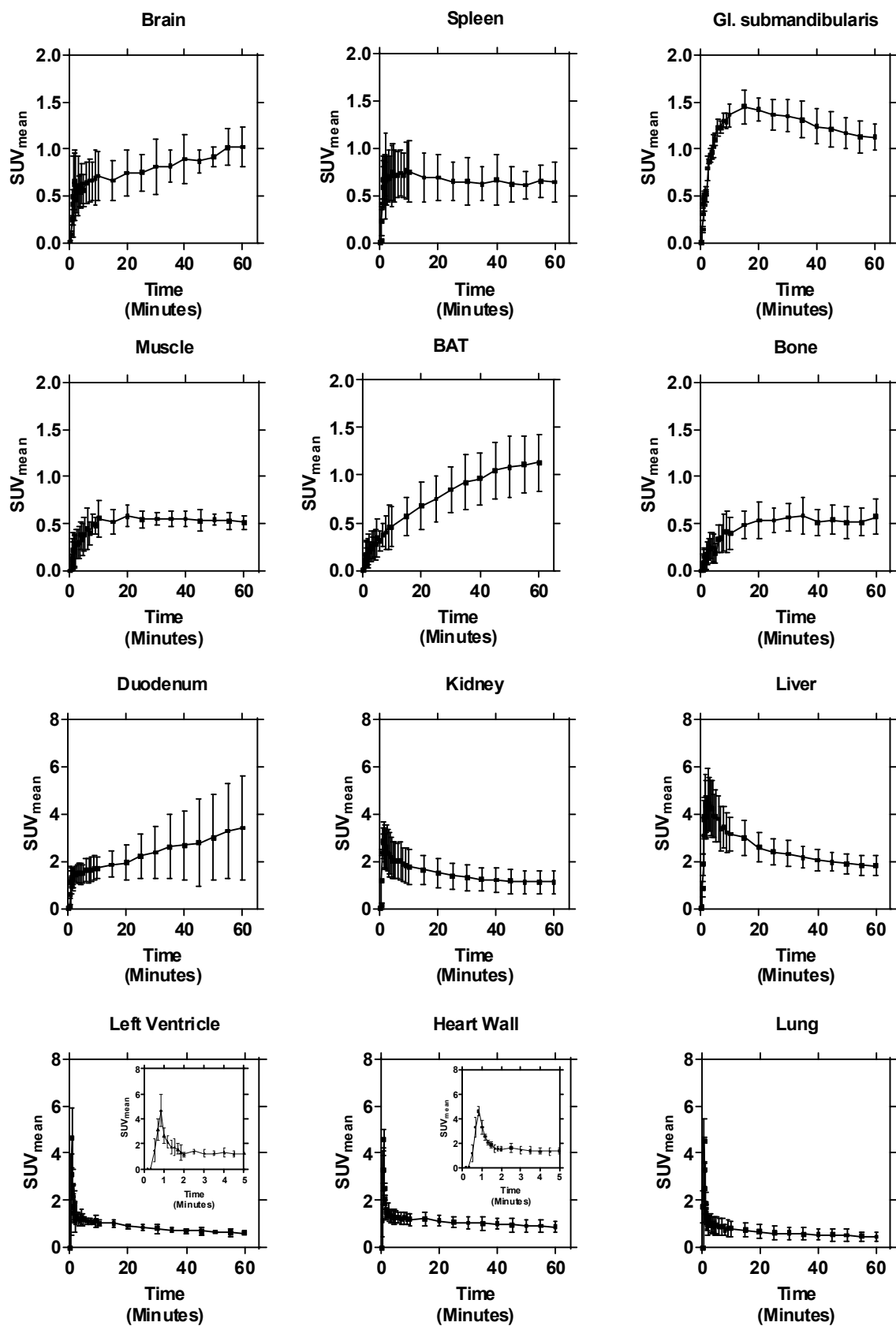


Figure S9. $[^{18}\text{F}]$ RM365 uptake over time after intravenous administration in indicated tissues of CD-1 mice represented in time activity curves. N = 4, mean $\pm$ SD.

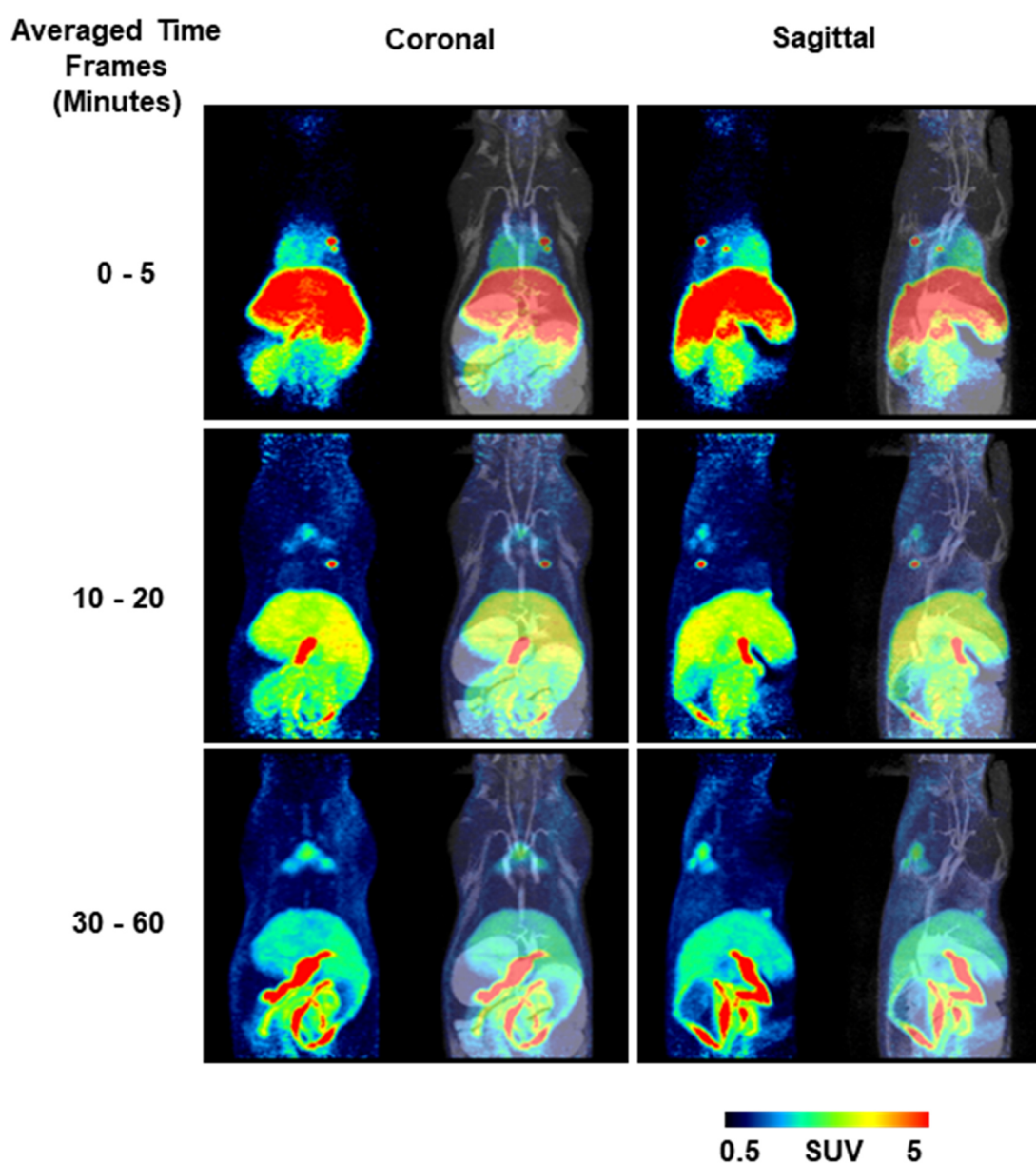


Figure S10. Thoracic and abdominal biodistribution of $[^{18}\text{F}]\text{RM365}$ after intravenous administration in an exemplary Wistar rat. Shown are maximal intensity projections (MIP) of the time averaged reconstructed PET recordings in coronal and sagittal view. MIPs of PET merged with T1w MR image (right).

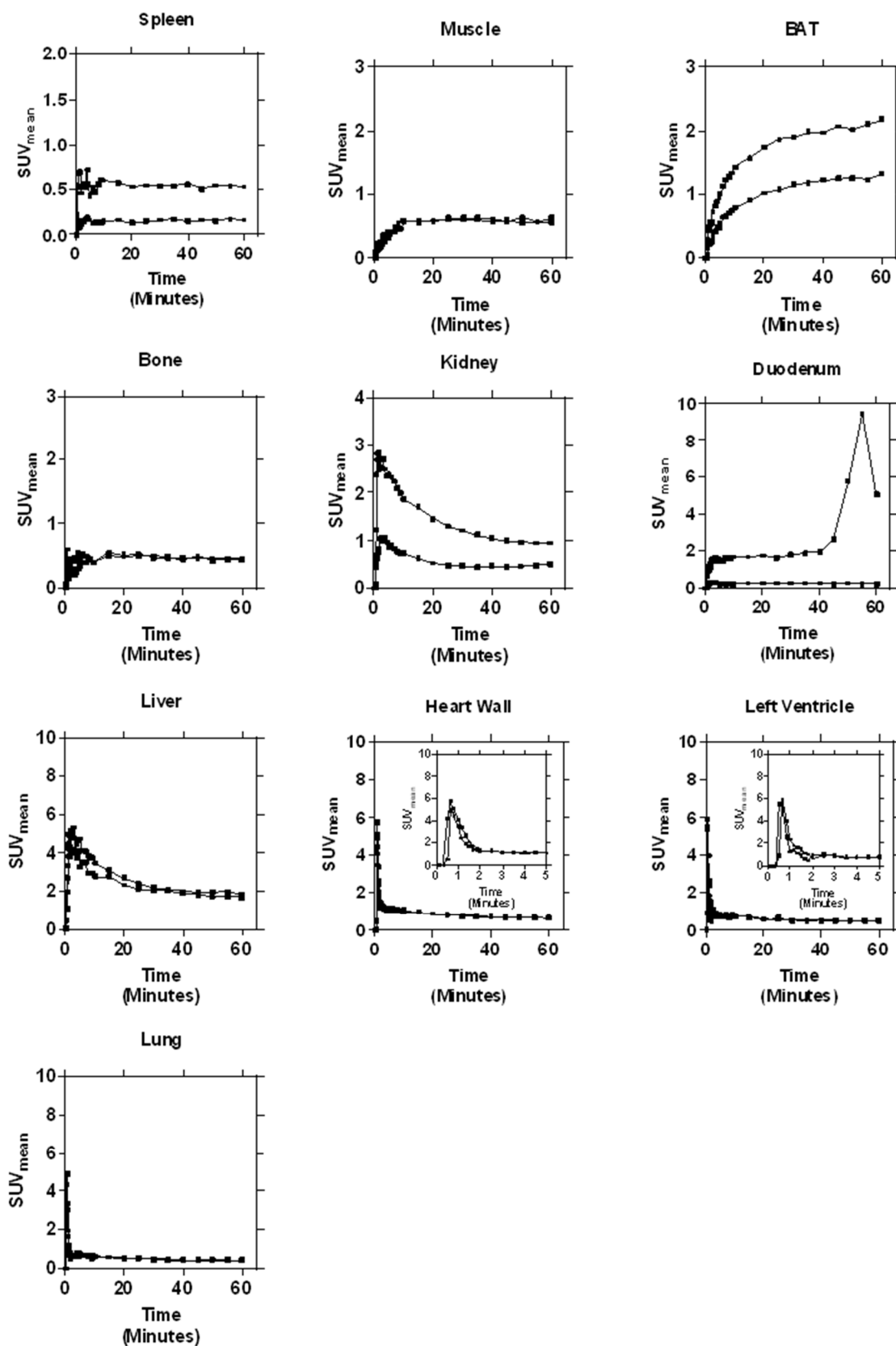


Figure S11. $[^{18}\text{F}]$ RM365 uptake over time after intravenous administration in indicated tissues of Wistar rats represented in time activity curves ($n = 2$).

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