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Supporting Information for

Small Molecule Recognition of Mephedrone using an Anthracene Molecular Clip

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General Procedure: All chemicals were used as purchased from Sigma Aldrich Company, Alfa Aesar or Cambridge Isotopes. Both ^{13}C-NMR and ^{1}H-NMR titration spectra were recorded on a JEOL 600 MHz spectrometer in the appropriate deuterated solvents. Chemical shifts are reported in parts per million (ppm) with tetramethylsilane (TMS) as the internal standard and coupling constants (*J*) are recorded in hertz (Hz). The multiplicities in the proton NMR spectra are reported as (br) broad, (s) singlet, (d) doublet, (dd) doublet of doublets, (ddd) doublet of doublet of doublets, (t) triplet, and (m) multiplet. All spectra are recorded at ambient temperature, unless specified otherwise. Mass spectrometry studies were conducted on a Varian Prostar 1200L quadrupole MS/MS via direct injection using an ESI source. UV/Vis spectra were recorded on a Cary 100 spectrometer, and fluorescence emission studies were carried out on a Perkin Elmer LS-55. IR spectra were recorded on a Nicolet Nexus 470 FT-IR paired with a Smart Orbit ATR attachment. The characteristic functional groups are reported in wavenumbers (cm^{-1}), and are described as weak (w), medium (m), strong (s), and very strong (vs). Elemental analysis was carried out by analytical and chemical consultancy services Medac LTD, Surry UK.

Pharmacophore explanation: After following the validation criteria set out in the Fig S1 below, the protein-ligand binding interactions of nine compounds (Table S1) were used to help develop the three-point pharmacophore (Fig S2). From this information it was found that the three functional groups of interest for binding are the carbonyl, benzyl and amine groups. From this, thiourea groups were chosen due to their strong hydrogen bonding properties as both donors and acceptors. Anthracene was chosen as the aromatic group for pi-stacking due in part to its strong fluorescent properties.

The spatial orientation of the binding interactions was imperative to develop a consensus probe design. From the pharmacophore work it is also found that caffeine is a promiscuous binder, as it has strong pi-stacking interactions with multiple proteins. Therefore it was decided that a flexible probe molecule, which will re-organise around mephedrone, could prevent caffeine binding due to steric effects obstructing the aromatic functionalities without hydrogen bonding occurring with the thiourea groups.

Computational Studies: Elucidation of a mephedrone-binding pharmacophore.

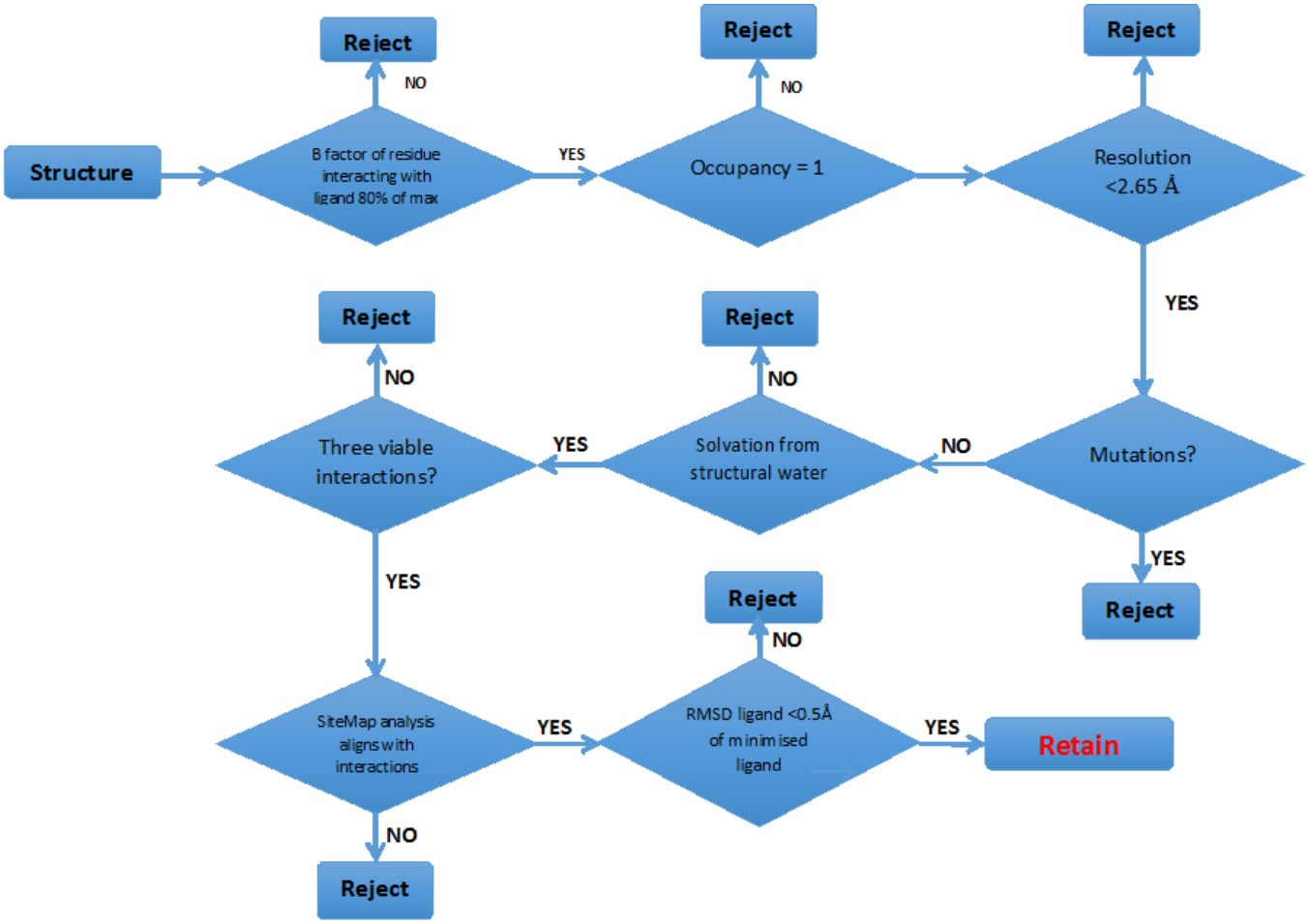


Fig S1. Flowchart summarizing how high quality experimental structures for the elucidation of the mephedrone-binding pharmacophore were obtained.

PDB accession code	Ligand ID
2A3B	Caffeine
3DDS	Caffeine
3DDW	Caffeine
3DD1	Caffeine
3GKZ	Methamphetamine
3GM0	MDMA
2DPZ	Paracetamol
3PY4	Paracetamol
3NK2	L-Dopamine

Table S1. The 9 protein-ligand complexes used to determine the consensus mephedrone binding pharmacophore.

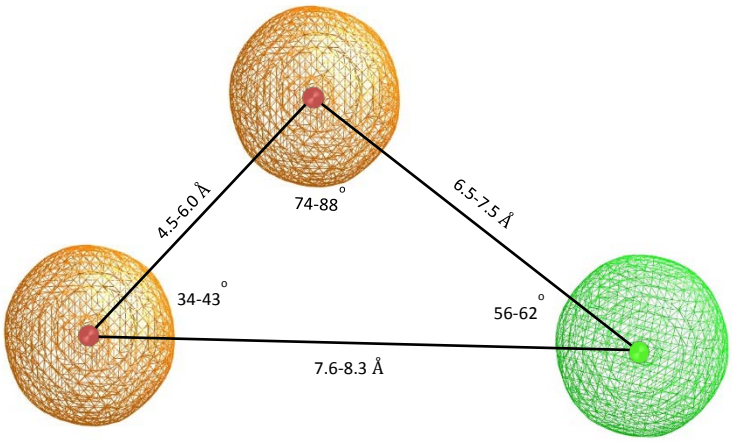
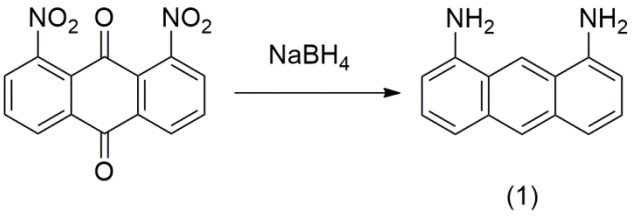


Fig S2. Consensus 3-point pharmacophore obtained for mephedrone binding showing expected ranges for bond distances and angles between pharmacophoric features. The binding features are portrayed as meshed spheres, (green represents hydrogen-bond acceptor, and orange represents aromatic interactions).

NMR Studies (see pages 5 to 18): ^{1}H-NMR titrations were carried out by preparing a 20.0 mM solution of molecular probe **2** or model compound **3** (1-benzyl-3-phenylthiourea) in acetone-d_6 (1.0 mL). A stock solution of the free-base form of the drugs was prepared in acetone-d_6 (1.0 mL). Aliquots of 2.5 µL (2.5 µL = 0.1 equivalence of drug to probe) were added, the sample tube was shaken to ensure mixing and the ^{1}H-NMR spectra were recorded after each addition.

Synthesis and Characterization:

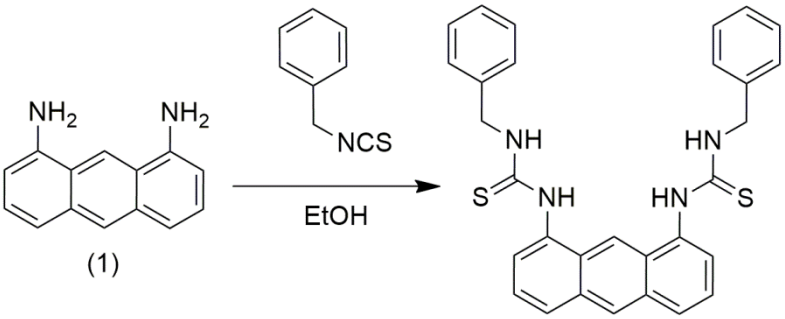
Preparation of 1,8-diaminoanthraquinone (1)[1]



(1)

1,8-Dinitroanthraquinone (1.2 g, 4.9 mmol) was dissolved in isopropanol (50 mL) and stirred under nitrogen for 15 minutes. Sodium borohydride (2.3 g, 59 mmol) was added and the reaction was refluxed for 36 hours. The reaction mixture was poured into iced water (100 mL) and a green precipitate formed, the solid was filtered and was washed with distilled water (50 mL) to yield a dark purple solid (**1**) (0.83 g, 3.99 mmol, 93%). ^{1}H NMR ($CDCl_3$) δ, ppm: 4.21 (*s*, 4H, NH), 6.96 (*d*, 2H, ArH), 7.42 (*t*, 2H, ArH), 7.81 (*d*, 2H, ArH), 8.22 (*s*, 1H, ArH), 8.37 (*s*, 1H, ArH).

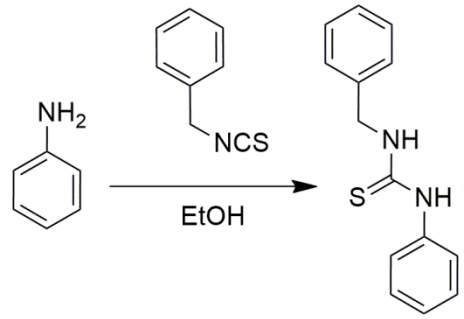
Preparation of dibenzylthiourea anthracene (2)[2]



(1) (2)

1,8-Diaminoanthracene (**1**) (0.88 g, 4.2 mmol) and benzylisothiocyanate (1.12 mL, 8.4 mml) were dissolved in ethanol (100 mL) and refluxed for two hours. The reaction was cooled in ice, and the precipitate was filtered. The solid was washed with excess water and ethanol, 25 mL each. The product was dried under vacuum, yielding a dark brown solid (**2**) (1.04 g, 2.05 mmol, 40 %). Mp; 213 °C. ^{1}H and ^{13}C NMR assignment are shown in table S2; IR (ATR solid) 3377, 3131, 2964, 1612, 1495, and 1250 cm^{-1}; ESI-MS (+'ve); $[M+H]^+$ = 507 m/z; Elemental analysis (%) calculated for $C_{30}H_{26}N_4S_2$: C, 71.11; H, 5.17; N, 11.06. Re-calculated for $C_{30}H_{26}N_4S_2 \cdot H_2O$ C, 68.67; H, 4.99; N, 10.68, Found: C, 68.93, H, 4.89; N, 10.34.

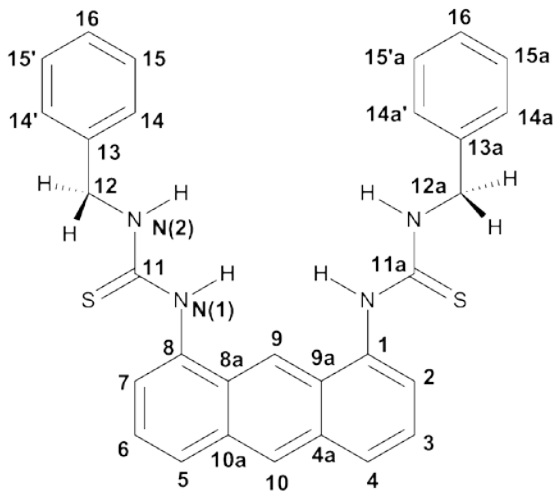
Preparation of benzylthiourea aniline (3)[3]



(3)

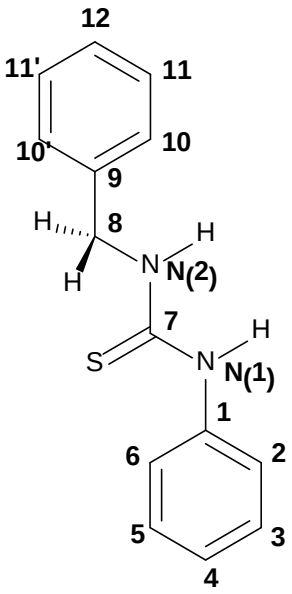
Model compound **3** was prepared based upon literature conditions. Aniline (400 mg, 392 µL, 4.3 mmol) and benzylisothiocyanate (0.64 g, 568 µL, 4.3 mmol) were taken up in ethanol (30 mL) and refluxed for two hours. Upon the formation of a precipitate, the reaction was allowed to cool to room temperature and filtered. The product, a cream coloured solid, was dried under vacuum, which yielded 0.62 g (59% yield) of compound **3**. See Table S3 for ^{1}H and ^{13}C-NMR assignments.

Table S2. ^{1}H NMR and ^{13}C NMR chemical shifts from molecular probe **2** (*disappears on D_2O shake)



Probe 2	^{1}H-NMR (DMSO-d_6, ppm, J)	^{13}C-NMR (DMSO-d_6, ppm)
1	N/A	139.0
2	8.02 (d, J = 8.1 Hz)	125.5
3	7.55 (m)	127.4
4	7.55 (m)	126.5
4a	N/A	132.1
5	7.55 (m)	126.5
6	7.55 (m)	127.4
7	8.02 (d, J = 8.1 Hz)	125.5
8	N/A	139.0
8a	N/A	126.7
9	8.78 (s)	116.8
9a	N/A	126.7
10	8.67 (s)	127.0
10a	N/A	132.1
11/11a	N/A	182.4
12/12a	4.78 (d, J = 5.5 Hz)	47.7
13/13a	N/A	134.9
14/14'	7.34 (m)	127.4
15/15'	7.34 (m)	128.2
16	7.34 (m)	127.0
N(1)H*	9.87 (br, s)	
N(2)H*	8.29 (br, s)	

Table S3. ^{1}H and ^{13}C shifts for model compound **3** (* *disappears on D_2O shake).*



	^{1}H-NMR (DMSO-d_6, ppm, ***J*** = Hz)	^{13}C-NMR (DMSO-d_6, ppm)
1	N/A	139.1
2	7.43 (d, J = 7.8 Hz)	128.2
3	7.38 – 7.30 (m)	128.6
4	7.29 – 7.23 (m)	124.3
5	7.38 – 7.30 (m)	128.6
6	7.43 (d, J = 7.8 Hz)	128.2
7	N/A	180.8
8	4.74 (d, J = 5.5 Hz)	47.2
9	N/A	139.0
10/10'	7.38 – 7.30 (m)	126.8
11/11'	7.38 – 7.30 (m)	127.4
12	7.12 (t, J = 7.3 Hz)	123.3
N(1)H*	9.61 (s)	
N(2)H*	8.16 (s)	

NMR Studies

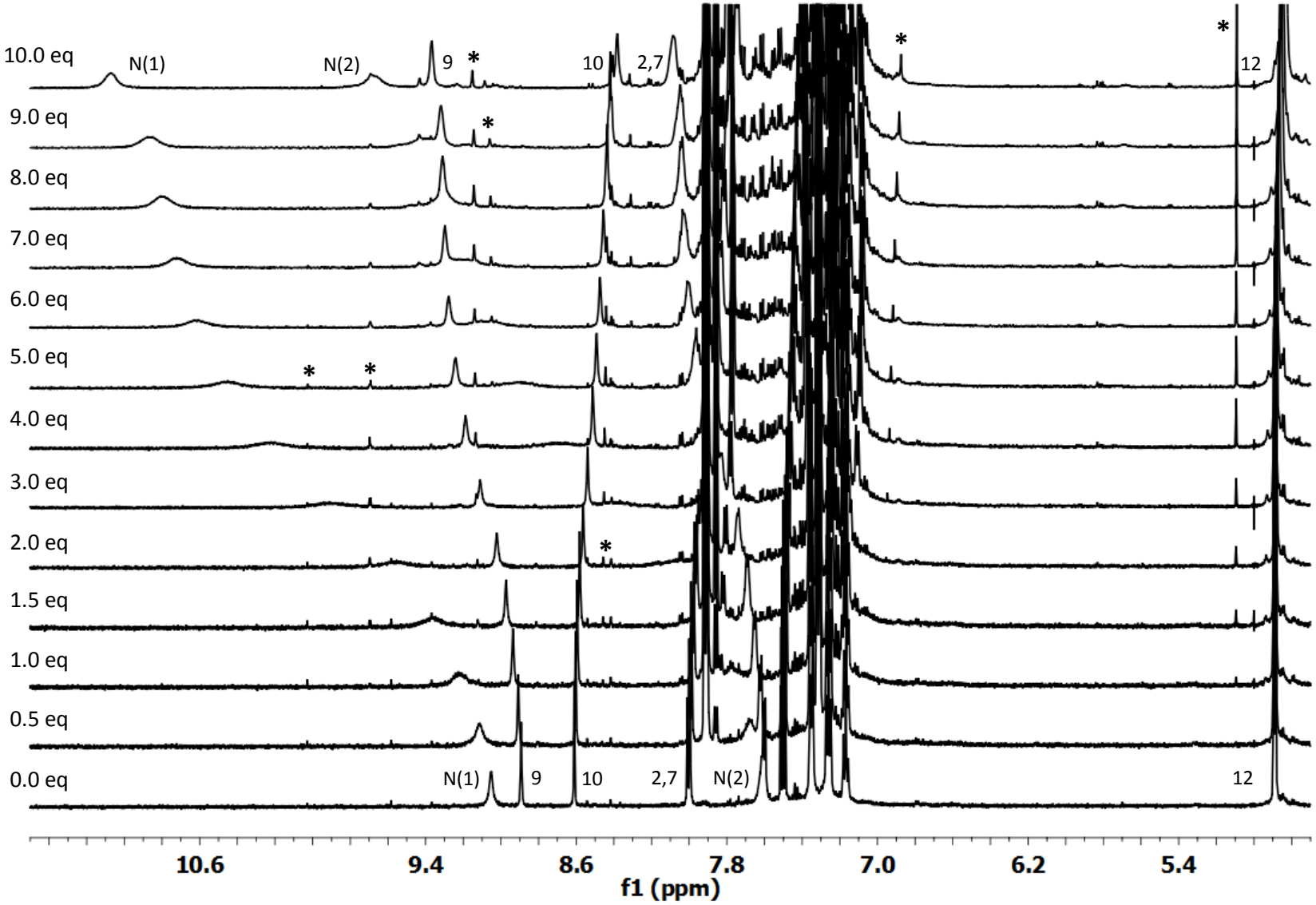


Fig S3. ^{1}H-NMR titration of probe **2** with of mephedrone in acetone-d_6.

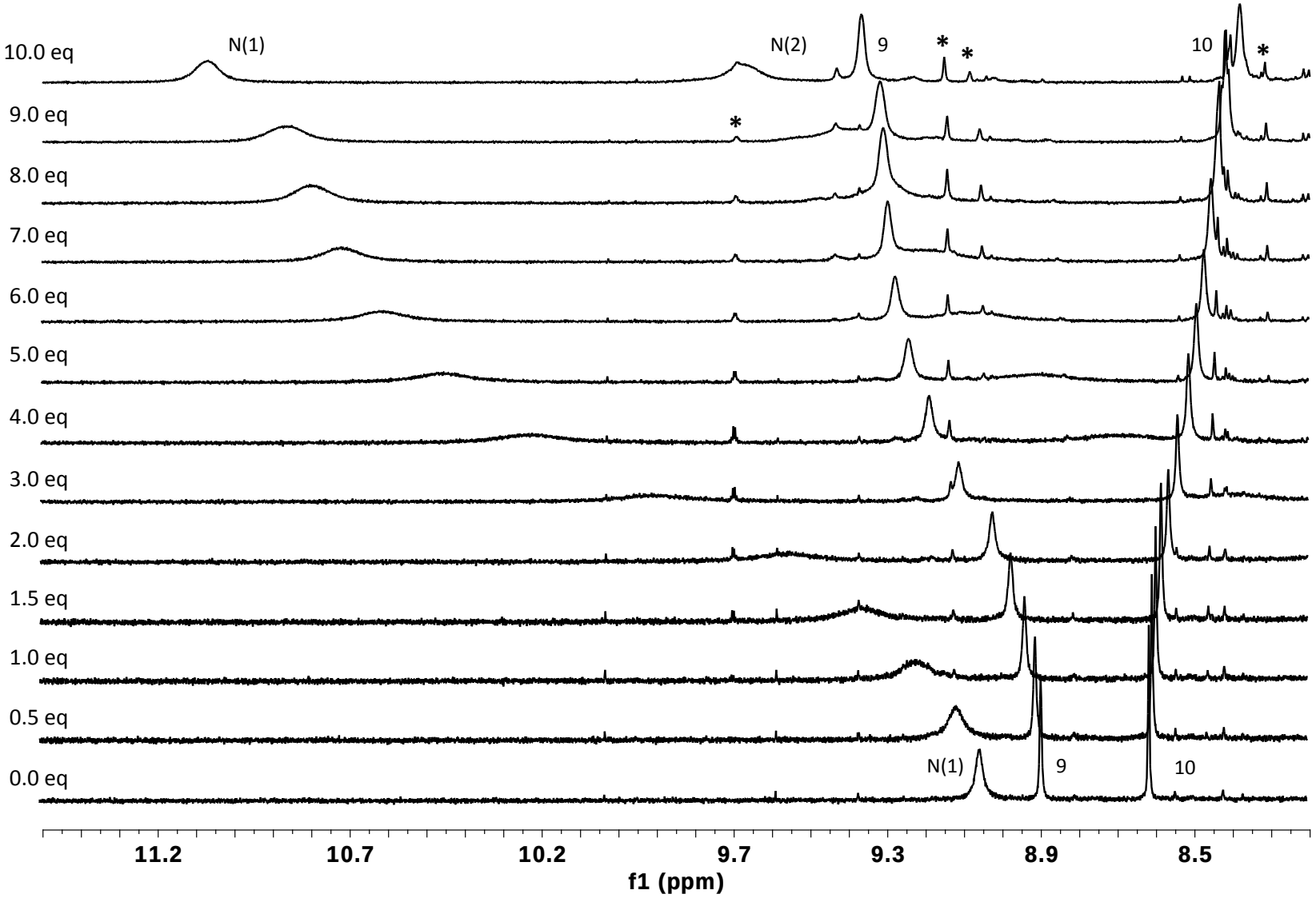


Fig S4. ^{1}H-NMR titration of probe **2** with of mephedrone in acetone-d_6 (expansion).

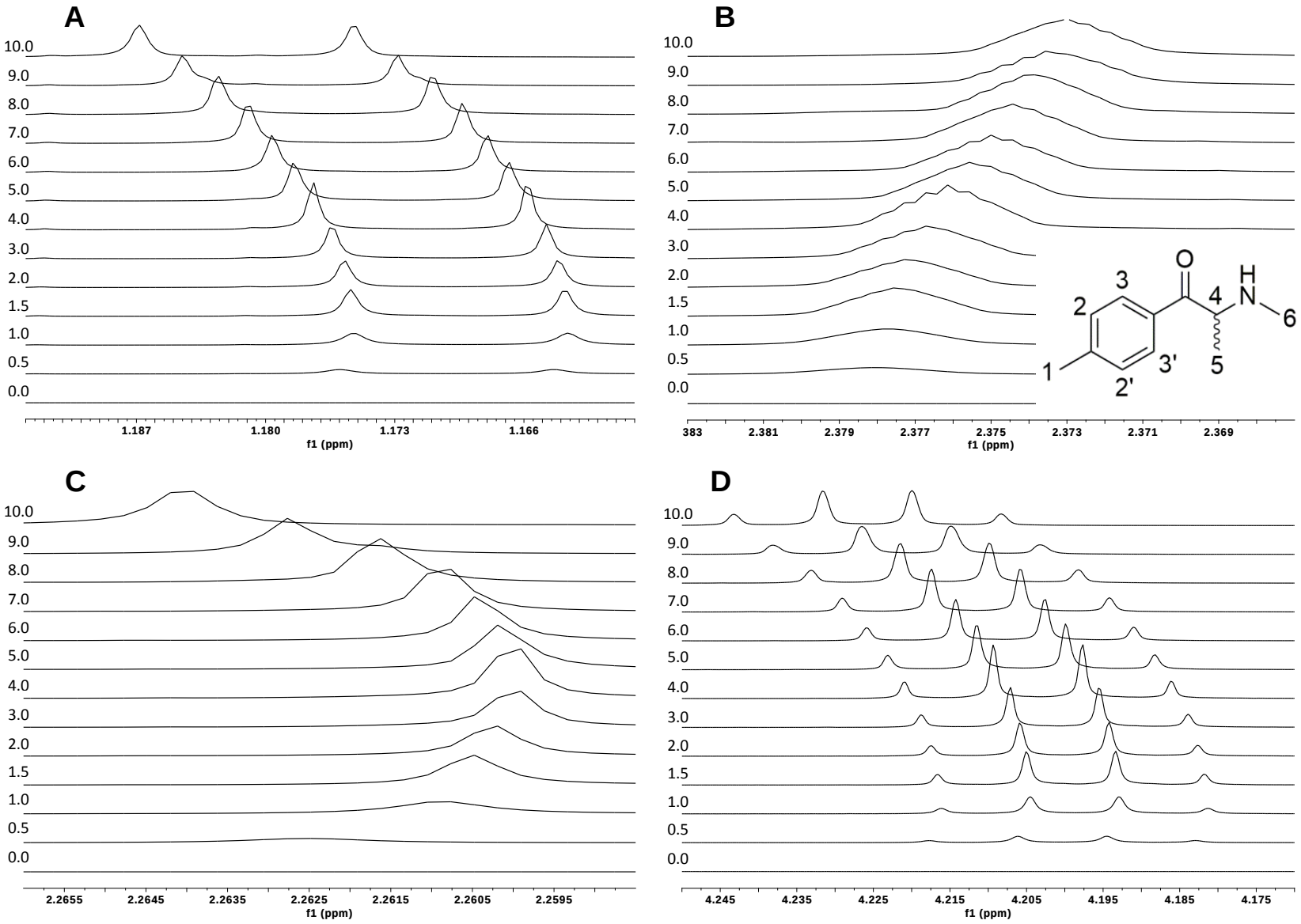


Fig S5. ^{1}H-NMR titration of probe **2** with mephedrone in acetone-d_6 highlighting the chemical shift changes of mephedrone signals (**A** methyl(5), **B** methyl (1), **C** methyl(6) and **D** methine(4)).

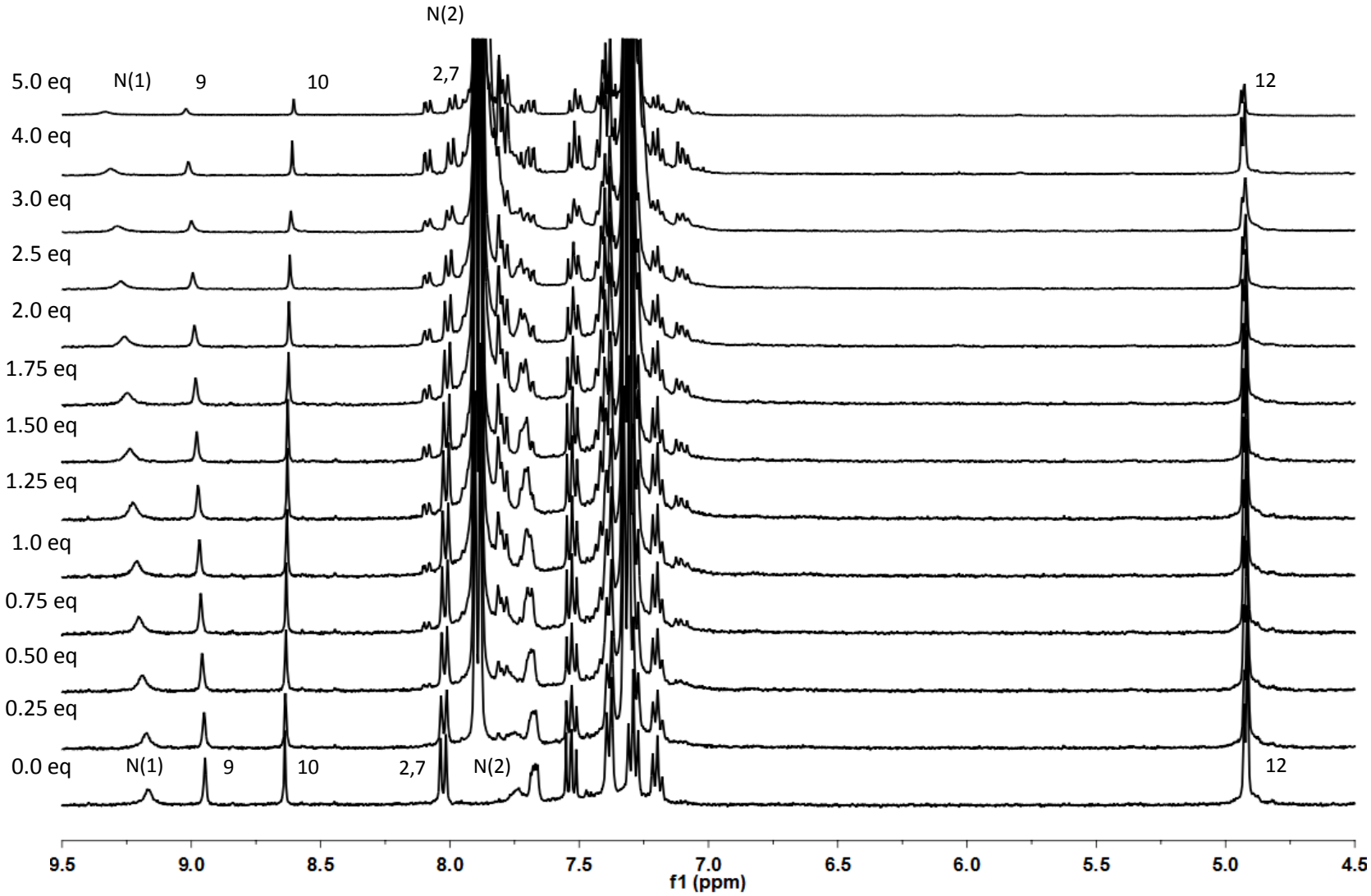


Fig S6. ^{1}H-NMR titration of probe **2** with mephedrone precursor (*p*-toyl butane-1-one) in acetone-d_6.

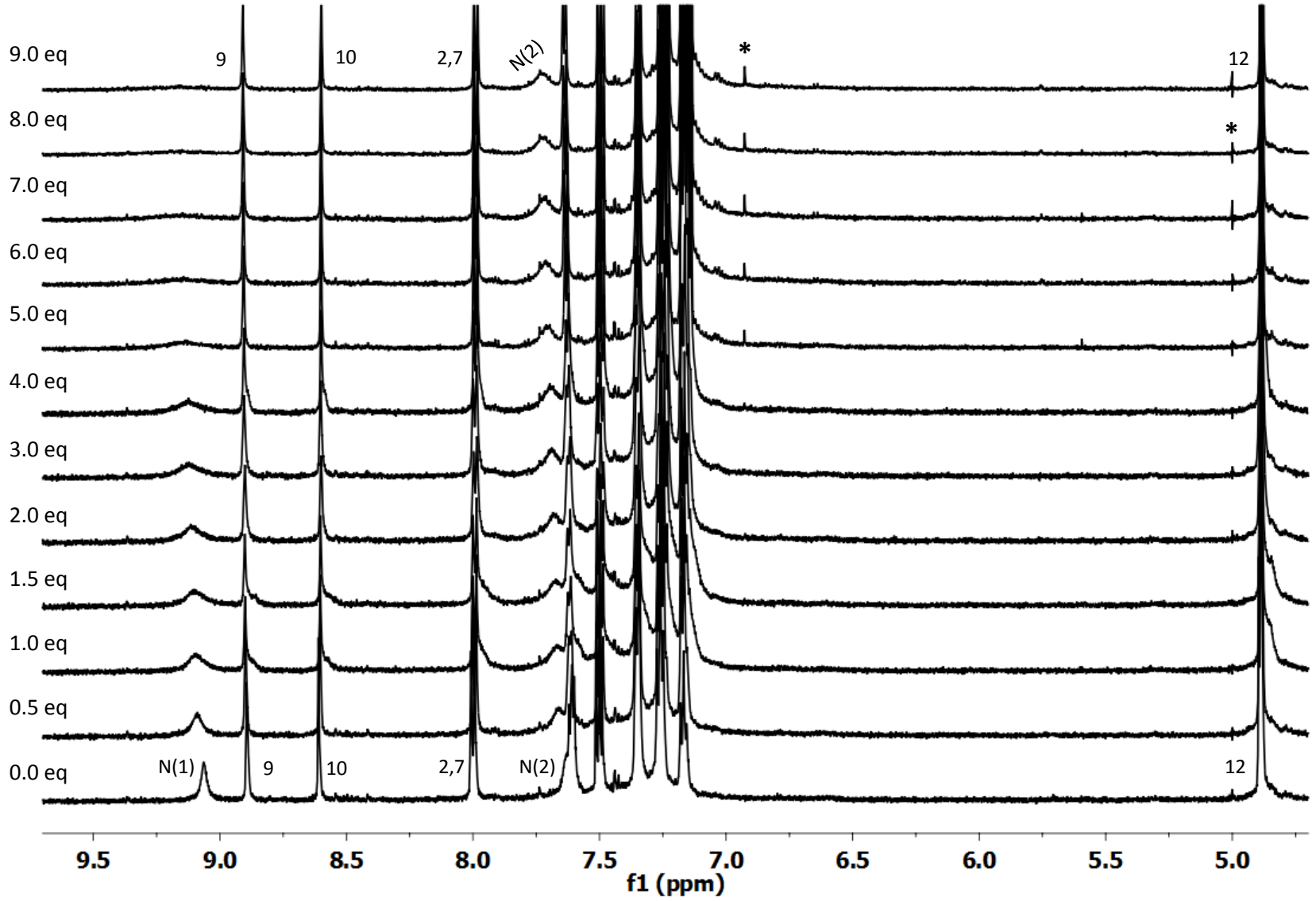


Fig S7. ^{1}H-NMR titration of probe **2** with methamphetamine in acetone-d_6.

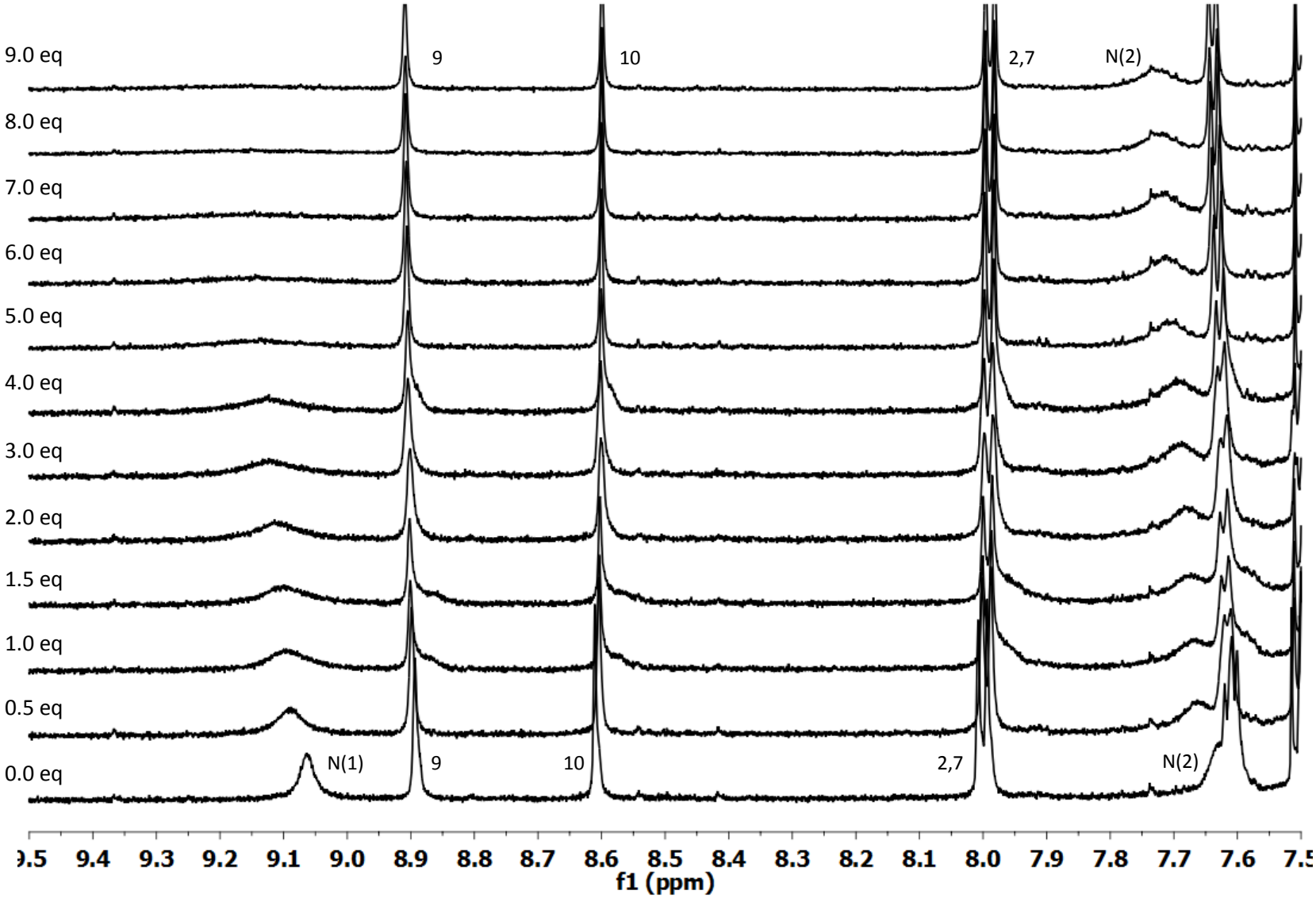


Fig S8. ^{1}H-NMR titration of probe **2** with methamphetamine in acetone-d_6 (expansion).

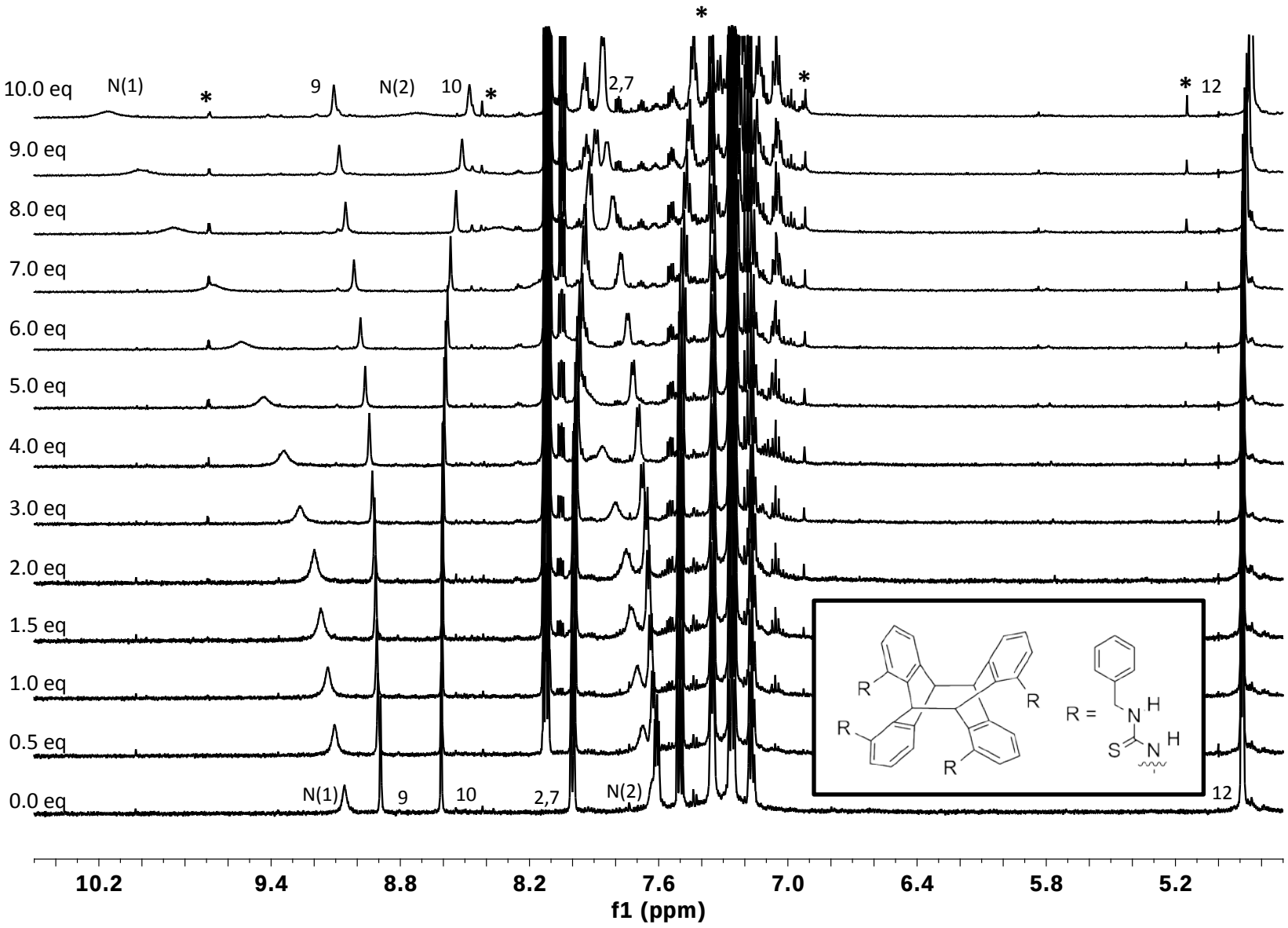


Fig S9. ^{1}H-NMR titration of probe **2** with flephedrone in acetone-d_6 (* cycloaddition product).

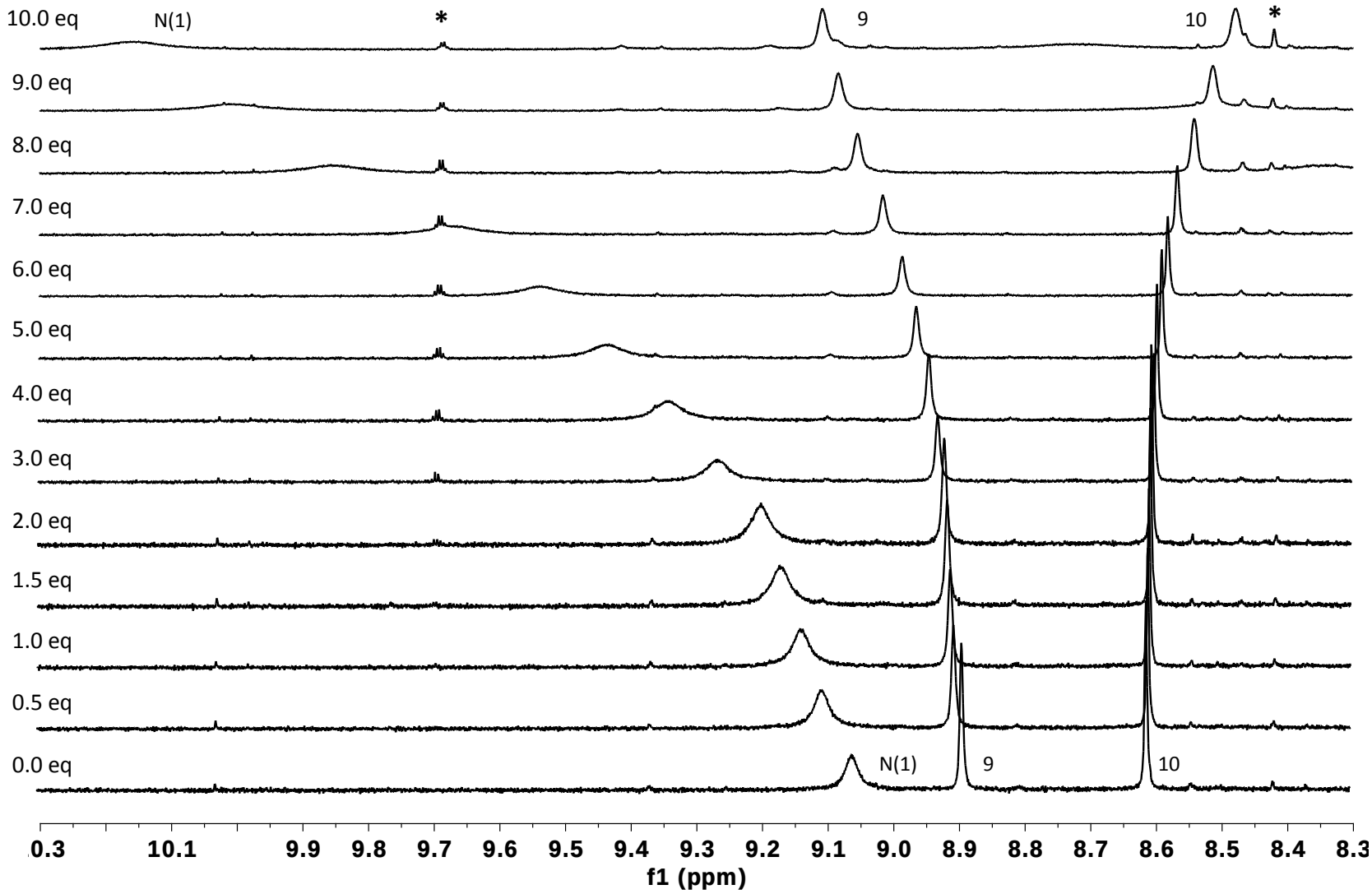


Fig S10. ^{1}H-NMR titration of probe **2** with flephedrone in acetone-d_6 (expansion).

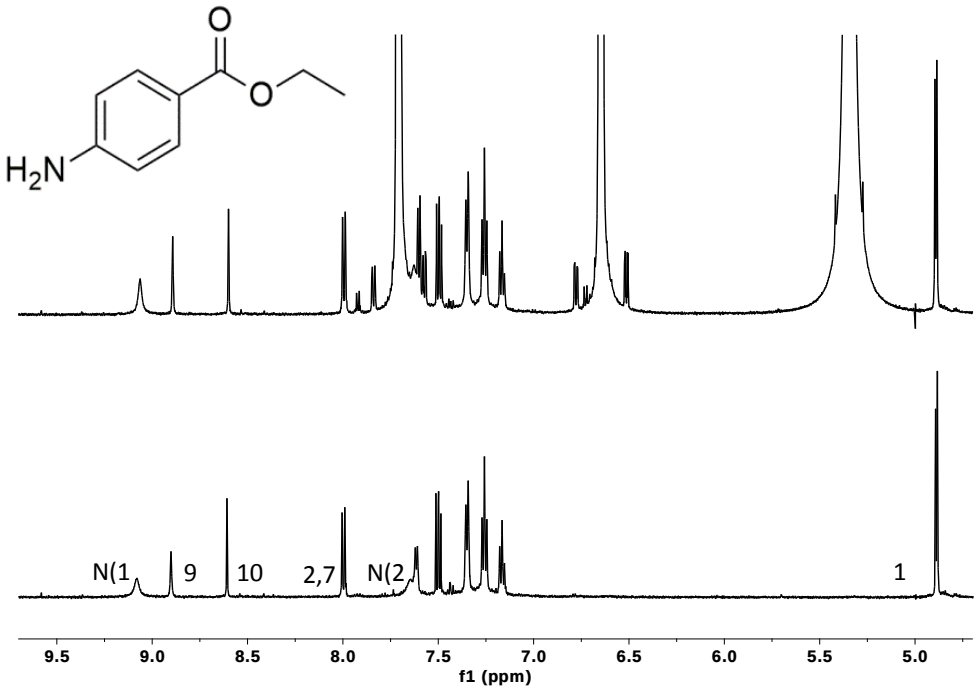


Fig S11. ^{1}H-NMR spectra of probe **2** (bottom) after the addition of ten equivalents of benzocaine (top) in acetone-d_6.

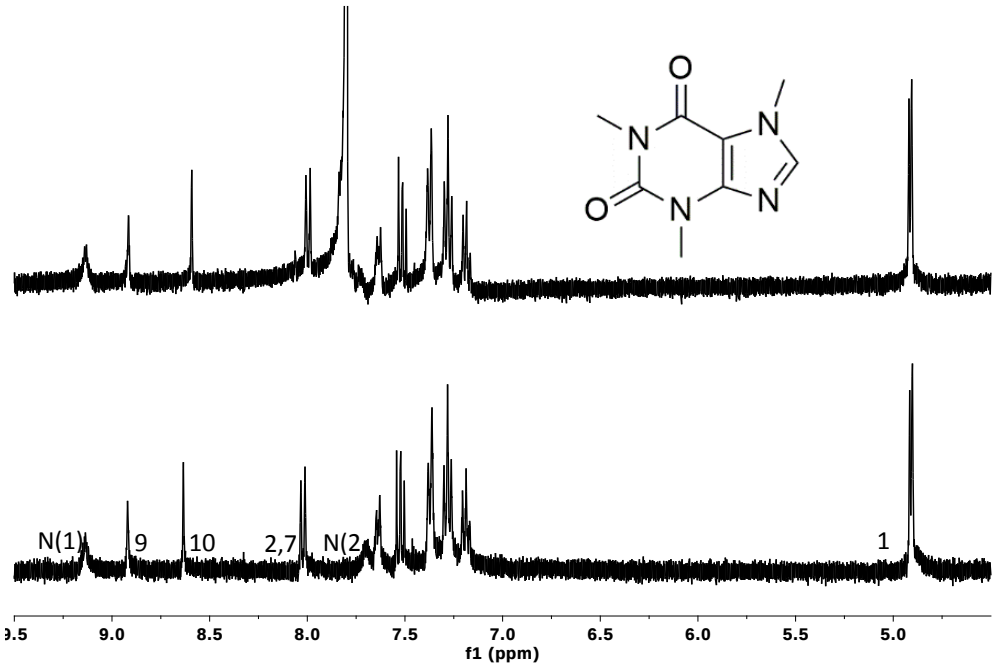


Fig. S12. ^{1}H-NMR spectra of probe **2** (bottom) after the addition of 10 equivalents of caffeine (top) in acetone-d_6.

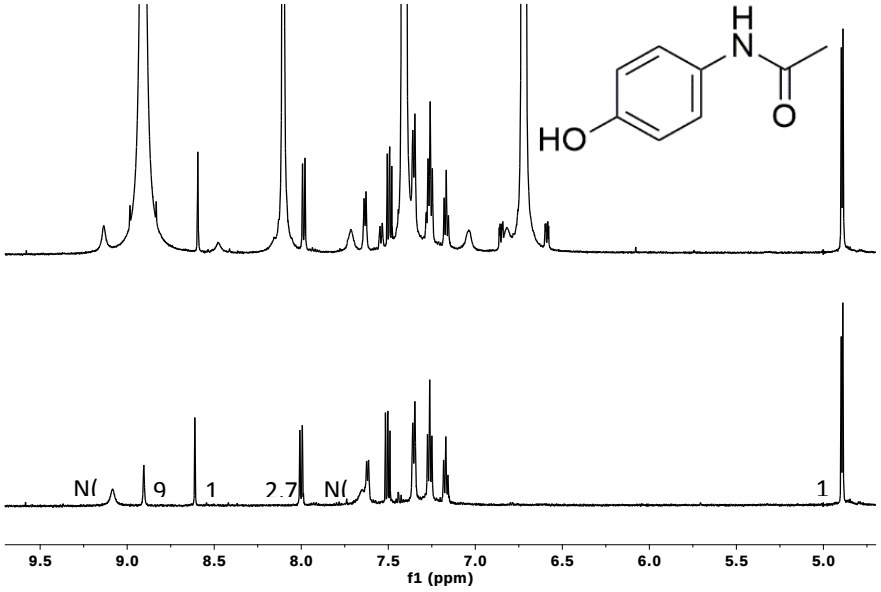


Fig. S13. ^{1}H-NMR spectra of probe **2** (bottom) after the addition of 10 equivalents of paracetamol (top) in acetone-d_6.

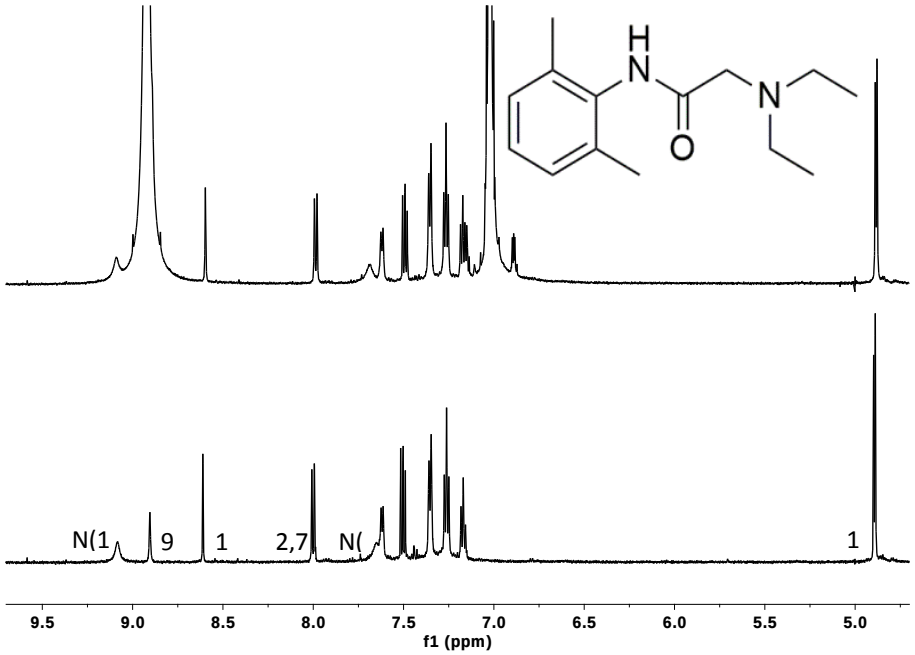


Fig. S14. ^{1}H-NMR spectra of probe **2** (bottom) with 10 equivalents of licocaine (top) in acetone-d_6.

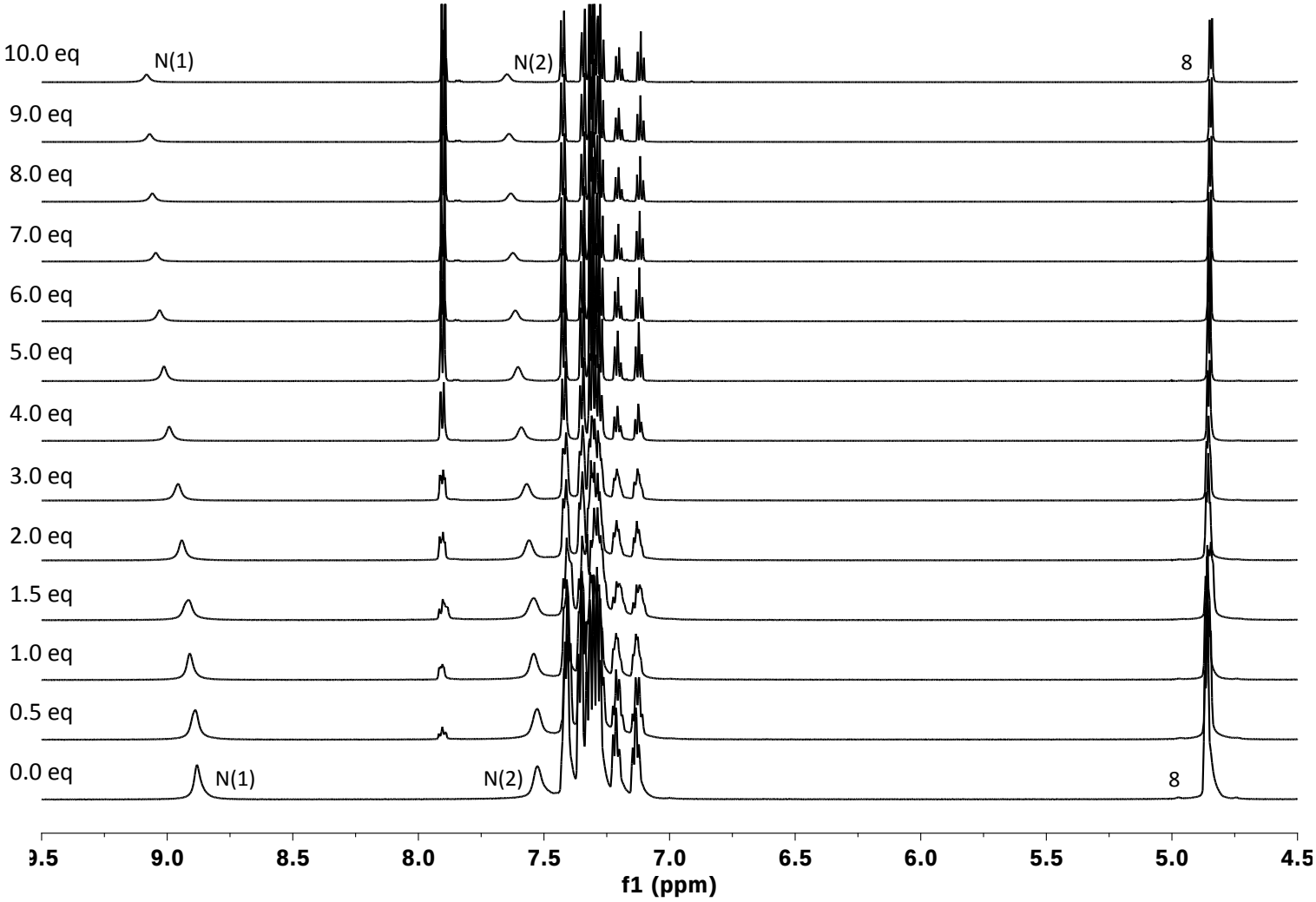


Fig. S15. ^{1}H-NMR titration of model compound **3** with mephedrone in acetone-d_6.

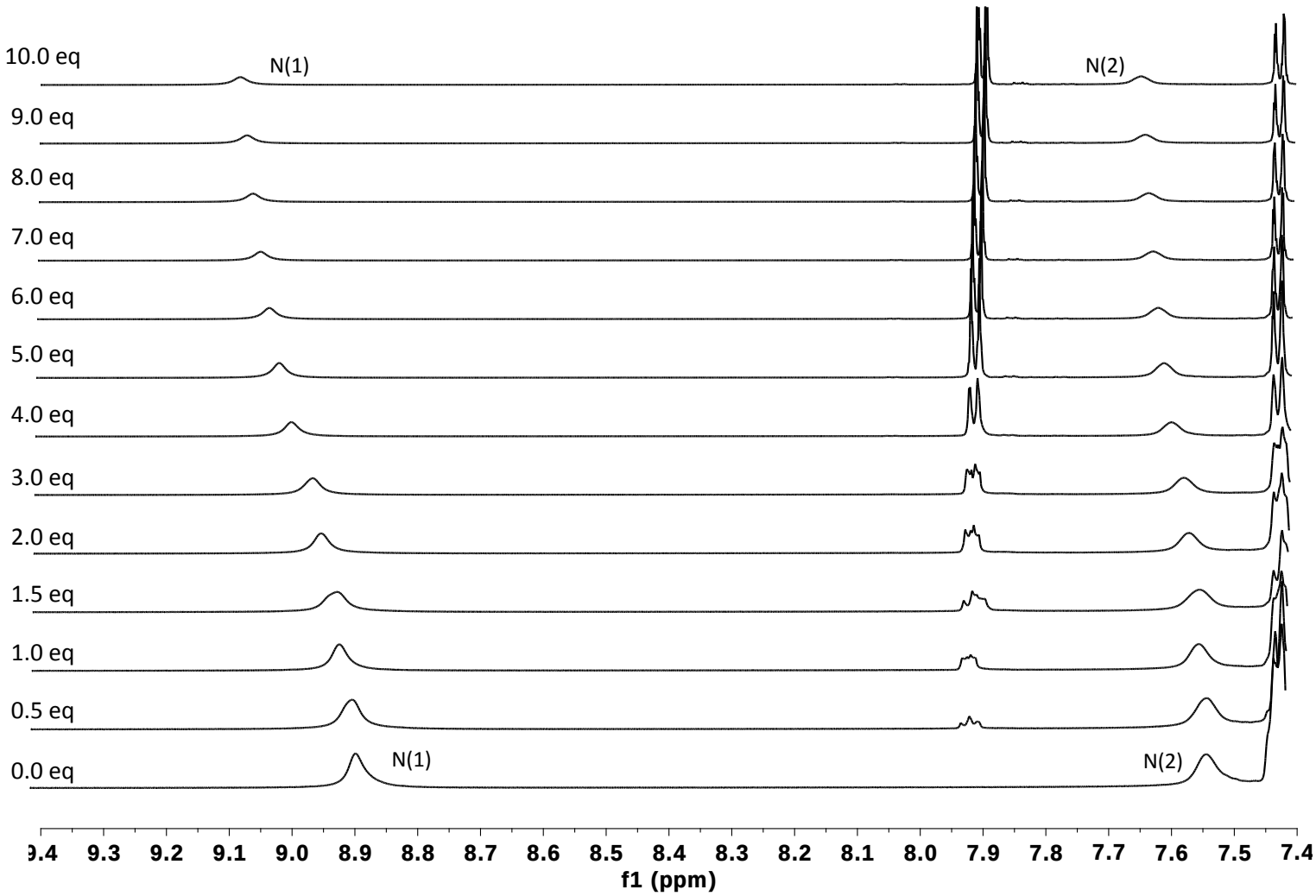


Fig. S16. ^{1}H-NMR titration of model compound **3** with mephedrone in acetone-d_6 (expansion).

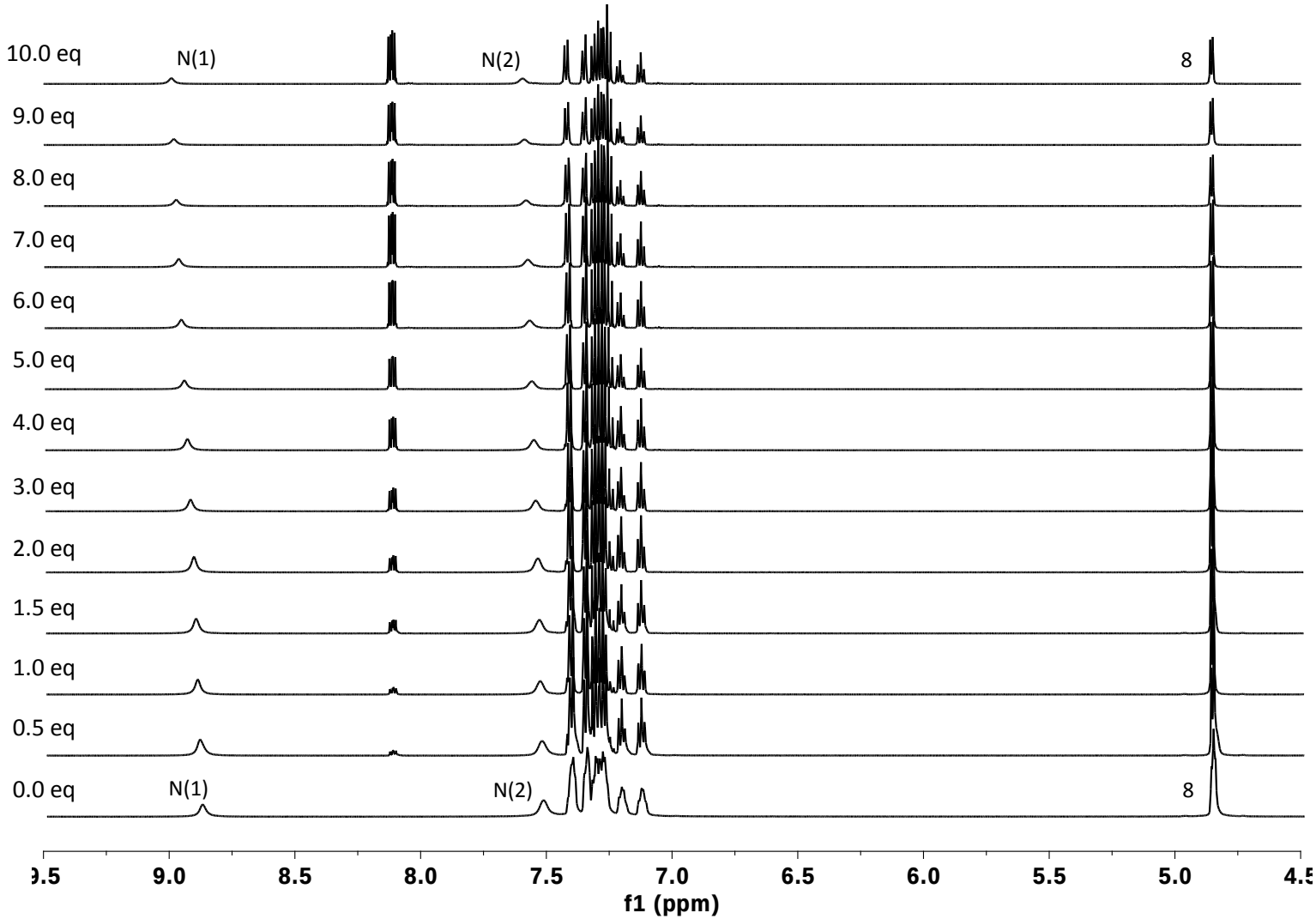


Fig. S17. ^{1}H-NMR titration of model compound **3** with flephedrone in acetone-d_6.

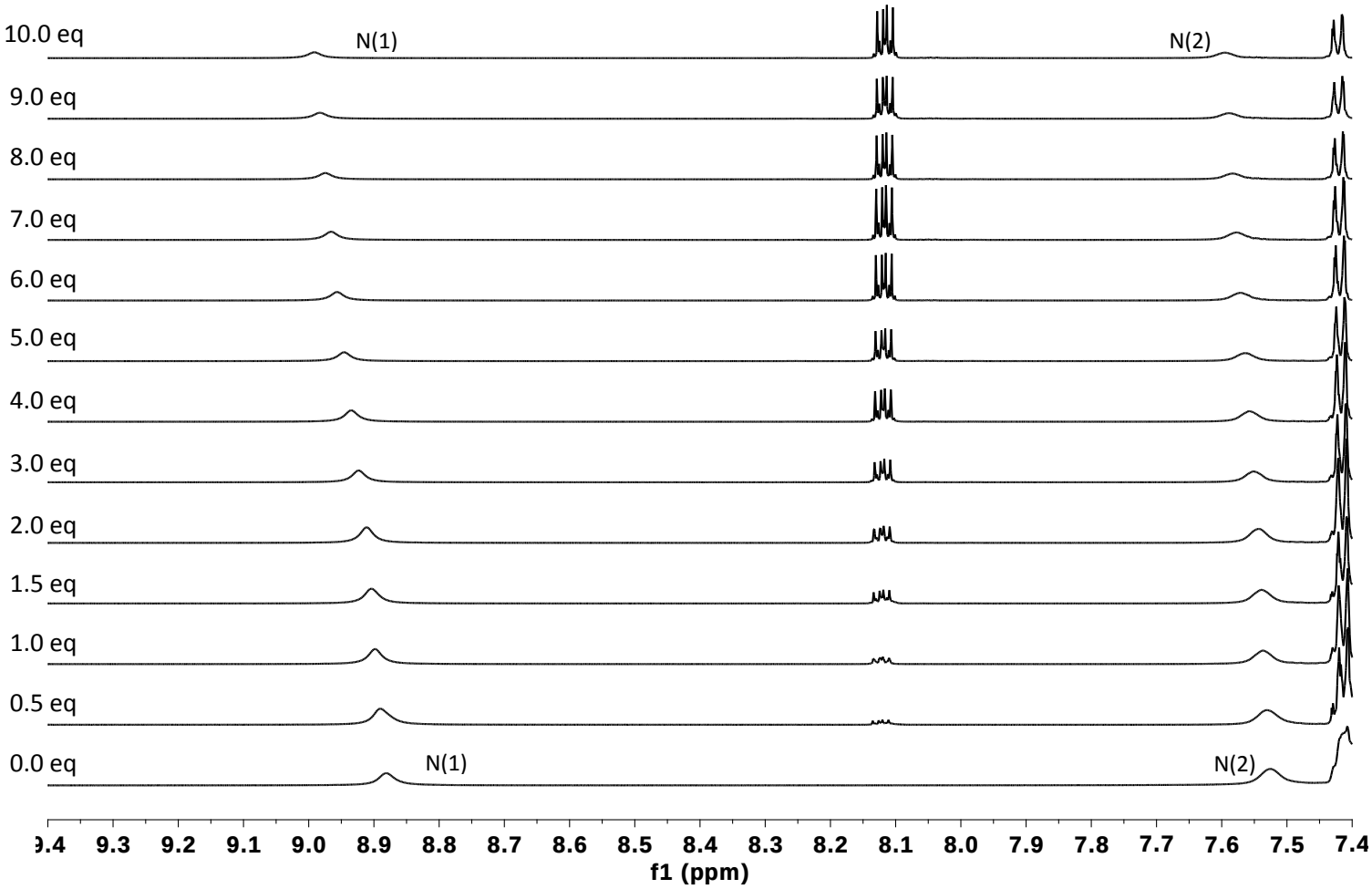


Fig. S18. ^{1}H-NMR titration of model compound **3** with flephedrone in acetone-d_6 (expansion).

Mass Spectrometry Studies

Samples were prepared in HPLC grade acetone at 0.5 mg·mL^{-1}. For analysis of the complexed sample, 10 equivalents of mephedrone freebase were added to a 0.5 mg·mL^{-1} solution of probe **2** in acetone. Formic acid (1% v/v) was added to each sample as an ionizing agent before injection. To ensure that a probe **2**-drug adduct is not an artifact, deuterated water was added to the sample to show an increase in the mass due to the exchange of the labile protons. Data was acquired in both the positive and negative ion mode. Needle voltage was 5000 V, drying gas temperature was 300°C, and nebulizer pressure was 24.6 psi.

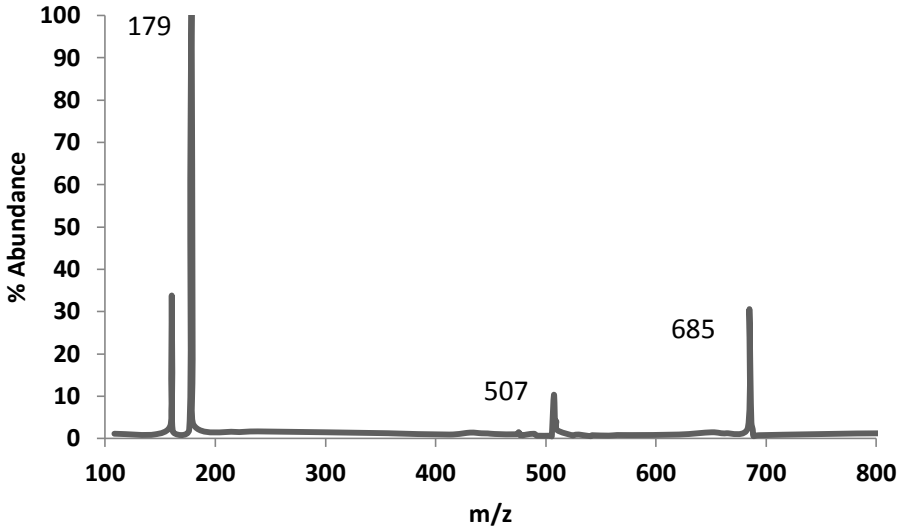


Fig. S19. ESI-MS (+ve mode) of probe **2** and mephedrone.

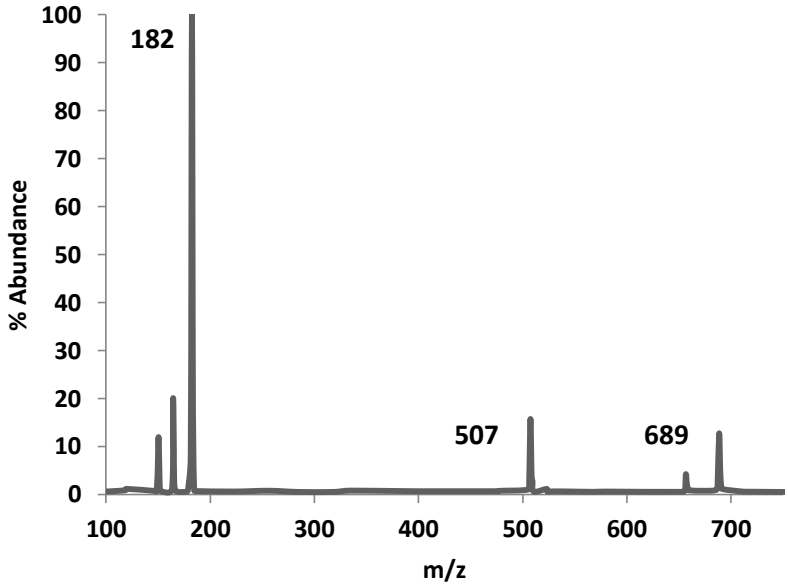


Fig. S20. ESI-MS (+ve mode) of probe **2** and flephedrone.

Computational DFT Studies

Conformational search was carried out for probe **2** alone and the Probe **2**-guest complexes using Hyperchem 8.10 and OPLS force field.[4] Five lowest energy unique structures/complexes were subjected to PM7 calculations using MOPAC2012.[5] The lowest energy complexes for each NPS with probe **2** was optimized at the DFT level using B3LYP 6-311++G(2d,2p)[6] basis set in Orca[7] (version 3.0.3), and that was followed by generating and optimizing alternative complexes for comparison, i.e. to ensure that the minimum conformation of both drugs was in fact achieved. The mephedrone and flephedrone were studied in their respective binding positions, i.e. mephedrone was positioned to bind to probe **2** outside of the binding pocket and vice versa. The interaction energy between probe **2** and guest was calculated using energies of respective most stable conformations according to the following:

$$\Delta E = E_{complex} - (E_{free\ \mathbf{2}} + E_{free\ guest})$$

where $E_{complex}$, $E_{free\ \mathbf{2}}$ and $E_{free\ guest}$ represents the total energy of the complex, the free optimized **2**, and the free optimized guest energy, respectively.

Computational Electrostatic Diagrams

Each of the electrostatic surfaces were calculated using VegaZZ using the minimized conformations previously calculated in the DFT studies.[7] Solid gradient surfaces were applied based on a four colour gradient, where red denotes low electrostatic potential and blue high electrostatic potential. The marching cubes method was implemented for surface calculations, which is based on surface facet approximation.

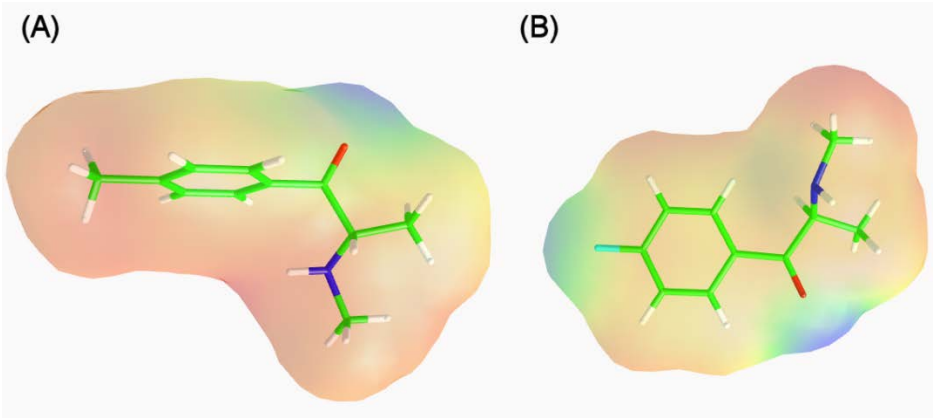


Fig. S21. Electrostatic surfaces potential for the free drugs (A) mephedrone and (B) flephedrone.

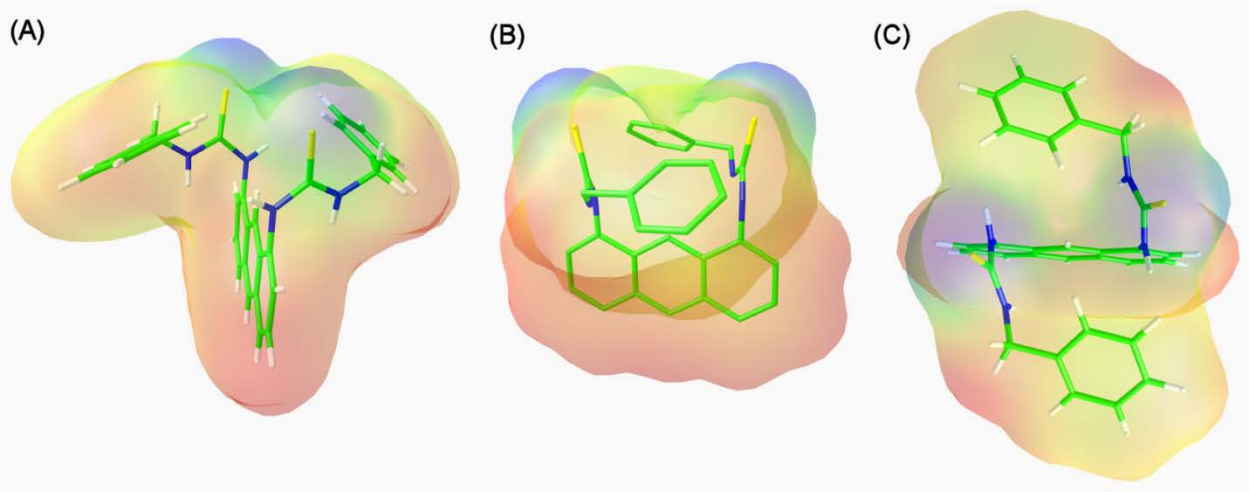


Fig. S2. Electrostatic surfaces potential for molecular probe (**2)** (lowest energy) highlighting different orientations (A) side on (B) front view and (C) top view.

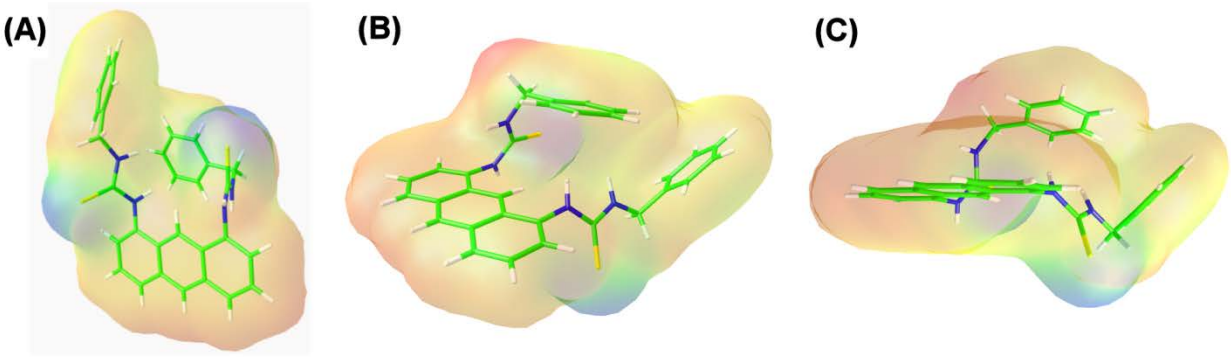


Fig. S23. Electrostatic surfaces potential for molecular probe **2** (second lowest energy), different views.

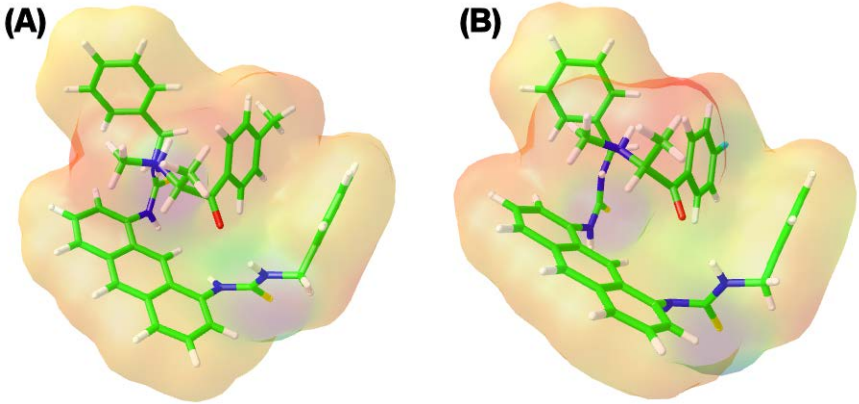


Fig. S24. Electrostatic surfaces potential for molecular probe **2** complexed with (A) mephedrone (B) flephedrone.

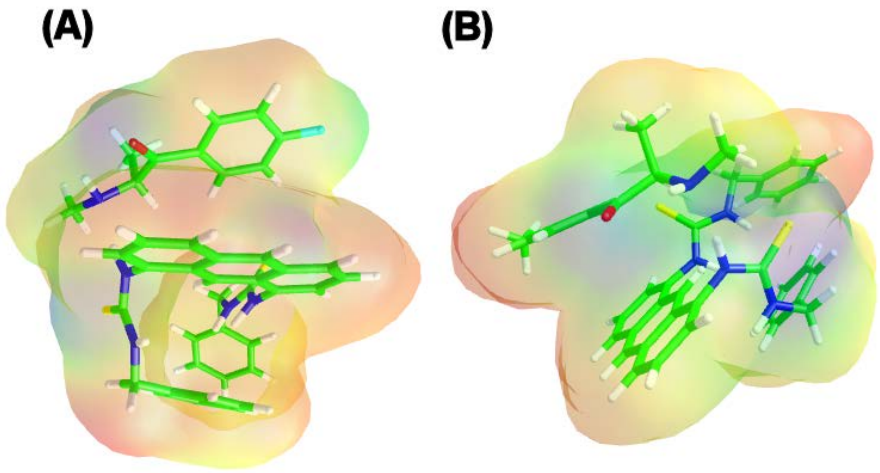


Fig. S25. Electrostatic surfaces potential for molecular probe (**2)** complexed with (A) flephedrone (B) mephedrone.

UV-Vis and Fluorescence Studies

A solution of probe **2**, 2.5 x 10^{-4} M, was prepared in HPLC grade acetone. An additional solution containing 0.01 M mephedrone freebase was prepared and aliquots of 25 µL (25 µL = 0.25 equivalence of mephedrone freebase to probe **2**) were added, spectra was recorded after each addition.

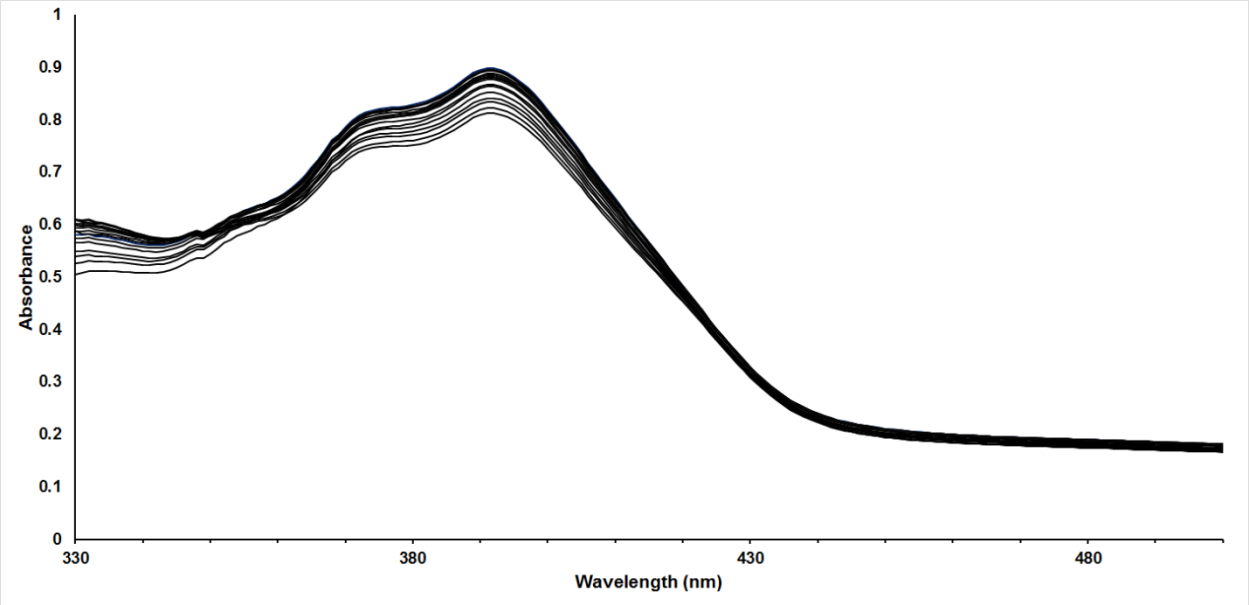


Fig. S26. UV-Vis titration of probe **2** plus mephedrone (2.5 x 10^{-6} M, acetone)

A solution of probe **2**, 5 x 10^{-6} M, was prepared in HPLC grade acetone. An additional solution containing 5 x 10^{-6} M probe **2** and 2.5 x 10^{-4} M mephedrone freebase was prepared and aliquots of 10 µL (10 µL = 0.1 equivalence of mephedrone freebase to probe 1) were added, spectra was recorded after each addition. Neat fluorescence titration involved adding the neat mephedrone and flephedrone free base in 5 µL aliquots and the emission spectra was recorded after each addition. The excitation wavelength was at 410 nm.

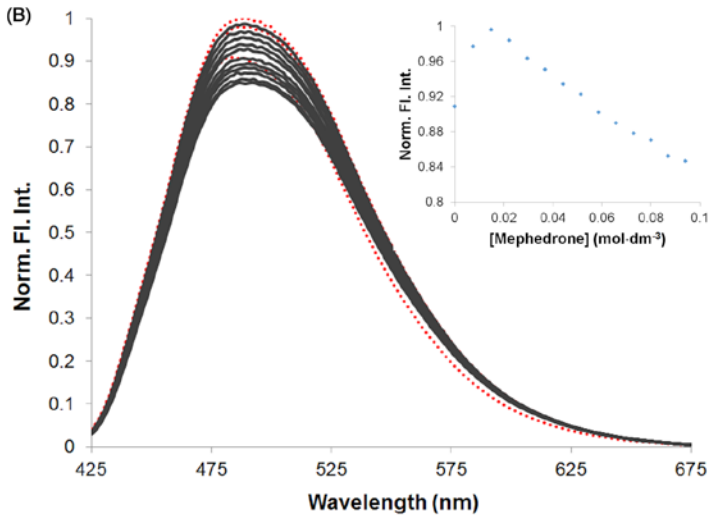
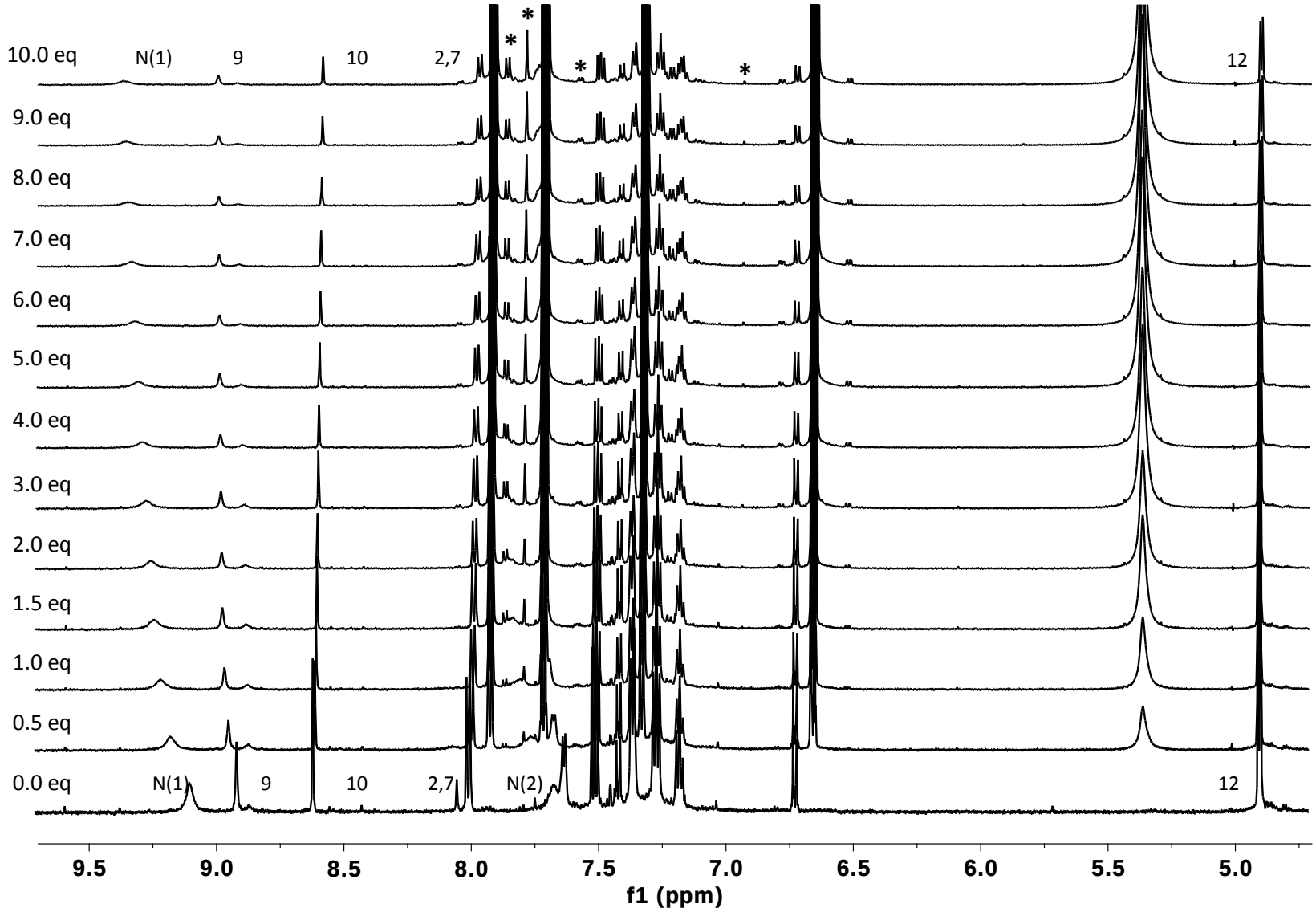


Fig. S27. Fluorescence titration of probe **2** plus neat mephedrone (5 x 10^{-6} M, acetone, λ_{ex} = 410 nm), red lines indicate mephedrone only and the first aliquot.

Simulated Street Protocol Studies: When found in street samples, mephedrone is found in combination with any number of cutting agents, the two most common being caffeine and benzocaine.[8] It has already been shown that caffeine and benzocaine do not bind to the sensor, but to ensure mephedrone can be detected in such mixtures a protocol was established to freebase mephedrone in the presence of benzocaine and caffeine.

Mephedrone hydrochloride, benzocaine and caffeine were combined in equal proportions and taken up in water. The mixture was filtered to remove the undissolved cutting agents; as caffeine is only sparingly soluble in water compared to benzocaine and mephedrone hydrochloride. The mephedrone freebase was liberated with ammonium hydroxide (pH~10) and extracted into diethyl ether. The organic layer was washed multiple times with water to ensure there were no residual anions present. The organic layer was dried over $MgSO_4$ and evaporated to dryness yielding a yellow oil with small amounts of white solid from the remaining cutting agents. The ^{1}H-NMR of the mixture shows that all the compounds are still present; however, larger proportions of mephedrone are present compared to the caffeine and benzocaine. NMR titration of probe **2** against the extracted street sample mixture shows that mephedrone is still binding in the presence of the caffeine and benzocaine.



FigS28. ^{1}H-NMR titration of simulated street sample (contains mephedrone, benzocaine, and caffeine) in 19.7 mM of probe **2** in acetone-d_6.

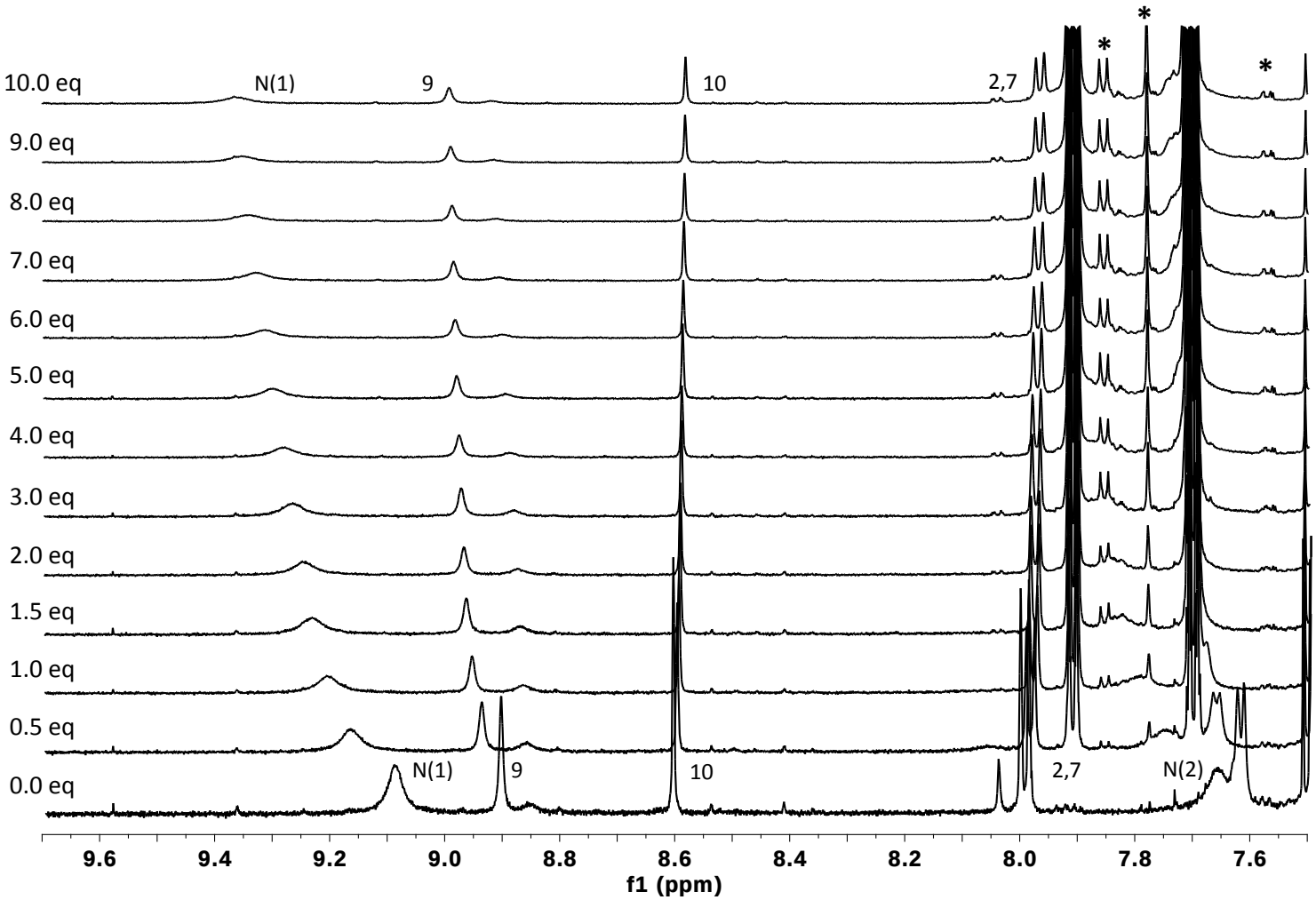


Fig S29: Simulated street sample (contains mephedrone, benzocaine, and caffeine) titration in 19.7 mM of probe **2** in acetone-d_6 (expansion).

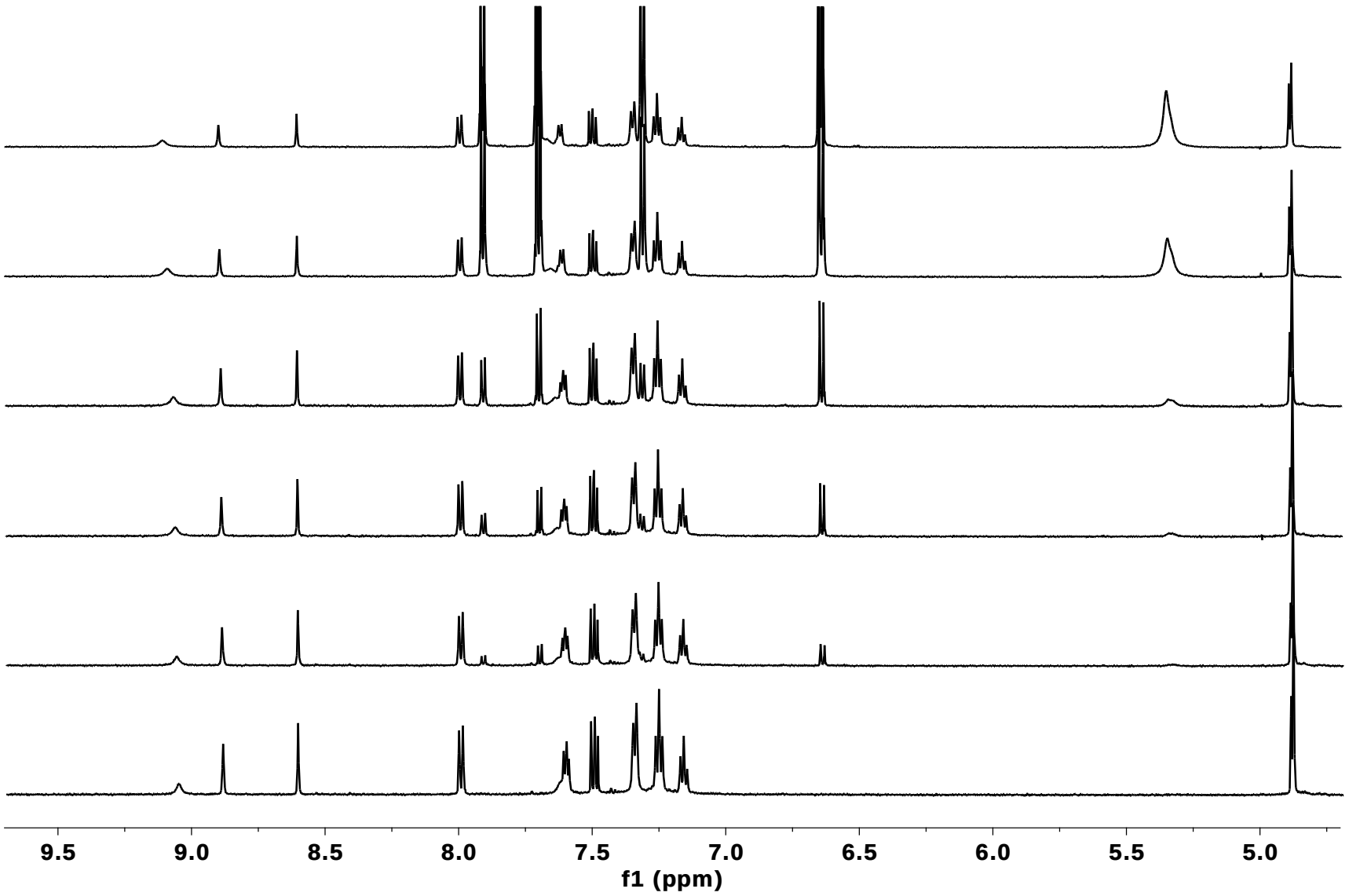


Fig S30. Simulated street titration (mephedrone and benzocaine only) in 19.7 mM of probe **2** in acetone-d_6.

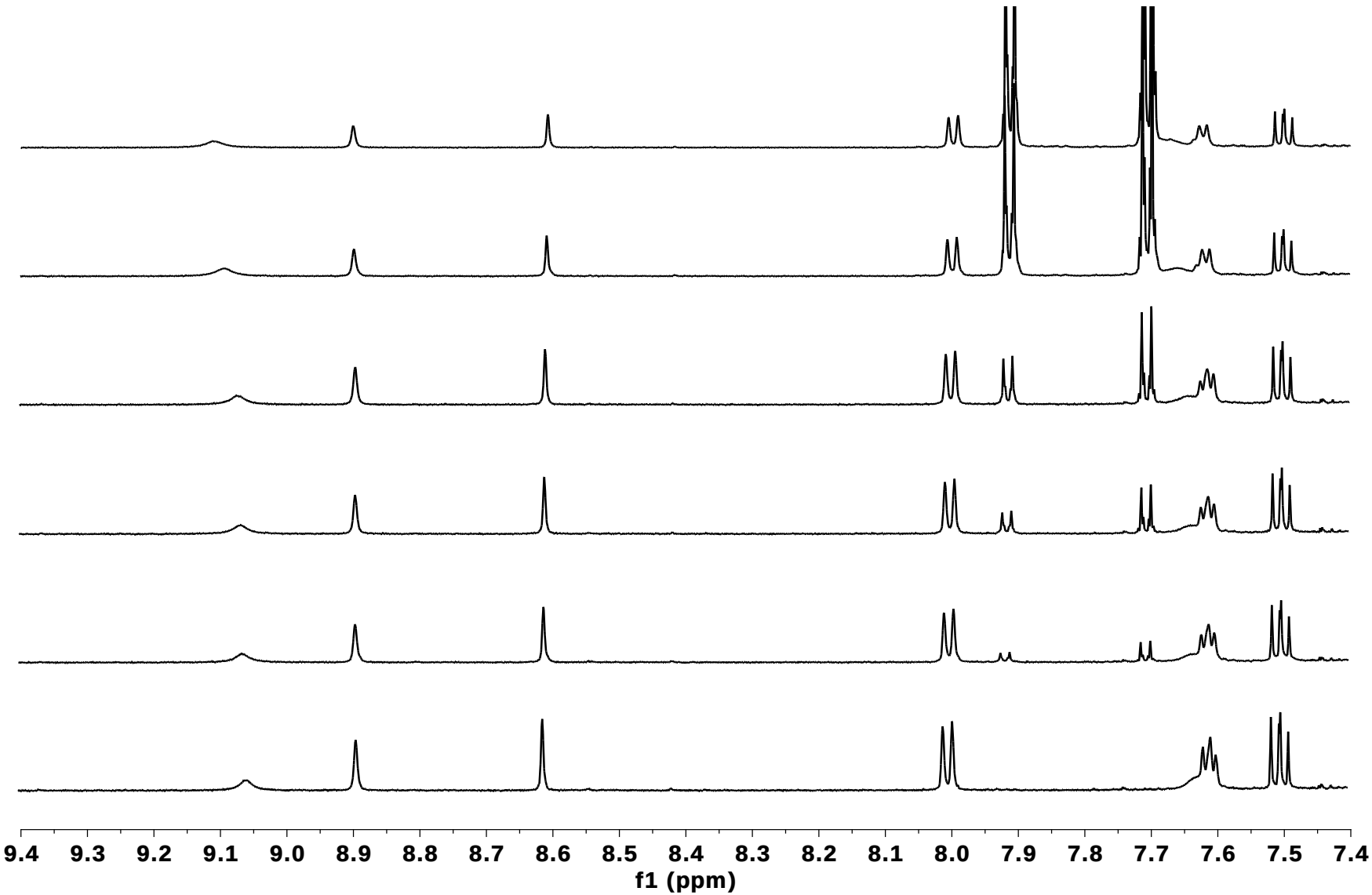


Fig S31: Simulated street titration (mephedrone and benzocaine only) in 19.7 mM of probe **2** in acetone-d_6 (expansion).

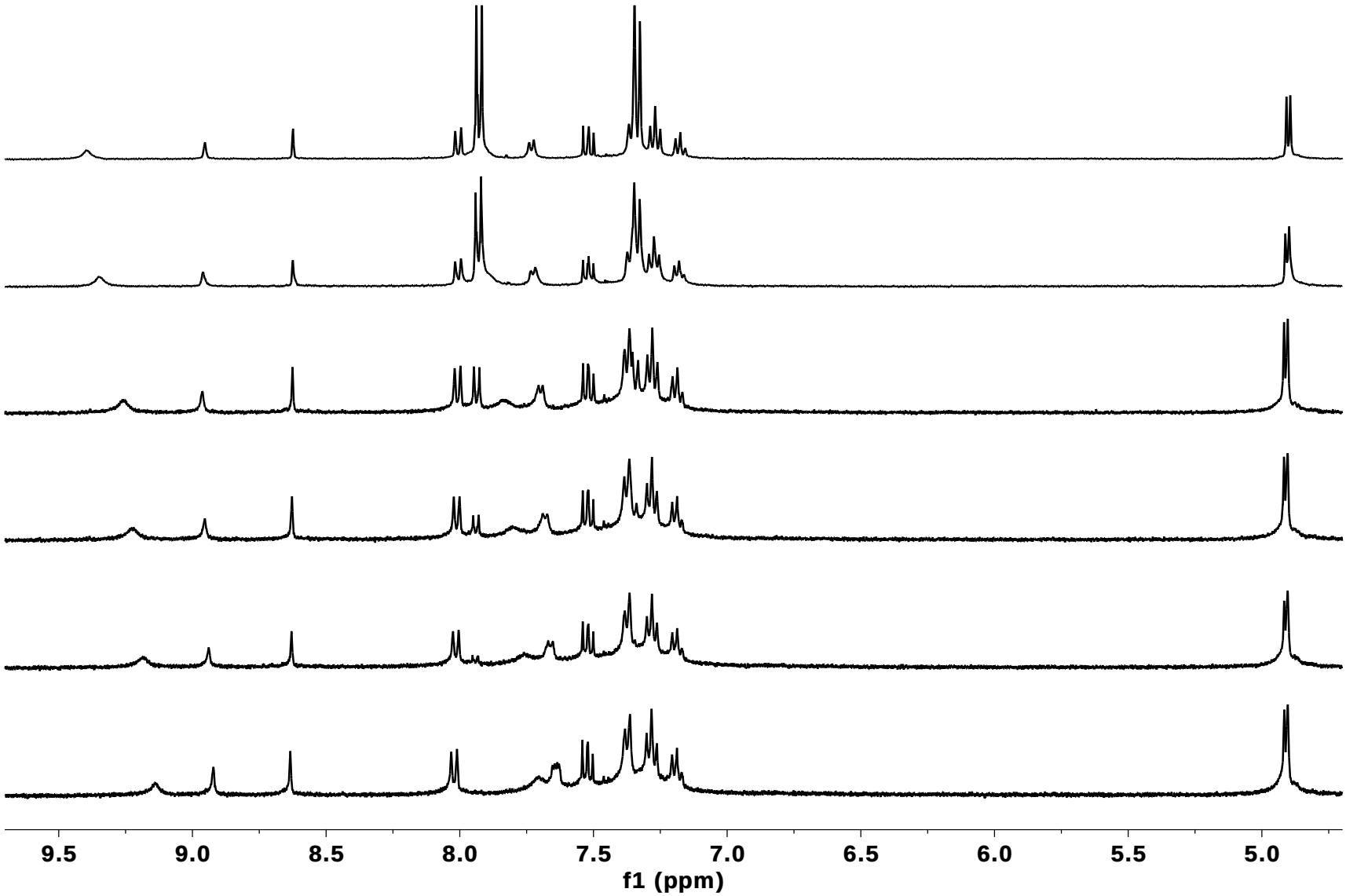
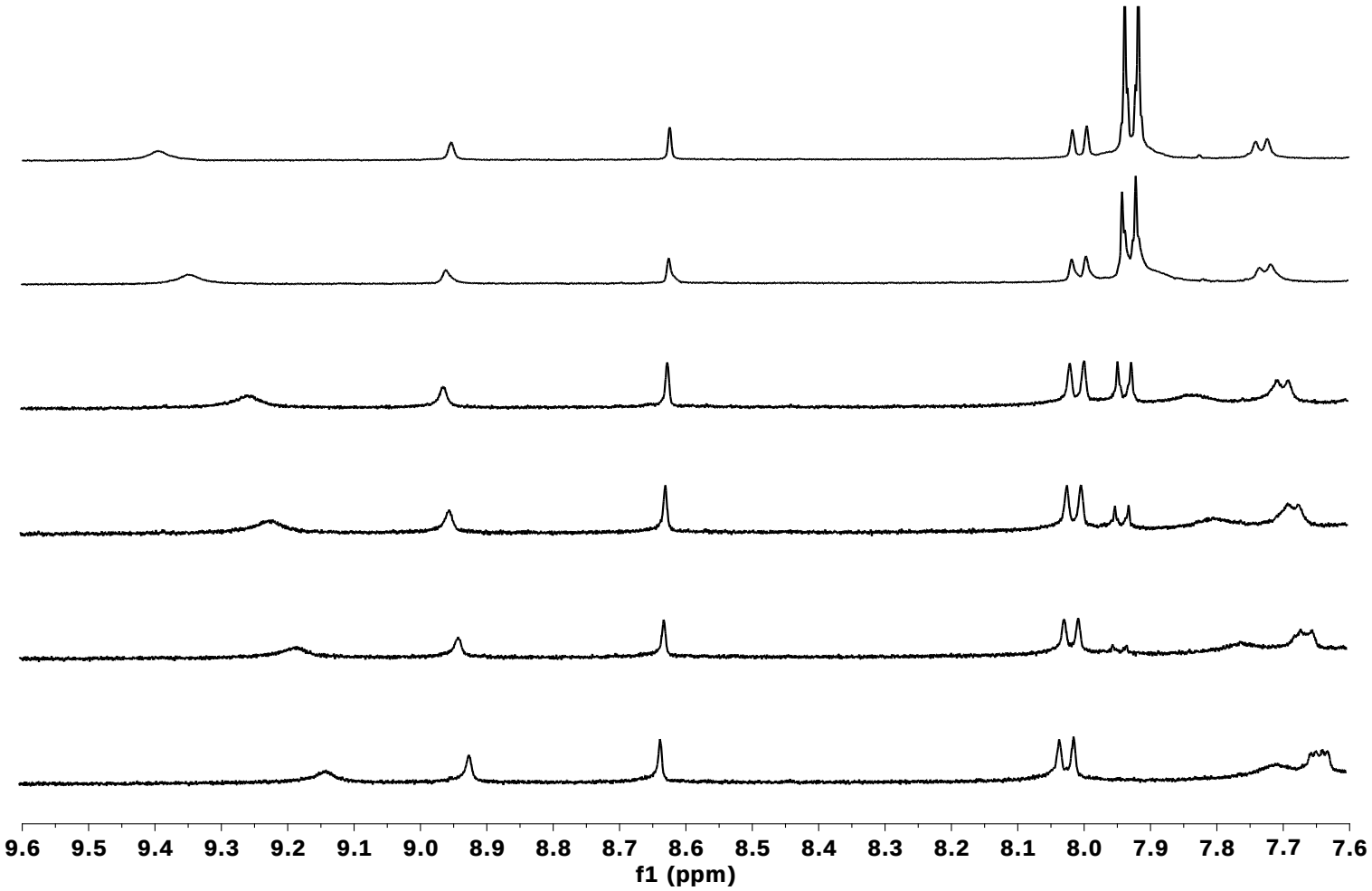


Fig S32: Simulated street titration (mephedrone and caffeine only) in 19.7 mM of probe **2** in acetone-d_6.



FigS33: Simulated street titration (mephedrone and caffeine only) in 19.7 mM of probe **2** in acetone-d_6 (expansion).

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