Supporting Information

Discovery of Small-Molecule Stabilizers of 14-3-3 Protein–Protein Interactions via Dynamic Combinatorial Chemistry

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Experimental

Materials and methods

Chemicals were purchased from commercial suppliers and used without pretreatment. Solvents used for the experiments were reagent-grade and dried, if necessary, according to standard procedures. The reactions were performed under nitrogen atmosphere, unless otherwise stated. The yields were calculated for the analytically pure compounds and were not optimized. The purifications were performed using column chromatography with Macherey-Nagel Silica 60 M 0.04–0.063 mm. Preparative high-performance liquid chromatography (HPLC, Ultimate 3000 UHPLC+ focused, Thermo Scientific) purification was performed on a reversed-phase column (C18 column, 5 µm, Macherey-Nagel, Germany). The solvents used for the chromatography were water (0.1% formic acid) and MeCN (0.1% formic acid), or EtOAc and DCM. ^{1}H-, ^{13}C and ^{31}P-NMR spectra were measured on a Bruker Fourier 500 spectrometer (500, 126 or 202 MHz, respectively). The chemical shifts were reported in parts per million (ppm) relative to the corresponding solvent peak. The coupling constants of the splitting patterns were reported in Hz and were indicated as singlet (s), doublet (d), triplet (t) and multiplet (m). Due to the presence of isomers for acylhydrazones, some of the signals are doubled. UPLC-MS and HRMS measurements were performed using Thermo Scientific systems.

DCC conditions

The corresponding hydrazides (each 3 µL, stock solutions 100 mM in DMSO) and the aldehydes (each 1 µL, stock solutions 100 mM in DMSO) were added to MES buffer (0.1 M, 2 mM $MgCl_2$, pH 6.5). Aniline (10 µL, stock solution 1 mM), synaptopodin (10 µL, stock solution 1 mM) and 14-3-3(ζ) (5.35 µL, stock solution 1.87 mM) were added accordingly. DMSO was added to reach a final concentration of DMSO in the DCL of 5%. The end-volume was 1 mL. Final concentrations in the DCLs are shown in Table S1. The reactions were performed in 1.5 mL Eppendorf cups and stirred on a Rotating Mixer (Benchmark Scientific). The DCL was left shaking at room temperature and was frequently monitored *via* UPLC-MS. To prepare samples for UPLC-MS analysis, 10 µL of the corresponding library was withdrawn and diluted in 90 µL acetonitrile, the pH was raised to pH > 8 by adding 2 µL NaOH (1.0 M) to freeze the reaction. The mixture was centrifuged at 10,000 rpm for 2 min, and the supernatant was analyzed *via* UPLC-MS.

Table S1. Final concentrations in the DCLs.

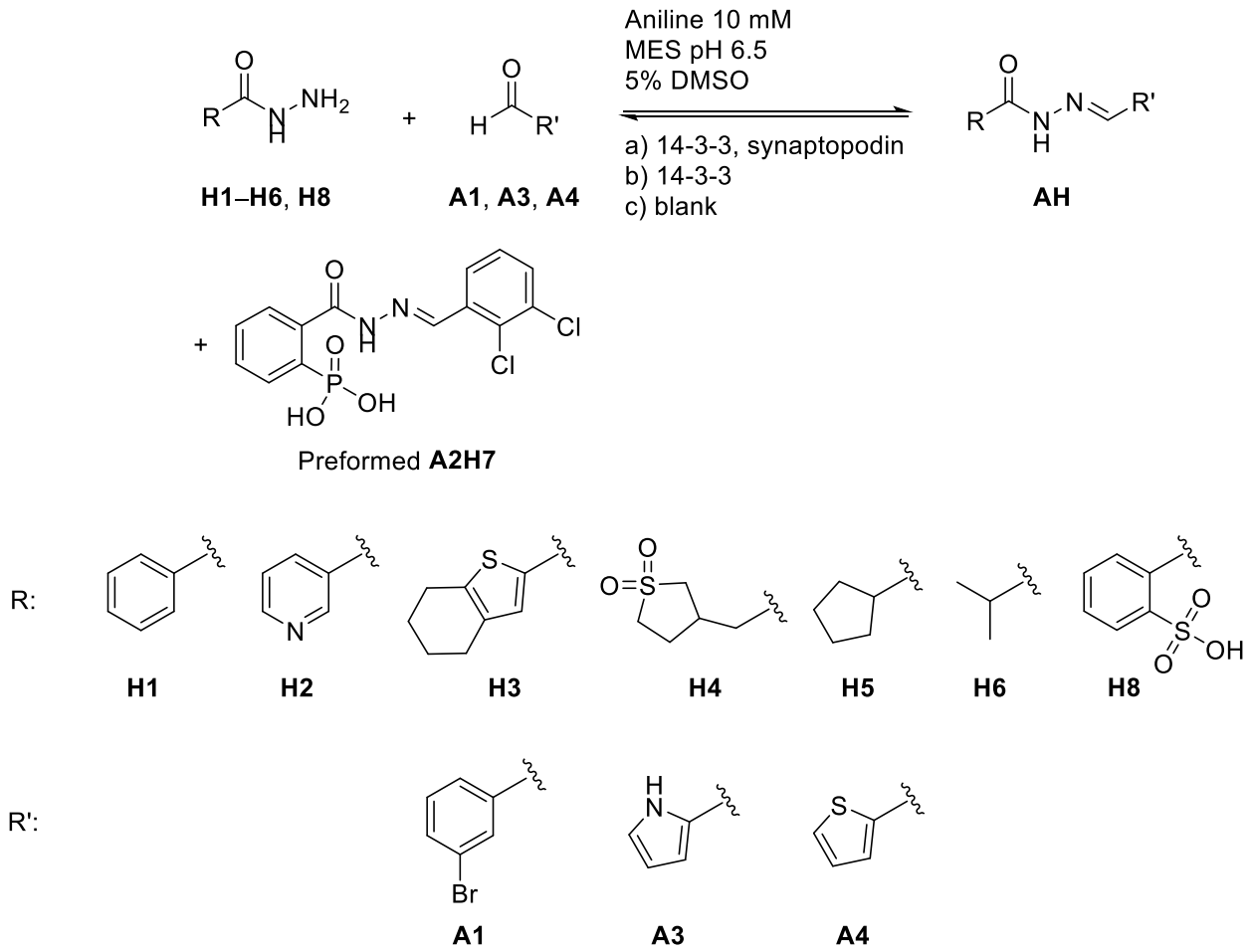
	14-3-3/synaptopodin	14-3-3	Blank
DMSO	5%	5%	5%
Aniline	10 mM	10 mM	10 mM
Aldehyde	100 µM (each)	100 µM (each)	100 µM (each)
Hydrazide	300 µM (each)	300 µM (each)	300 µM (each)
Protein	10 µM	10 µM	-
Synaptopodin	10 µM	-	-

UPLC-MS analysis of DCC

UPLC-MS was carried out on a ThermoScientific Dionex Ultimate 3000 UHPLC System coupled to a ThermoScientific Q Exactive Focus with an electrospray ion source. An Acquity Waters Column (BEH, C8 1.7 µm, 2.1 x 150 mm, Waters, Germany) equipped with a VanGuard Pre-Column (BEH C8, 5 x 2.1 mm, 1,7 µm, Waters, Germany) was used for separation. At a flow rate of 0.250 mL/min, the gradient of H_2O (0.1% formic acid) and acetonitrile (0.1% formic acid) was held at 5% acetonitrile for 1 min and then increased to 95% over 16 min. It was held there for 1.5 min before the gradient was decreased to 5% over 0.1 min where it was held for 1.9 min. The mass spectrum was measured in positive mode in a range from 100 – 700 m/z.

HRMS analysis

High-resolution mass spectra were recorded with a ThermoScientific system where a Dionex Ultimate 3000 RSLC was coupled to a Q Exactive Focus mass spectrometer with an electrospray ion source. An Acquity UPLC® BEH C8, 150 x 2.1 mm, 1.7 µm column equipped with a VanGuard Pre-Column BEH C8, 5 x 2.1 mm, 1,7 µm (Waters, Germany) was used for separation. At a flow rate of 250 µL/min, the gradient of H2O (0.1% FA) and ACN (0.1% FA) was held at 10% B for 1 min and then increased to 95% B over 4 min. It was held there for 1.2 min before the gradient was decreased to 10% B over 0.3 min where it was held for 1 min. The mass spectrum was measured in positive mode in a range from 120 – 1000 m/z. UV spectrum was recorded at 254 nm.



Scheme S1. Original dynamic combinatorial library (DCL), from which not all products could be observed. Aldehydes (100 μM each), hydrazides (100 μM each) and preformed **A2H7** (compound **2**) (100 μM), DMSO (5%), aniline (10 mM), and a) blank without protein; b) library with 14-3-3(ζ) (10 μM). See Figure S1 for the chromatograms.

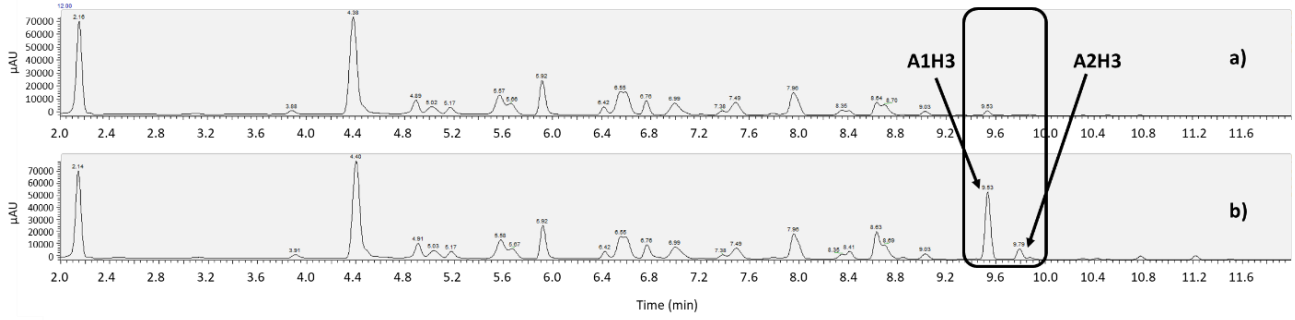
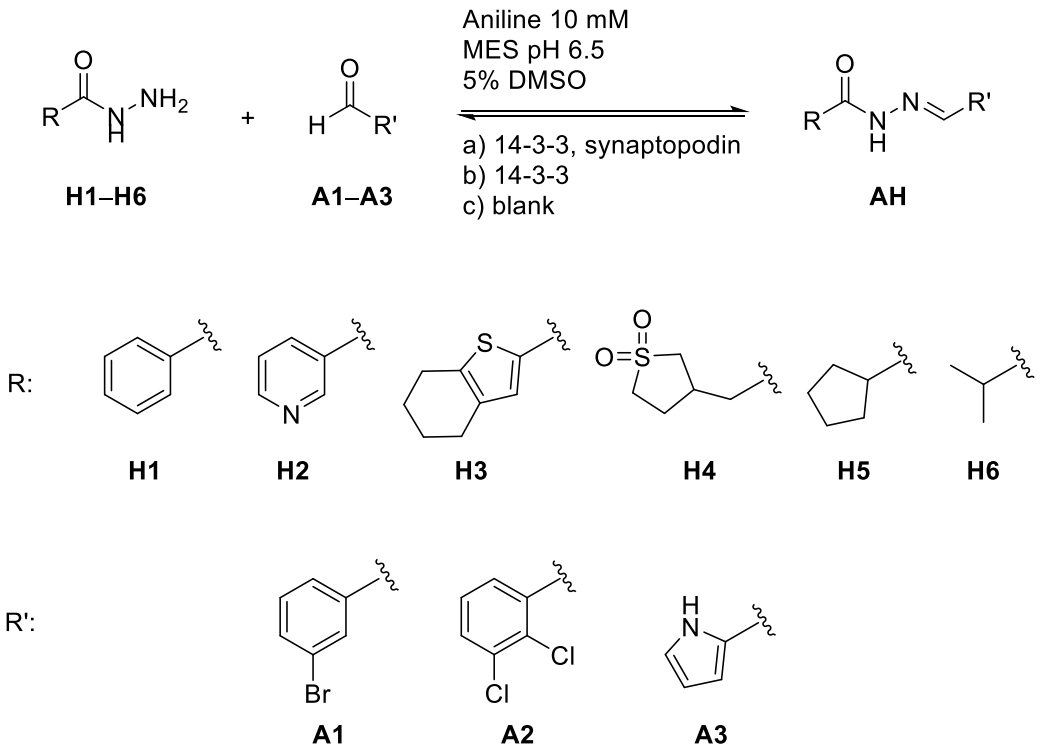


Figure S1. UV-chromatograms at 290 nm of dynamic combinatorial libraries of a) blank without protein; and b) library with 14-3-3(ζ) (10 μM). Data obtained from single experiment.

Products with **H7** and **H8** could not all be observed, however **A1H3** and **A2H3** showed amplification in protein compared to blank. Therefore, we modified the DCL and omitted these two troublesome compounds, resulting in the DCL shown in Scheme S2.



Scheme S2. Adapted dynamic combinatorial library with aldehydes (100 μM each), hydrazides (300 μM each), DMSO (5%), aniline (10 mM) and: a) 14-3-3(ζ) (10 μM) and synaptopodin (10 μM); b) control with 14-3-3(ζ) (10 μM); and c) control without protein or synaptopodin. See figure S2 for the chromatograms in duplicate.

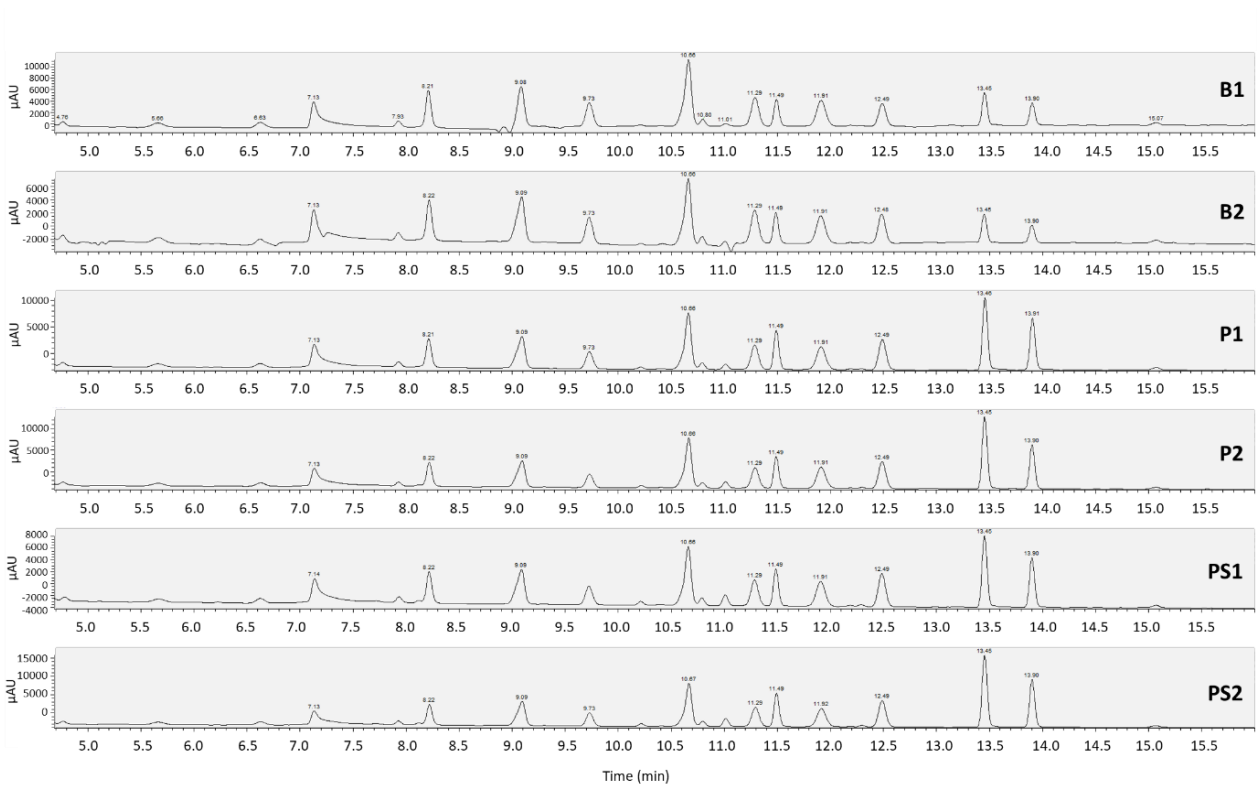


Figure S2. UV-chromatograms at 290 nm of dynamic combinatorial libraries in duplicate, blank (B), protein (P) and protein plus synaptopodin (PS) at 6 h.

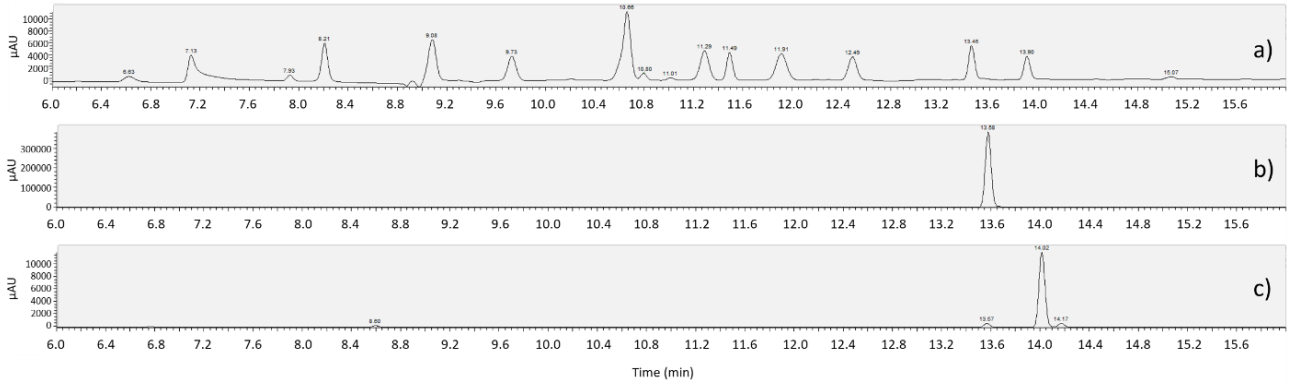


Figure S3. UV-chromatograms at 290 nm of a) the blank DCL at 6 h, b) synthesized **A1H3**, and c) synthesized **A2H3**. The synthesized compounds both have a bit longer retention time (0.12 min) due to being dissolved in pure MeCN, whereas the DCL also contains water and DMSO. Data obtained from single experiments.

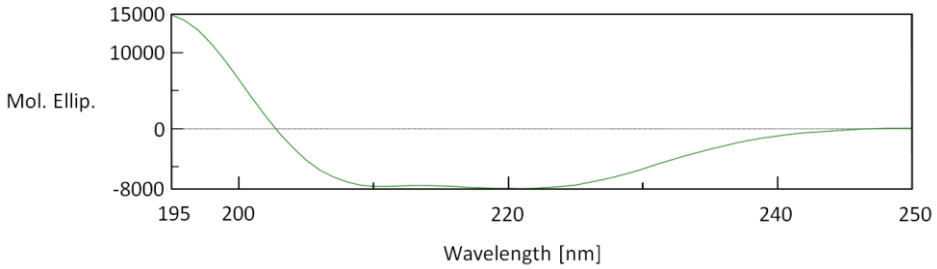


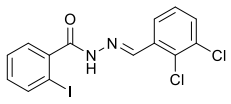
Figure S4. UV CD spectra of 14-3-3(ζ) 1 µM protein in Milli-Q water. Measured on a J-1500 CD Spectrometer (Jasco). In agreement with Ghosh et al.[5] Data obtained from single experiment.

Synthesis

General procedure for acylhydrazone formation[1]

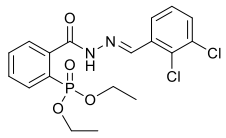
To the hydrazide (1 eq.) dissolved in MeOH, the corresponding aldehyde (1.2 eq.) was added. The reaction mixture was stirred at room temperature or refluxed until completion. After cooling to room temperature, the reaction mixture was concentrated *in vacuo*. Purification was performed by column chromatography, affording the corresponding acylhydrazone in 60 – 99% yield.

(*E*/*Z*)-*N*'-(2,3-Dichlorobenzylidene)-2-iodobenzohydrazide (3)



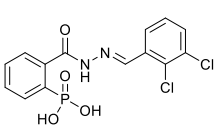
The acylhydrazone was synthesized at room temperature following the general procedure, using 2-iodobenzohydrazide (1.00 g, 3.82 mmol) in MeOH (7.6 mL) and 2,3-dichlorobenzaldehyde (0.607 g, 3.47 mmol). After purification by column chromatography (DCM/EtOAc 1:0 → 0:1), the acylhydrazone **3** was obtained as a mixture of *E* and *Z* isomers (*E*:*Z* = 3:2) as a white solid (866 mg, 60%). ^{1}H-NMR (500 MHz, DMSO-d_6) δ= 12.22 – 12.15 (m, 1H), 8.73 – 8.49 (m, 1H), 8.07 – 7.14 (m, 7H); ^{13}C-NMR (126 MHz, DMSO-d_6) (combined peaks of *E*/*Z*) δ= 171.0, 165.1, 143.6, 141.9, 140.9, 139.8, 139.3, 138.4, 133.8, 133.7, 132.4, 131.8, 131.6, 131.4, 131.1, 130.8, 130.6, 128.6, 128.6, 128.5, 128.3, 128.2, 127.8, 125.6, 124.9, 94.0, 93.6; HRMS (ESI) calcd for $C_{14}H_{10}Cl_2IN_2O$ [*M*+H]$^+$: 418.9215, found 418.9190.

Diethyl (*E*/*Z*)-(2-(2-(2,3-dichlorobenzylidene)hydrazine-1-carbonyl)phenyl)phosphonate (4)



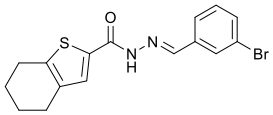
The phosphonic ester was synthesized according to an adapted protocol of Benin *et al.*[2] Triethyl phosphite (1.3 mL, 7.6 mmol) was added to [$PdCl_2$] (16.9 mg, 0.10 mmol) under N_2 atmosphere and heated at 150 °C for 3 h. The mixture was cooled to 100 °C, and compound **3** (400 mg, 0.95 mmol) was added. The reaction mixture was stirred for two days until complete conversion was observed. Water and DCM were added after the mixture had cooled down to room temperature. The organic layer was washed with water (3 x 5 mL), a saturated aqueous solution of $NaHCO_3$ (1 x), dried over $MgSO_4$, filtered, and the solvent was evaporated *in vacuo*. The crude material was purified by column chromatography (SiO_2; DCM/EtOAc 3:1 → 0:1). The product **4** was obtained as a mixture of *E* and *Z* isomers (*E*:*Z* = 3:2) as a colorless oil (113 mg, 28%). ^{1}H-NMR (500 MHz, DMSO-d_6) δ= 12.11 – 12.01 (m, 1H), 8.69 – 8.41 (m, 1H), 8.03 – 7.18 (m, 7H), 4.12 – 3.77 (m, 4H), 1.16 (dt, *J* = 53.0, 7.0 Hz, 6H); ^{13}C-NMR (126 MHz, DMSO-d_6) (combined peaks of *E*/*Z, and coupling with* ^{31}*P*) δ= 170.9, 170.9, 164.6, 164.6, 142.9, 140.2, 140.1, 139.3, 139.2, 138.7, 134.0, 133.8, 133.0, 133.0, 132.5, 132.4, 132.4, 132.4, 132.3, 132.0, 132.0, 131.7, 131.1, 131.0, 130.6, 129.8, 129.7, 128.8, 128.7, 128.6, 128.3, 128.2, 127.6, 127.5, 127.4, 126.1, 125.9, 125.5, 124.7, 124.6, 62.0, 61.9, 61.7, 61.6, 16.1, 16.0, 15.9, 15.9; ^{31}P-NMR (202 MHz, DMSO-d_6) δ= 15.55 (dd, *J* = 45.1, 4.5 Hz); HRMS (ESI) calcd for $C_{18}H_{20}Cl_2N_2O_4P$ [*M*+H]$^+$: 429.0538, found 429.0516.

(*E*/*Z*)-(2-(2-(2,3-Dichlorobenzylidene)hydrazine-1-carbonyl)phenyl)phosphonic acid (2)



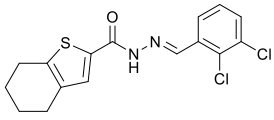
The phosphonic acid was obtained by deprotection of the ester by TMSBr. Compound **4** (93 mg, 0.2 mmol) was dissolved in dry MeCN (2.2 mL) under N_2 atmosphere. To this mixture, was added TMSBr (143 µL, 1.08 mmol), and the reaction mixture was stirred at 35 °C for 3 h. The solvent was evaporated *in vacuo*, water/THF (1:1) was added, and the mixture was stirred at room temperature for 1 h. The crude mixture was lyophilized and purified by reversed-phase prep-HPLC (water (0.1% formic acid)/MeCN (0.1% formic acid), 9:1 → 1:9). Product **2** was obtained as a mixture of *E* and *Z* isomers (*E*:*Z* = 7:3) as a white solid (21.2 mg, 24%). ^{1}H-NMR (500 MHz, DMSO-d$_6$) δ= 12.33 (s, 1H), 11.94 (s, 1H), 8.68 – 8.37 (m, 1H), 8.05 – 7.18 (m, 7H); ^{13}C-NMR (126 MHz, DMSO-d$_6$) δ= 171.6, 168.0, 142.9, 138.2, 137.6, 134.0 (d, *J* = 11.0 Hz), 132.4, 132.2, 131.6, 130.9 (d, *J* = 16.6 Hz), 129.7 (d, *J* = 12.6 Hz), 128.5, 128.2, 125.4; ^{31}P-NMR (202 MHz, DMSO-d$_6$) δ= 10.6 (s); HRMS (ESI) calcd for $C_{14}H_{12}Cl_2N_2O_4P$ [*M*+H]$^+$: 372.9912, found 372.9902.

(*E*/*Z*)-*N*'-(3-Bromobenzylidene)-4,5,6,7-tetrahydrobenzo[b]thiophene-2-carbohydrazide (A1H3)



The acylhydrazone was synthesized following the general procedure using 4,5,6,7-tetrahydro-1-benzothiophene-2-carbohydrazide (43.5 mg, 0.22 mmol) in MeOH (1.6 mL) and 3-bromobenzaldehyde (40 µL, 0.34 mmol) and heating to reflux. After purification *via* column chromatography (DCM/EtOAc, 9:1 → 1:9), the acylhydrazone was obtained as a mixture of *E* and *Z* isomers (*E*:*Z* = 3:2) as a white solid (81 mg, quantitative). ^{1}H-NMR (500 MHz, $CDCl_3$) δ= 9.35 (s, 1H), 7.92 (s, 1H), 7.89 – 7.74 (m, 1H), 7.70 (d, *J* = 7.7 Hz, 1H), 7.53 (d, *J* = 8.0 Hz, 1H), 7.31 (t, *J* = 7.8 Hz, 1H), 2.86 (t, *J* = 5.8 Hz, 2H), 2.67 (t, *J* = 5.8 Hz, 2H), 1.92 – 1.79 (m, 4H); ^{1}H-NMR (500 MHz, DMSO-d$_6$) δ= 11.87 – 11.81 (m, 1H), 8.35 – 8.06 (m, 1H), 7.93 (s, 1H), 7.77 – 7.55 (m, 3H), 7.44 (t, *J* = 6.8 Hz, 1H), 2.78 (m, 2H), 2.61 (t, *J* = 5.9 Hz, 2H), 1.85 – 1.71 (m, 4H); ^{13}C-NMR (126 MHz, $CDCl_3$) δ=136.0, 133.2, 130.6, 130.5, 126.3, 123.1, 25.4, 25.4, 23.4, 22.8; HRMS (ESI) calcd for $C_{16}H_{16}BrN_2OS$ [*M*+H]$^+$: 363.0167, found 363.0158.

(*E*/*Z*)-*N*'-(2,3-Dichlorobenzylidene)-4,5,6,7-tetrahydrobenzo[b]thiophene-2-carbohydrazide (A2H3)



The acylhydrazone was synthesized following the general procedure using 4,5,6,7-tetrahydro-1-benzothiophene-2-carbohydrazide (34.4 mg, 0.18 mmol) in MeOH (1.6 mL) and 2,3-dichlorobenzaldehyde (27.8 mg, 0.16 mmol) and heating to reflux. After purification *via* column chromatography (DCM/EtOAc, 9:1 → 1:9), the acylhydrazone was obtained as a mixture of *E* and *Z* isomers (*E*:*Z* = 3:2) as a white solid (38.2 mg, 68%). ^{1}H-NMR (500 MHz, $CDCl_3$) δ= 8.97 (s, 1H), 8.27 (s, 1H), 8.10 (d, *J* = 7.9 Hz, 1H), 7.85 (s, 1H), 7.51 (dd, *J* = 7.9, 1.4 Hz, 1H), 7.31 (t, *J* = 7.9 Hz, 1H), 2.85 (t, *J* = 6.0 Hz, 2H), 2.66 (t, *J* = 6.0 Hz, 2H), 1.92 – 1.77 (m, 4H); ^{1}H-NMR (500 MHz, DMSO-d$_6$) δ= 12.03 – 11.95 (m, 1H), 8.82 – 8.52 (m, 1H), 8.05 – 7.96 (m, 1H), 7.73 – 7.61 (m, 2H), 7.47 (s, 1H), 2.78 (m, 2H), 2.61 (t, *J* = 5.9 Hz, 2H), 1.83 – 1.70 (m, 4H); ^{13}C-NMR (126 MHz, $CDCl_3$) δ=133.5, 132.5, 131.7, 127.8, 126.2, 25.5, 25.4, 23.4, 22.8; HRMS (ESI) calcd for $C_{16}H_{15}Cl_2N_2OS$ [*M*+H]$^+$: 353.0282, found 353.0274.

Protein expression and purification

14-3-3ζ was expressed according to the general protocol as published by Andrei *et al.*.[3]

Peptide synthesis and purification

Peptides based on the Ser137 phosphorylation site of Synaptopodin (Sequence QVGKELLRLAYpS137EPCGLRGALLDVCVEQG) were synthesized via Fmoc solid phase peptides synthesis (SPPS) on an Intavis MultiPep RSi peptide synthesizer. Peptide synthesis was performed on a 100 µmol scale on a Tentagel R RAM resin (Rapp Polymere). All reactions were performed in *N*-Methyl-2-Pyrrolidinone (NMP). Fmoc deprotection was performed in 20% (v/v) piperidine and acid activation in a solution of 1.2 M diisopropyl ethylamine (DIPEA) and 0.4 M (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU, 8-fold excess). The coupling reaction was subsequently performed twice for one hour in 0.5 M amino acid solution (10-fold excess) and followed by capping through acetylation (pyridine/acetic anhydride/DMF, 1/1/1, v/v/v). The last N-terminal amino acid was either capped through acetylation, or coupled to a β-alanine linker and fluorescein isothiocyanide (FITC). The final peptides were cleaved off the resin in trifluoracetic acid (TFA), H_2O, triisopropylsilane (TIS) and ethanedithiol (EDT) (92.5/2.5/2.5/2.5, v/v/v/v) for 5 hours at room temperature and precipitated in 20 mL diethyl ether (Et_2O) at -30 °C. The precipitated peptide suspension was stored at -30 °C for ten minutes, centrifuged at 2000 rpm and decanted. Twenty milliliters of fresh Et_2O at -30 °C was added, the suspension stored at -30 °C for another ten minutes and centrifuged again. The solvent was decanted and the remaining pellet dried in the open air. Peptides were then purified by mass triggered HPLC on an appropriate gradient of H_2O/acetonitrile + 0.1% TFA. The resulting peptide solutions were lyophilized and stored at -30 °C until further use.

Fluorescence polarization (FP) assay

The performed FP assays were based on the protocol that was reported by Andrei *et al.*.[3] FP assays were performed in 10 mM HEPES, pH 7.4, 150 mM NaCl, 1.0 mg/mL BSA and 0.01% v/v Tween-20 buffer. Fluorescently labelled peptides were dissolved to 10 nM in FP buffer as a mastermix. The desired compounds were then added from DMSO stock as required, to a final DMSO content of 1%. This solution was used to fill Corning Low-binding Black Round Bottom 384-well plates (Corning #4514) with a final volume of 10 µL per well. A two-fold dilution series was then performed with 14-3-3(ζ), starting from 243 µM. FP was then measured in a Tecan Infinite F500 plate reader, using 485 (20) nm excitation and 535 (20) nm emission filters. The obtained anisotropy values were then plotted and fitted against a 4-parameter one-site binding model in GraphPad Prism.

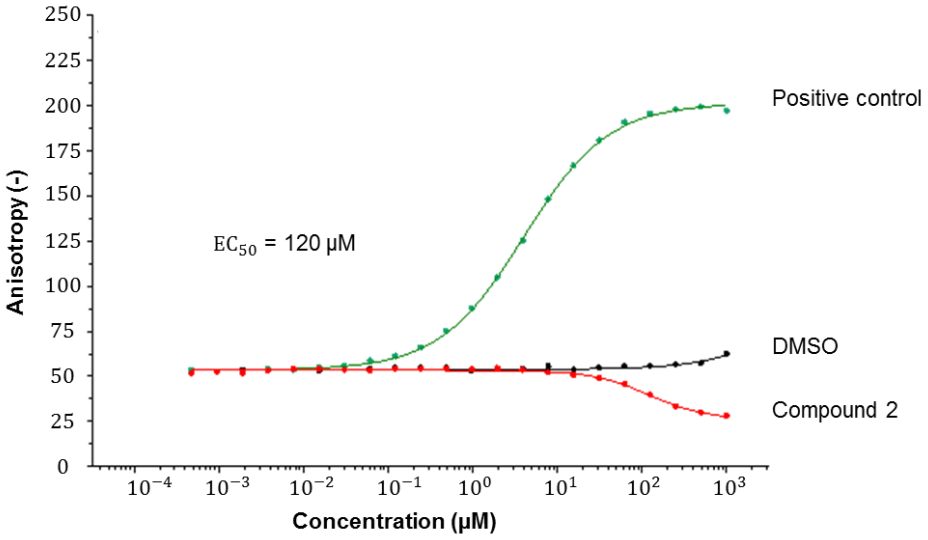


Figure S5. Fluorescent polarization assay of compound **2**: Titration of **2** to 400 nM 14-3-3ζ with 10 nM fluorescently labelled peptide synaptopodin, resulting in the displacement of the peptide (EC_{50} 120 µM). Error bars indicate SEM from a triplicate experiment.

Binding studies by surface plasmon resonance (SPR)

The SPR experiments were performed using a Reichert SR7500DC surface plasmon resonance spectrometer (Reichert Technologies, Depew, NY, USA), and medium density carboxymethyl dextran hydrogel CMD500M sensor chips (XanTec Bioanalytics, Düsseldorf, Germany). Doubly distilled (dd) water was used as the running buffer for immobilization. HBS-EP buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% v/v tween 20, pH 7.4) containing 5% v/v DMSO was used as the running buffer for binding study. All running buffers were filtered and degassed prior to use. The 14-3-3 (30.3 kDa) was immobilized in one of the two flow cells according to a standard amine-coupling protocol.[4] The other flow cell was left blank to serve as a reference. The system was initially primed with borate buffer 100 mM (pH 9.0), then the carboxymethyldextran matrix was activated by a 1:1 mixture of *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) 100 mM and *N*-hydroxysuccinimide (NHS) 100 mM at a flow rate of 10 µL/min for 7 min. The 14-3-3 was diluted to a final concentration of 57 µg/mL in 10 mM sodium acetate buffer (pH 4.5) and was injected at a flow rate of 10 µL/min for 7 min. Non-reacted surface was quenched by 1 M ethanolamine hydrochloride (pH 8.5) at a flow rate of 25 µL/min for 3 min. A series of 10 buffer injections was run initially on both reference and active surfaces to equilibrate the system resulting in a stable immobilization level of approximately 2500 µ refractive index unit (µRIU) (Figure S13). Binding experiments were performed at 20 °C. Compounds dissolved in DMSO were diluted with HBS-EP buffer (final DMSO concentration of 5% v/v) and were injected at a flow rate of 30 µL/min. Single-cycle kinetics were applied for K_D determination. The association time was set to 60 s, and the dissociation phase was recorded for 120 s. Ethylene glycol 80% in the running buffer was used for regeneration of the surface. Differences in the bulk refractive index due to DMSO were corrected by a calibration curve (nine concentrations: 3–7% v/v DMSO in HBS-EP buffer). Data processing and analysis were performed by Scrubber software (Version 2.0c, 2008, BioLogic Software). Sensorgrams were calculated by sequential subtractions of the corresponding curves obtained from the reference flow cell and the running buffer (blank). SPR responses are expressed in resonance unit (RU). The K_D values were calculated by global fitting of the kinetic curves.

SPR competition assays

The compounds were prepared as 100 µL samples in the same manner as mentioned above in the SPR binding assays. For each competition experiment, a series of 12 injections was applied including two blanks, the acylhydrazone, a blank for surface regeneration, synaptopodin, ethylene glycol 80% in buffer for surface regeneration, two blanks, acylhydrazone/synaptopodin mixture with the same concentrations of the single compounds, ethylene glycol 80% in buffer, and two blanks, respectively. Samples were injected at a flow rate of 30 µL/min for 60 s and the dissociation phase was recorded for 120 s. Data processing and analysis were performed as mentioned above.

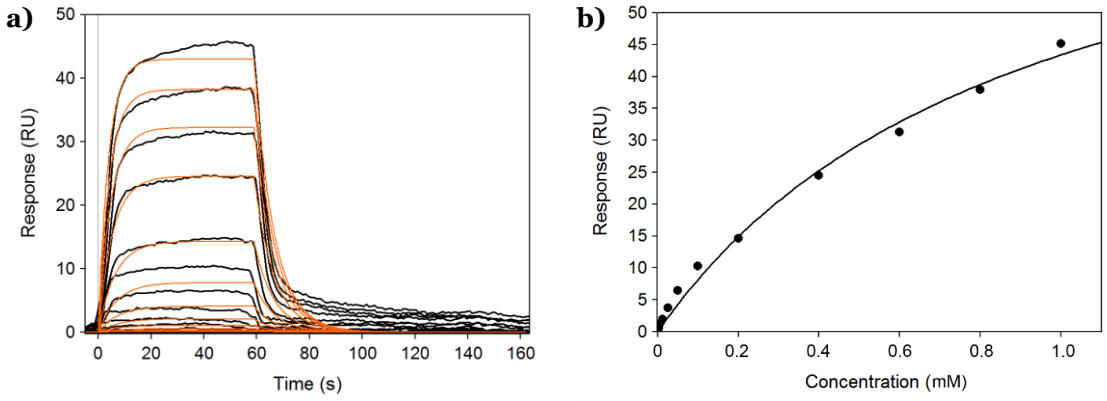


Figure S6. SPR binding assay of **2**: a) Overlay of sensorgrams (black) of **2** at concentrations of 1.6–1000 µM running over an immobilized 14-3-3(ζ). Global fitting of the association and dissociation curves (red), b) Langmuir binding isotherm (K_D = 1.01 ± 0.03 mM). The RU values are MW normalized. Data obtained from single experiment.

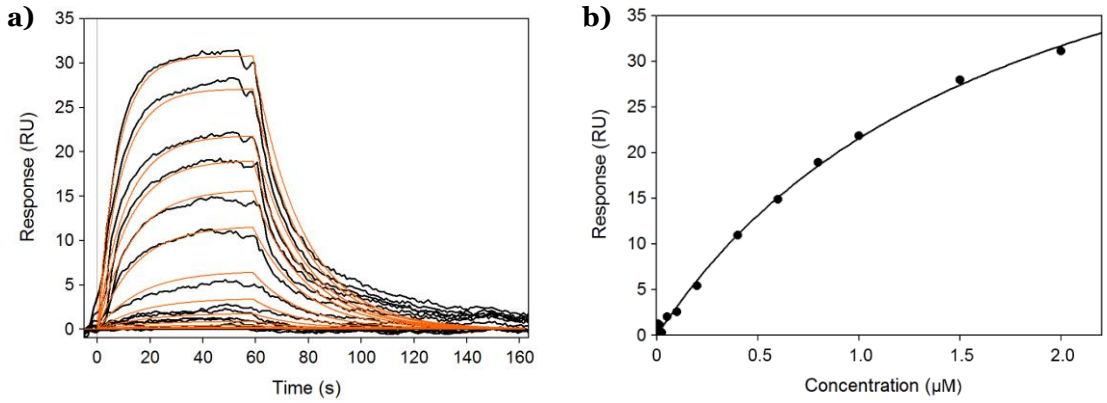


Figure S7. SPR binding assay of synaptopodin: (a) Overlay of sensorgrams (black) of synaptopodin at concentrations of 0.003–2.0 µM running over an immobilised 14-3-3(ζ). Global fitting of the association and dissociation curves (red). (b) Langmuir binding isotherm (K_D = 1.38 ± 0.02 µM). The RU values are MW normalized. Data obtained from single experiment.

Table S2. The kinetic parameters of compound **2** and synaptopodin binding to 14-3-3. The RU values are MW normalized. Data obtained from single measurement. R_{max}: maximum analyte binding capacity; k_{on}: association rate constant; k_{off}: dissociation rate constant; K_D: equilibrium dissociation constant; Res sd: residual standard deviation.

Compound	R_{max} (RU)	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_D (µM)	Res sd
2	86.0 ± 1.0	1.32 ± 0.04 ×10²	0.133 ± 0.002	1010 ± 30	1.61
Synaptopodin	52.0 ± 5.0	3.92 ± 0.06 ×10⁴	0.0542 ± 0.0004	1.38 ± 0.02	0.90

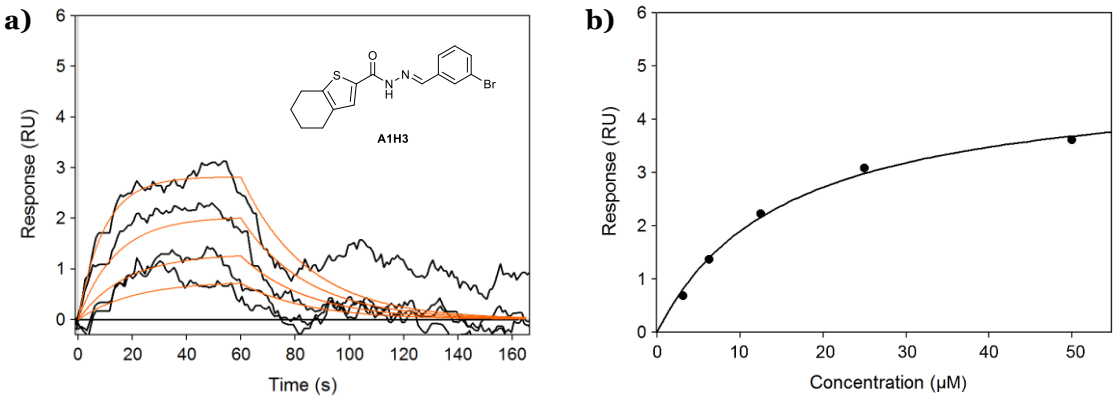


Figure S8. SPR binding assay of **A1H3**: a) Overlay of sensorgrams (black) of **A1H3** at concentrations of 3.1–50 μM running over an immobilized 14-3-3(ζ). Global fitting of the association and dissociation curves (red), b) Langmuir binding isotherm of **A1H3** (K_D = 16 ± 1 μM). The RU values are MW normalized. Data obtained from two independent experiments.

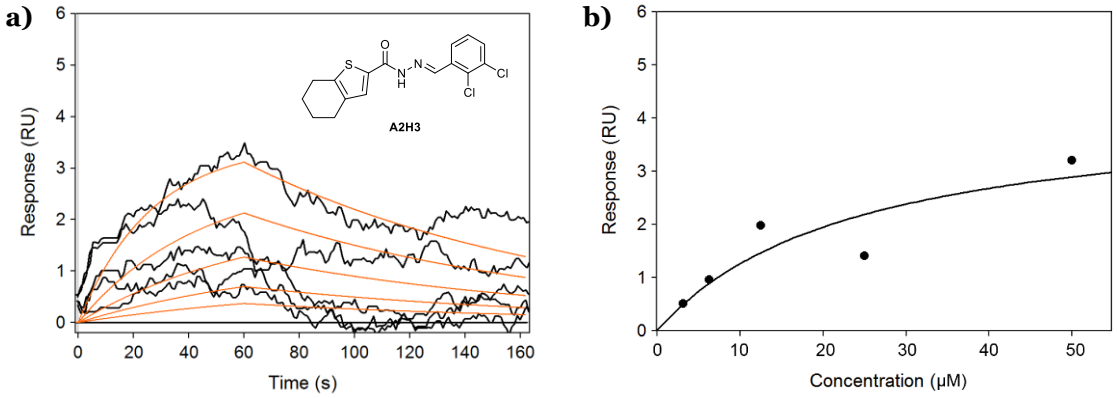
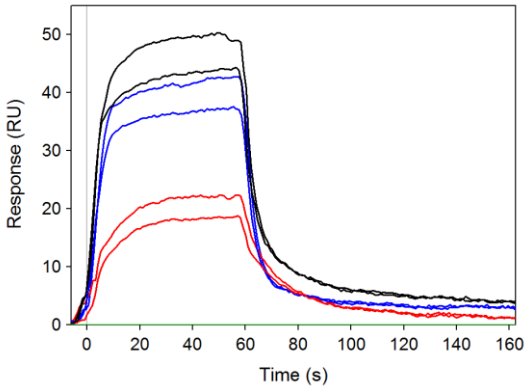
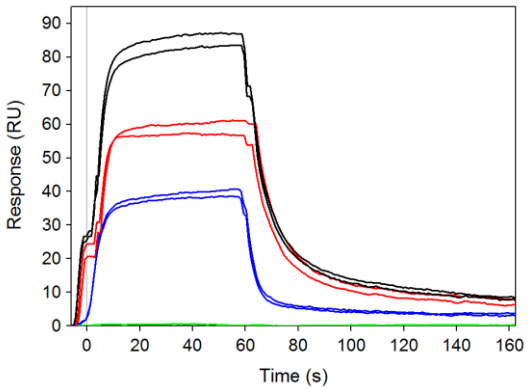


Figure S9. SPR binding assay of **A2H3**: a) Overlay of sensorgrams (black) of **A2H3** at concentrations of 3.1–50 μM running over an immobilized 14-3-3(ζ). Global fitting of the association and dissociation curves (red), b) Langmuir binding isotherm of **A2H3** (K_D = 15 ± 1 μM). The RU values are MW normalized. Data obtained from two independent experiments.



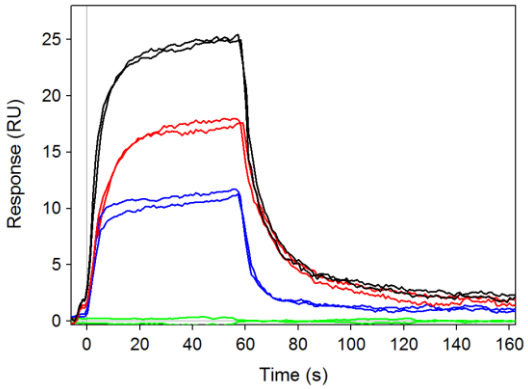
	Compound **2** (1000 μM)	Synaptopodin (1 μM)	**2** (1000 μM) + Synaptpodin (1 μM)
$RU_{(experiment\ 1)}$	42.08	22.21	49.67
$RU_{(experiment\ 2)}$	36.93	18.39	43.55
$RU_{(average)}$	39.51 (± 3.6)	20.30 (± 2.7)	46.61 (± 4.3)

Figure S10 and **Table S3.** SPR competition assay: Overlay of sensorgrams of **2** (1000 μM, blue), synaptopodin (1 μM, red) and **2**–synaptopodin mixture (black) in duplicate. RU values of the mixture are less than the sum of the individual RU responses (29–33% decrease of **2** response and 60–65% decrease of synaptopodin response), indicating a competitive effect.



	Compound **2** (1000 μM)	Synaptopodin (25 μM)	**2** (1000 μM) + Synaptpodin (25 μM)
$RU_{(experiment\ 1)}$	39.99	60.35	87.03
$RU_{(experiment\ 2)}$	37.18	55.73	81.94
$RU_{(average)}$	38.59 (± 1.9)	58.04 (± 3.3)	84.49 (± 3.6)

Figure S11 and Table S4. SPR competition assay: Overlay of sensorgrams of **2** (1000 μM, blue), synaptopodin (25 μM, red) and **2**–synaptopodin mixture (black) in duplicate. RU values of the mixture are less than the sum of the individual RU responses (30–33% decrease of **2** response and 20–22% decrease of synaptopodin response), indicating a competitive effect.



	Compound **2** (200 μM)	Synaptopodin (1 μM)	**2** (200 μM) + Synaptpodin (1 μM)
$RU_{(experiment\ 1)}$	10.55	16.90	24.85
$RU_{(experiment\ 2)}$	11.20	17.69	24.69
$RU_{(average)}$	10.88 (± 0.5)	17.30 (± 0.6)	24.77 (± 0.1)

Figure S12 and **Table S5.** SPR competition assay: Overlay of sensorgrams of **2** (200 μM, blue), synaptopodin (1 μM, red) and **2**–synaptopodin mixture (black) in duplicate. RU values of the mixture are less than the sum of the individual RU responses (25–37% decrease of **2** response and 15–24% decrease of synaptopodin response), indicating a competitive effect.

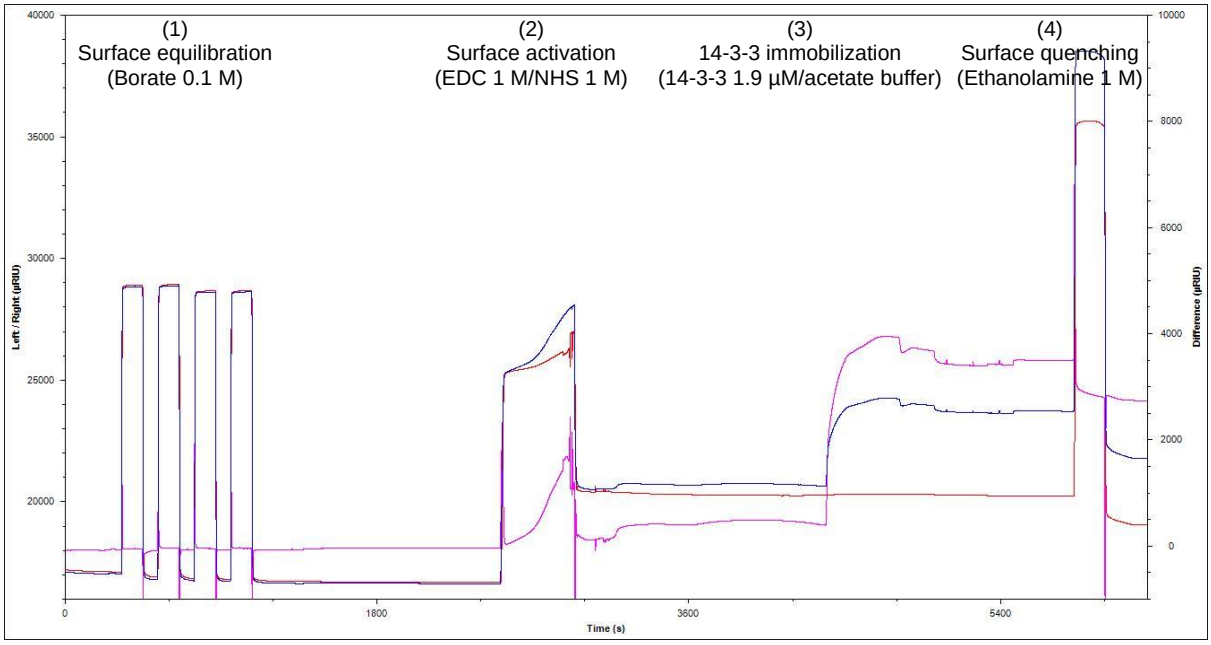


Figure S13. Sensorgram of the immobilization procedure for 14-3-3 on CMD500M sensor chip: (1) Four injections of cleaning solution, (2) activation solution, (3) 14-3-3, and (4) quenching solution. The blue, red, and magenta curves represent the left (active) channel, right (reference) channel, and the difference, respectively.

NMR and UV spectra

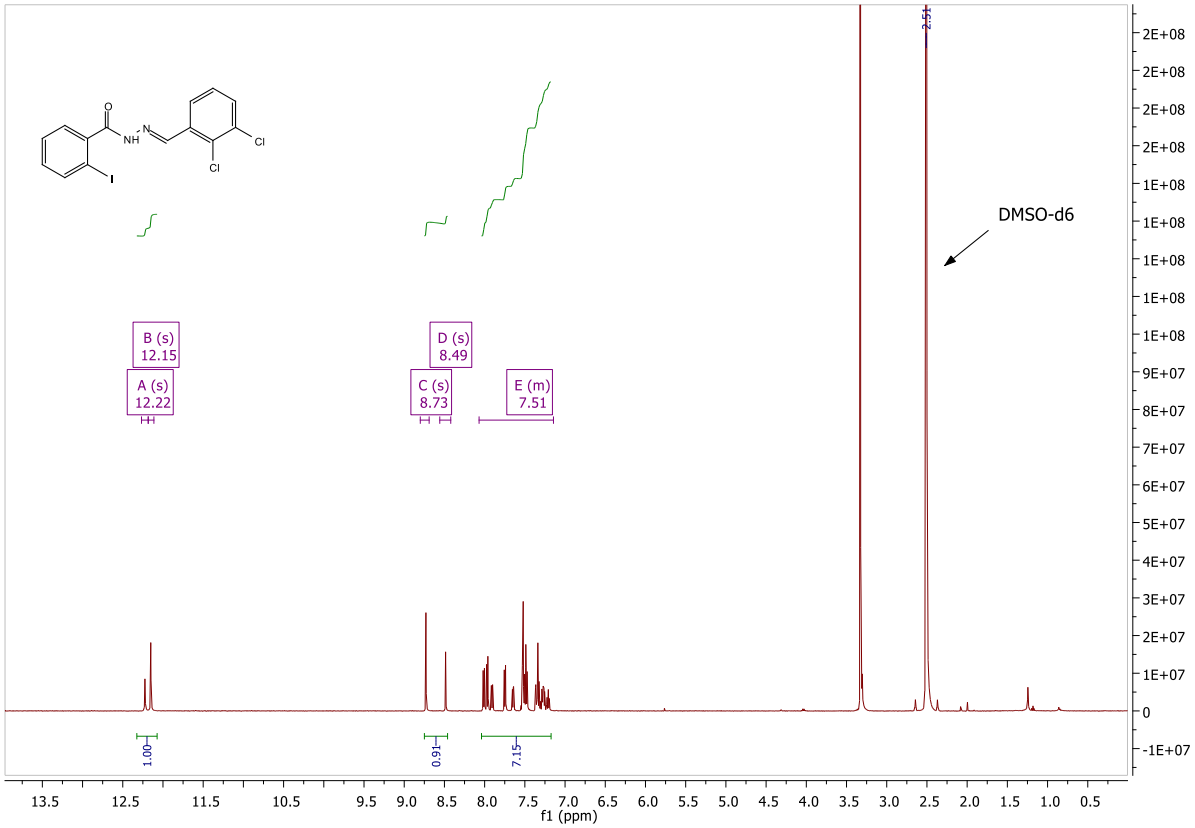


Figure S14. ^{1}H-NMR spectrum of compound **3**.

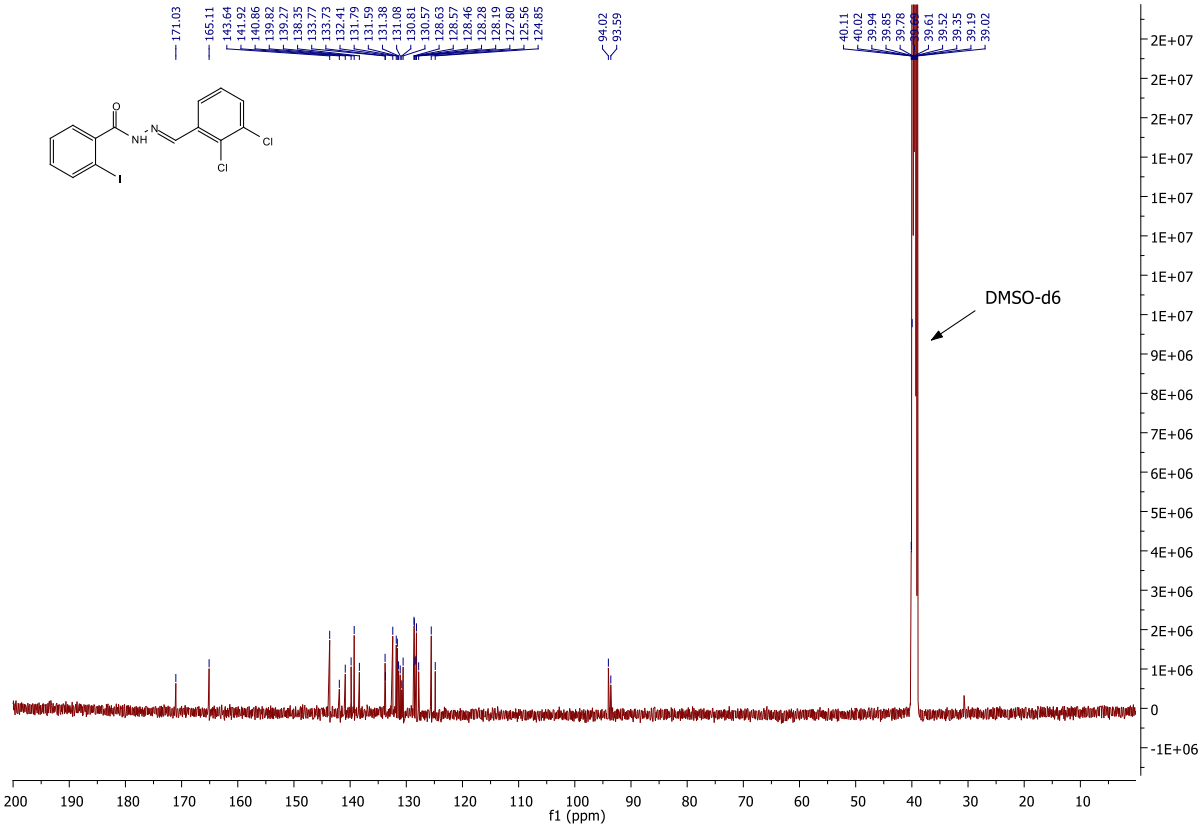


Figure S15. ^{13}C-NMR spectrum of compound **3**.

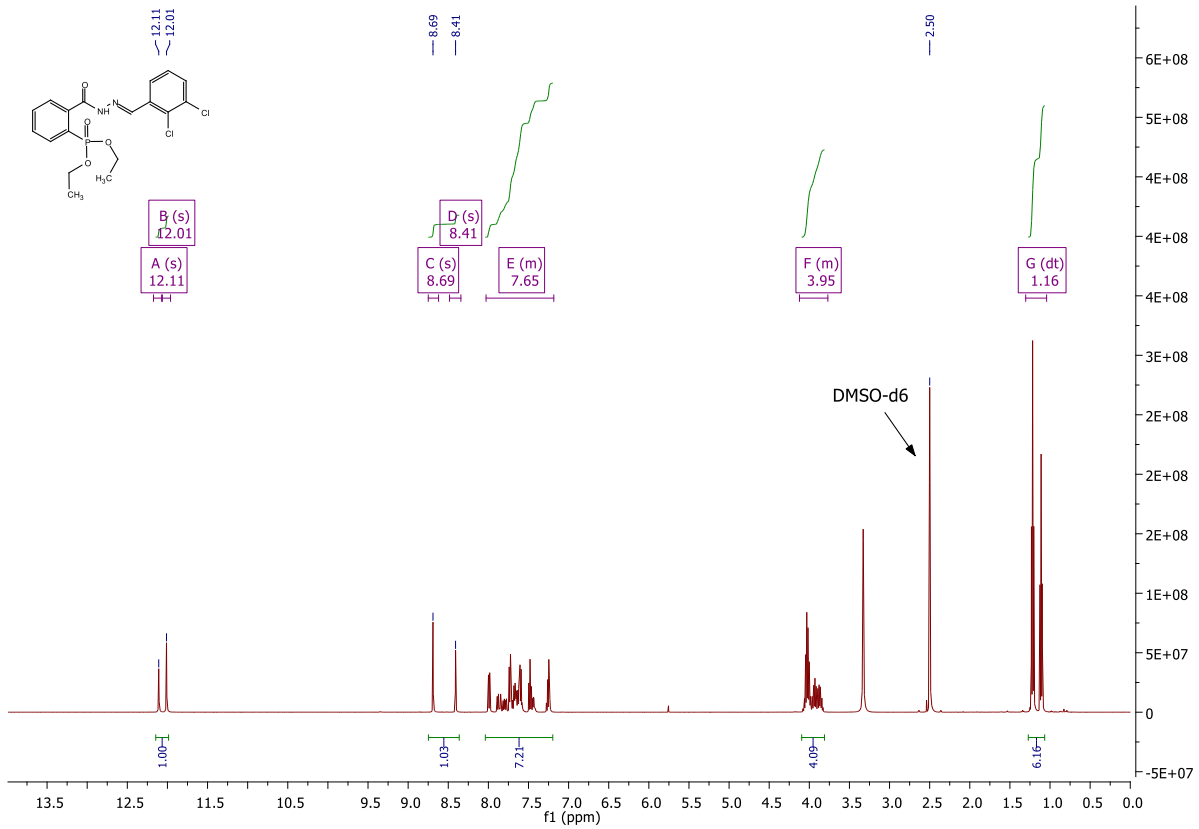


Figure S16. ^{1}H-NMR spectrum of compound **4**.

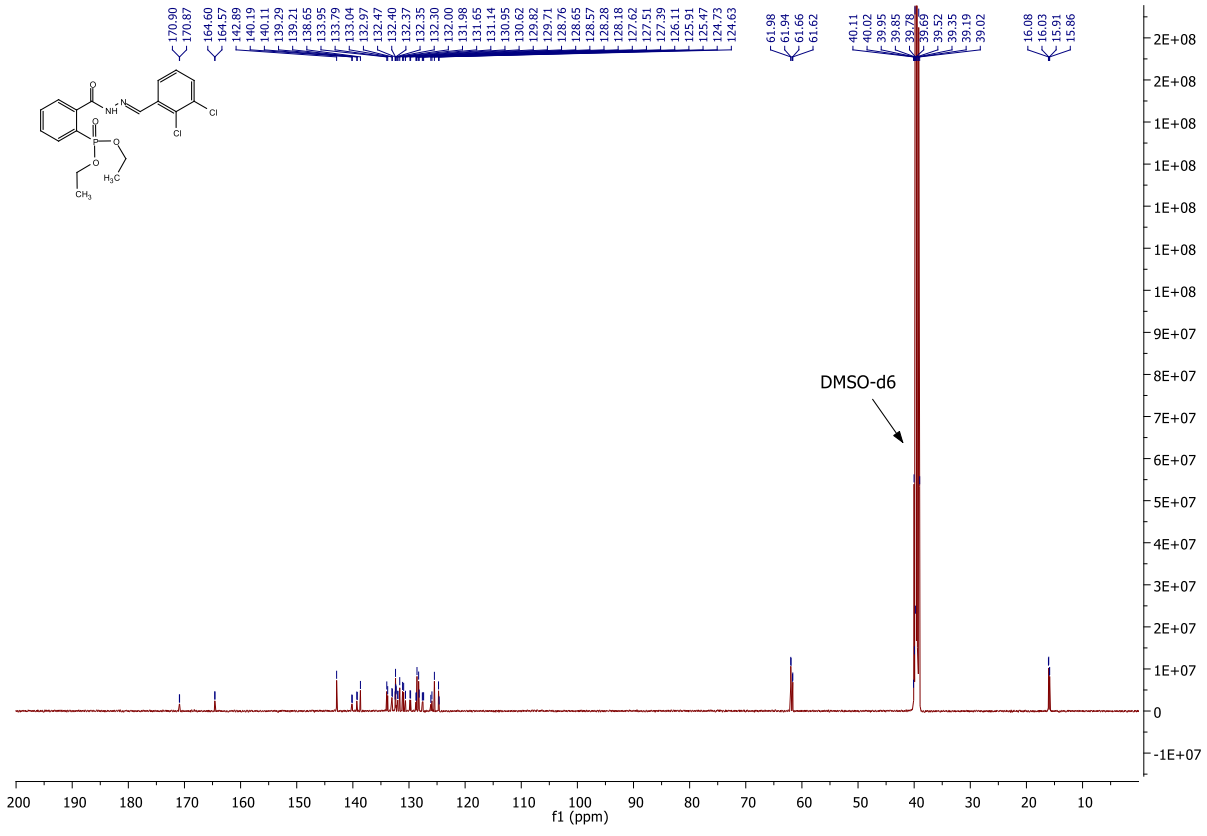


Figure S17. ^{13}C-NMR spectrum of compound **4**.

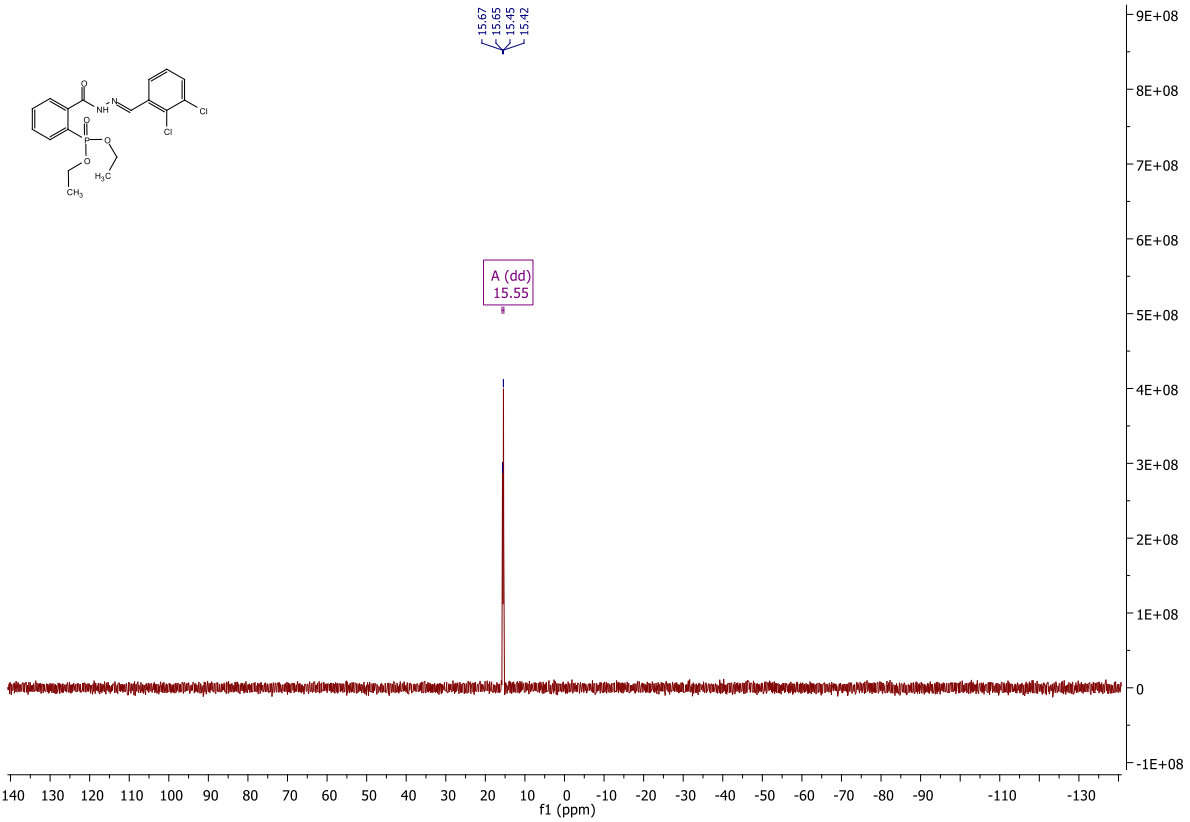


Figure S18. ^{31}P-NMR spectrum of compound **4**.

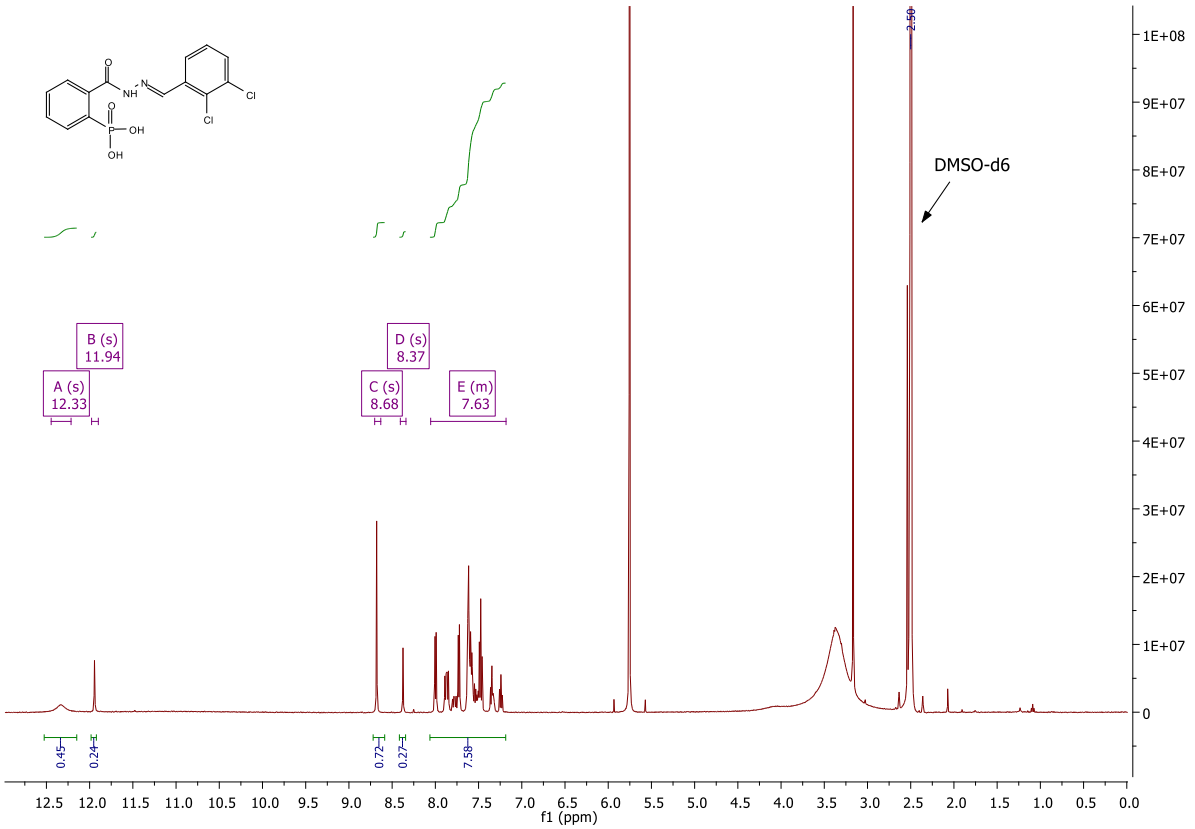


Figure S19. ^{1}H-NMR spectrum of compound **2.**

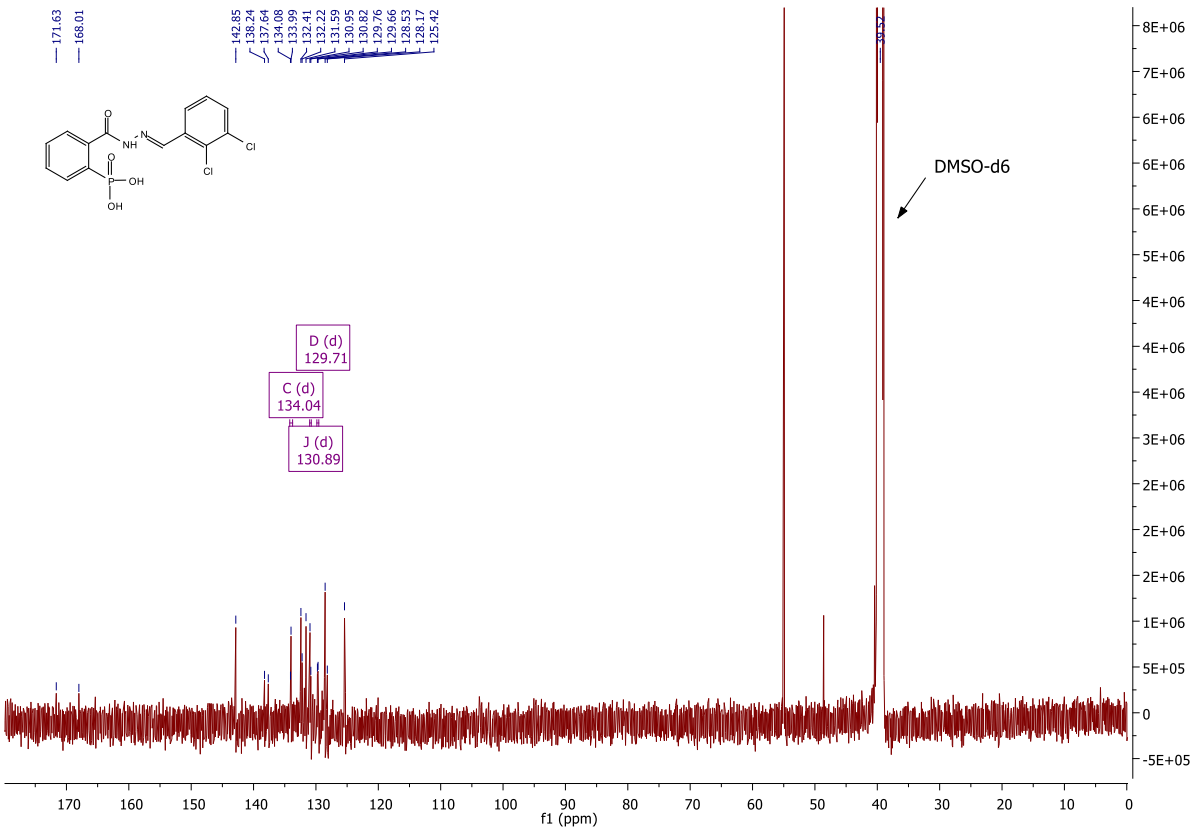


Figure S20. ^{13}C-NMR spectrum of compound **2**.

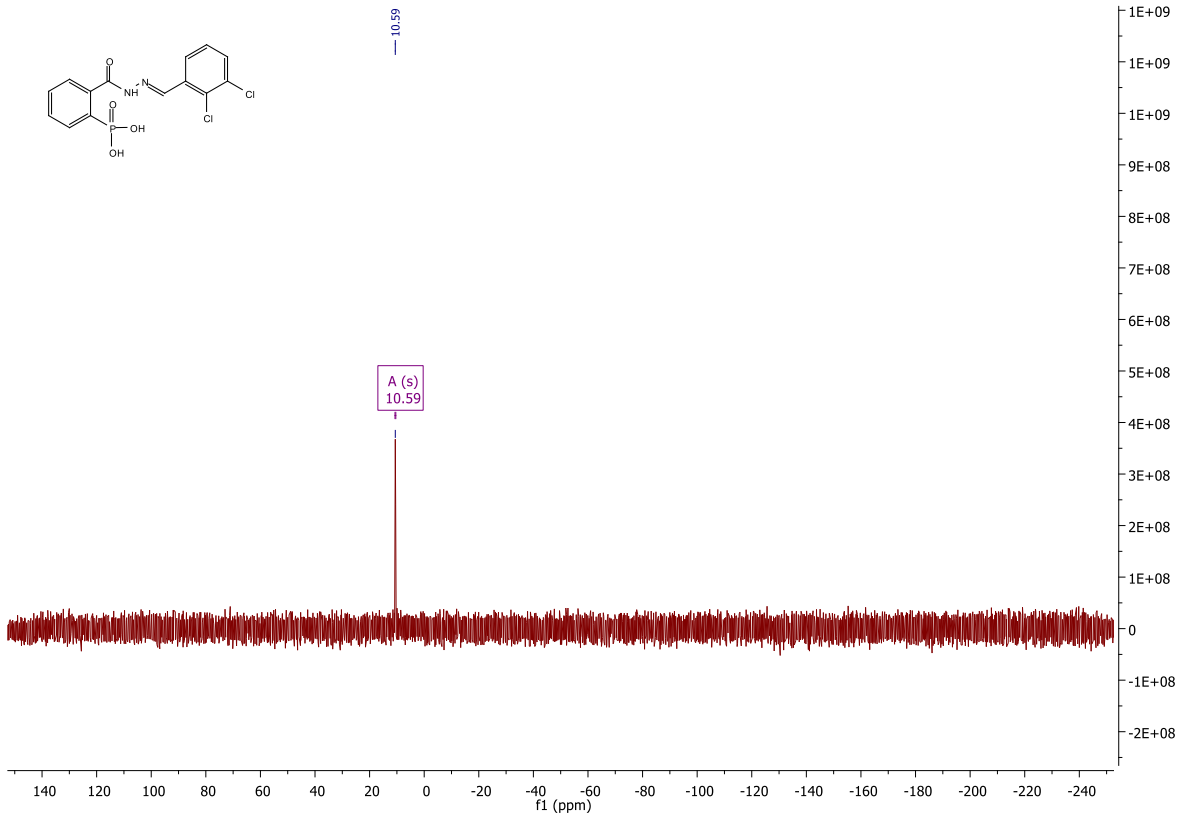


Figure S21. ^{31}P-NMR spectrum of compound **2**.

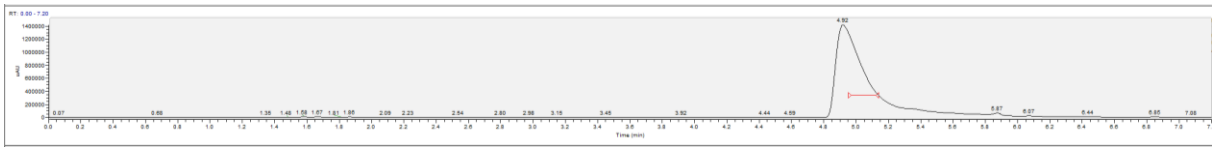


Figure S22. UV-chromatogram at 290 nm of compound **2**, > 95% pure

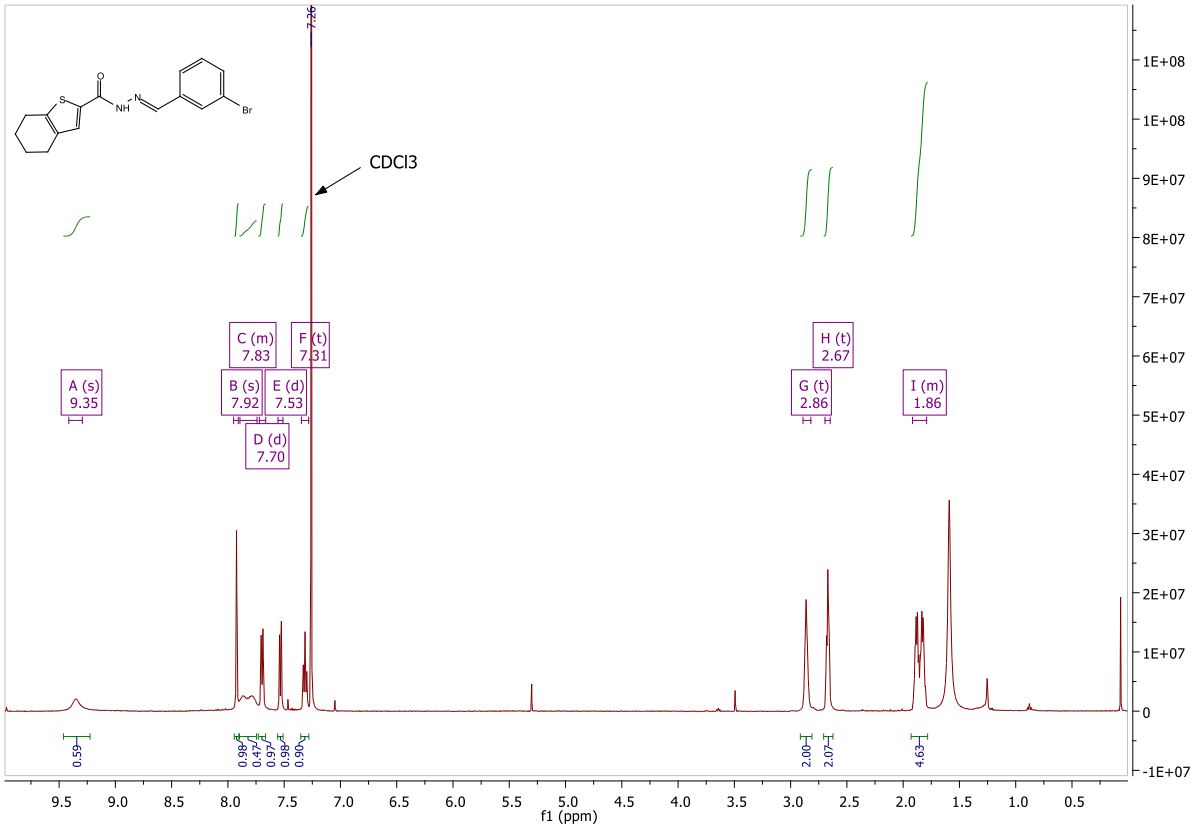


Figure S23. ^{1}H-NMR spectrum of **A1H3** in $CDCl_3$.

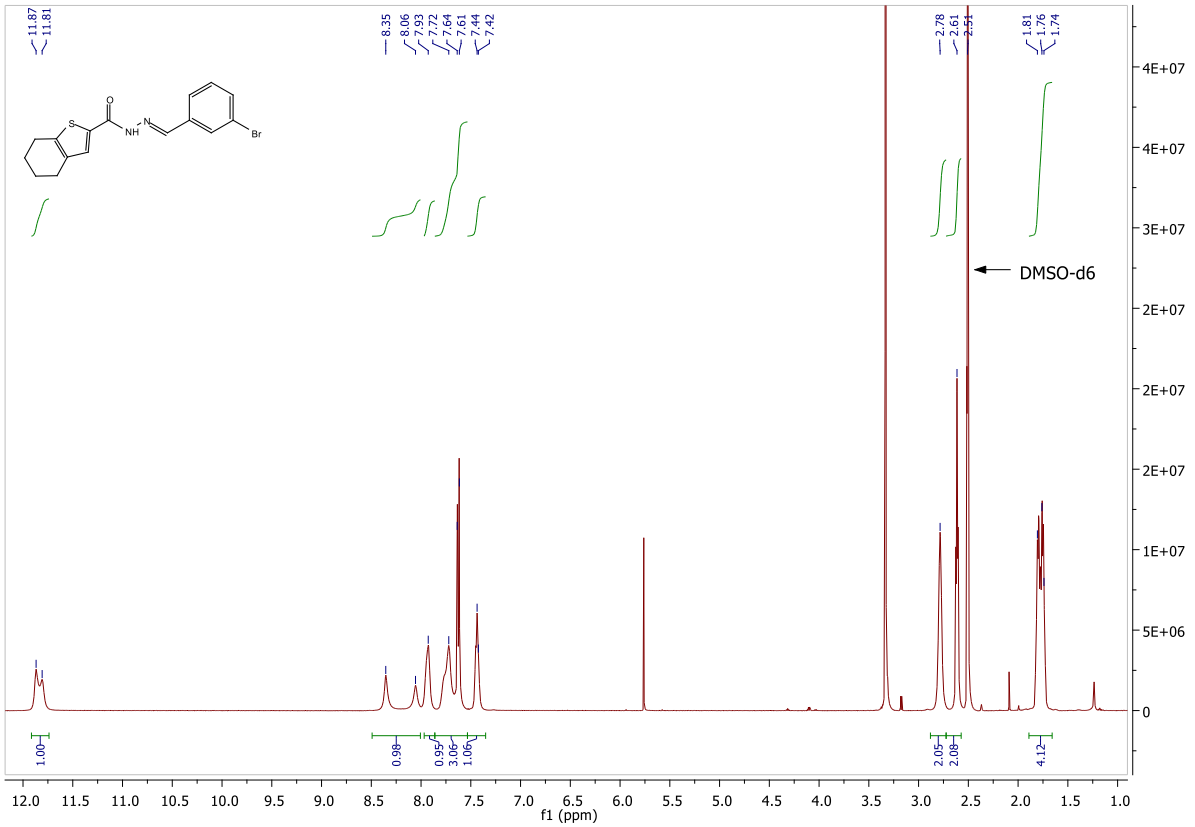


Figure S24. ^{1}H-NMR spectrum of **A1H3** in DMSO-d_6.

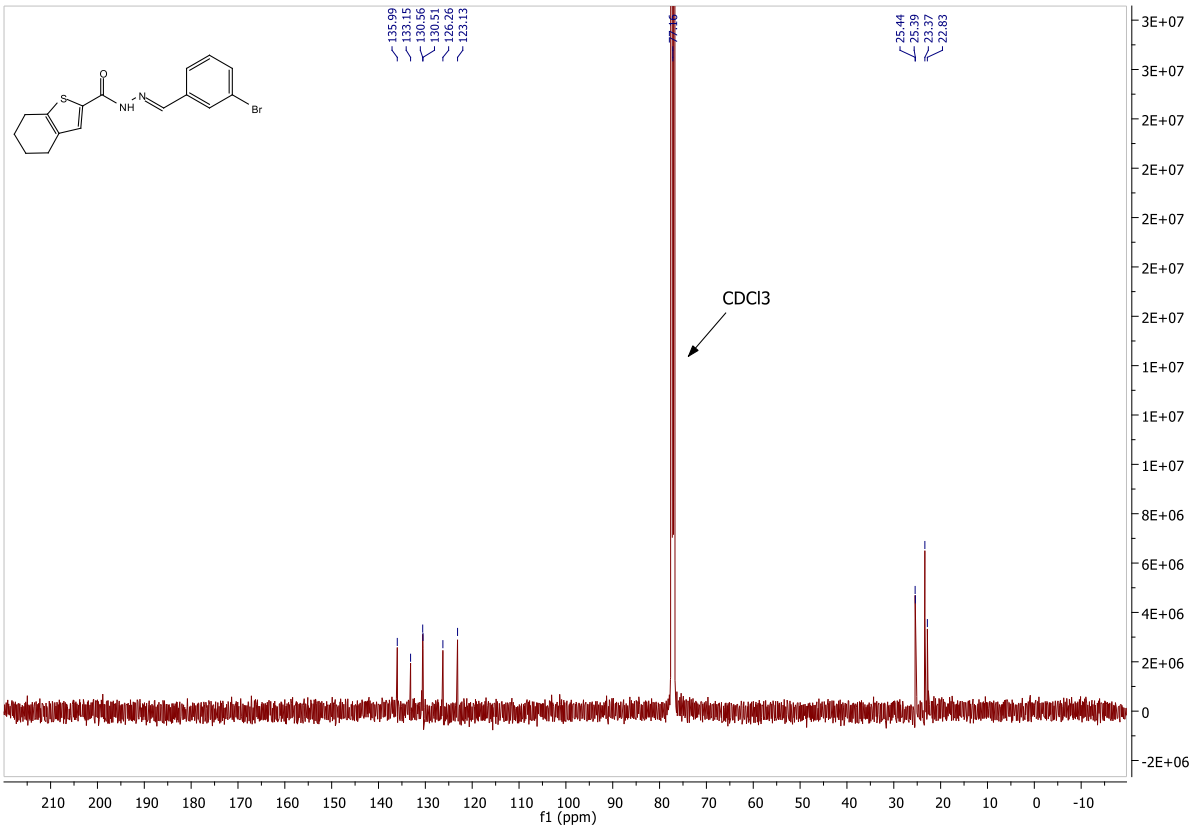


Figure S25. ^{13}C-NMR spectrum of compound **A1H3**.

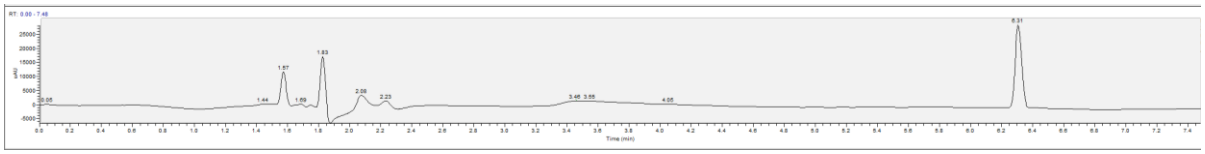


Figure S26. UV-chromatogram at 290 nm of compound **A1H3**, > 95% pure.

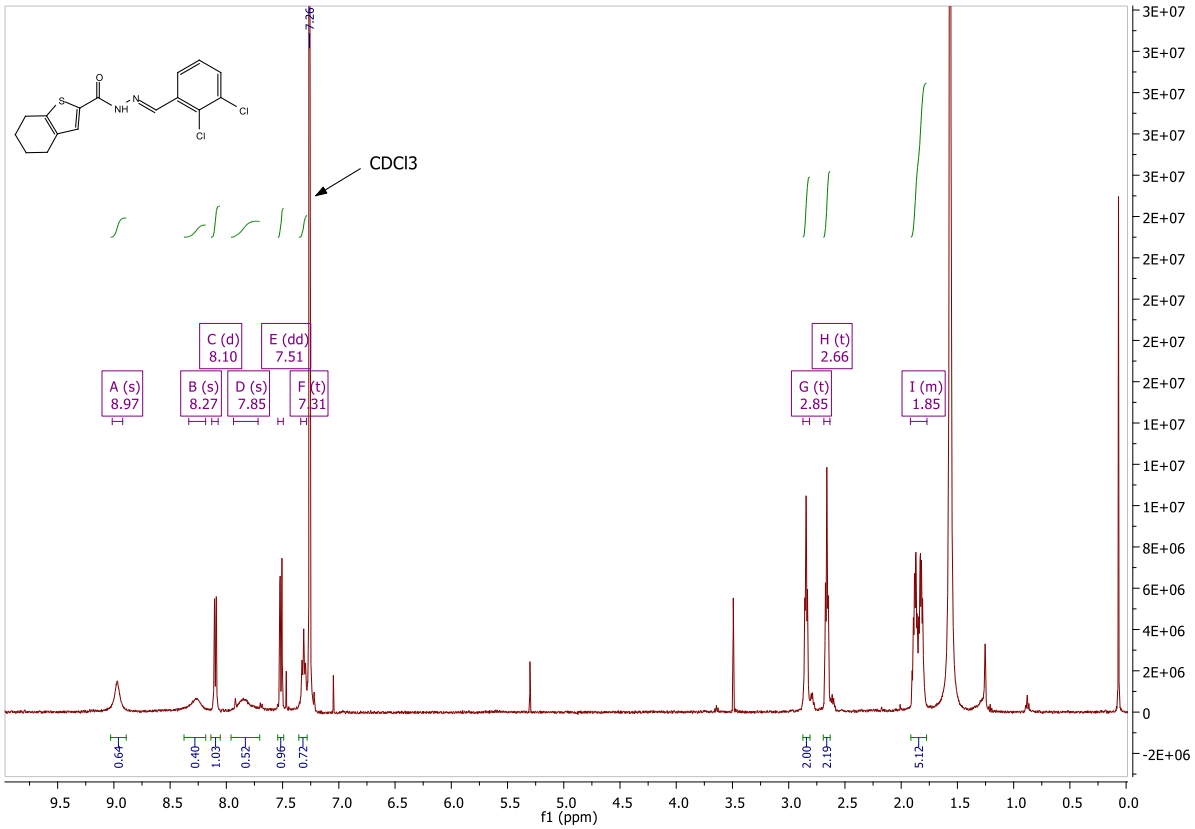


Figure S27. ^{1}H-NMR spectrum of compound **A2H3** in $CDCl_3$.

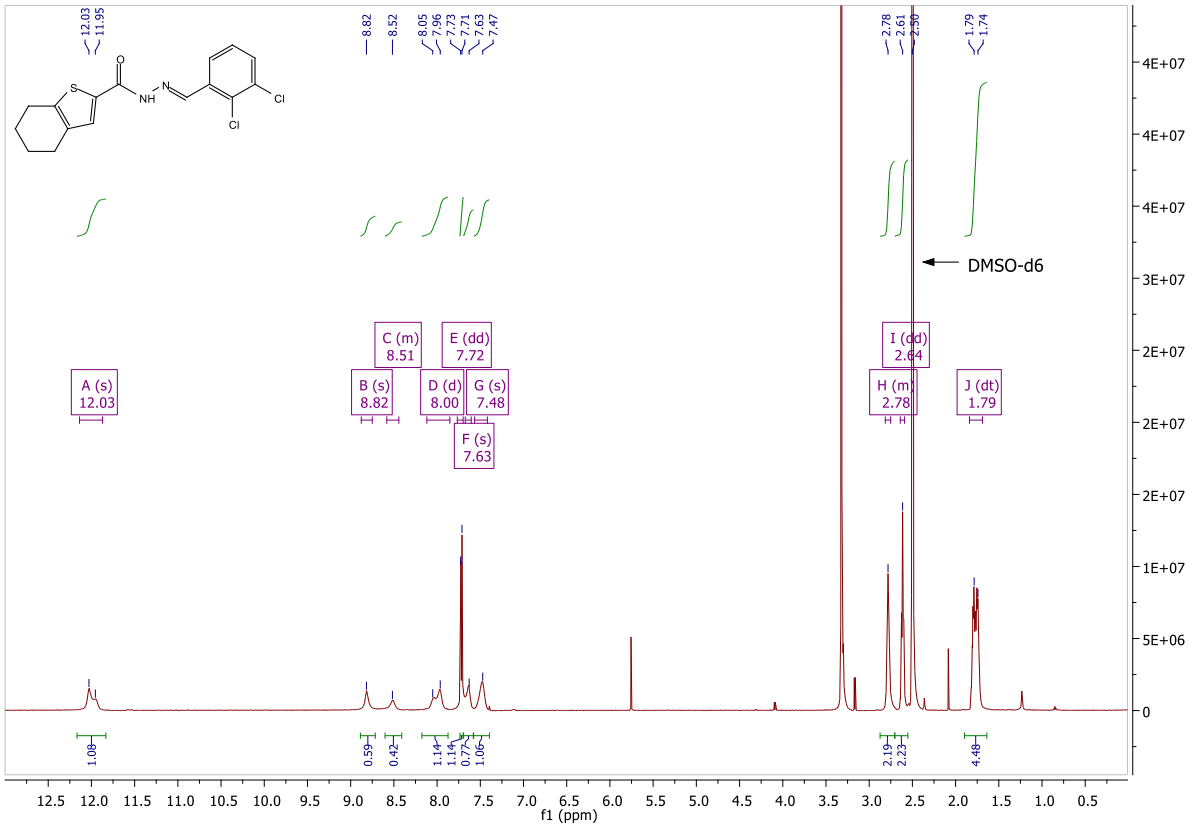


Figure S28. ^{1}H-NMR spectrum of compound **A2H3** in DMSO-d_6.

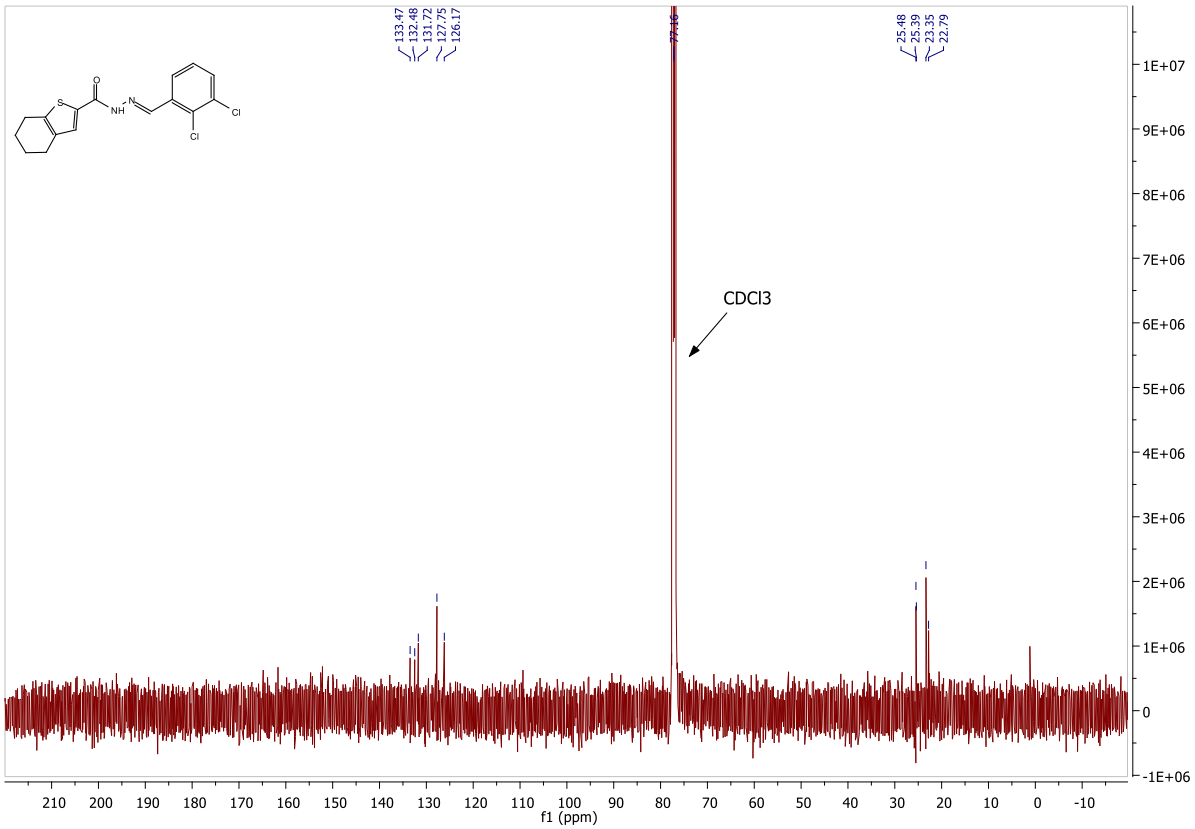


Figure S29. ^{13}C-NMR spectrum of compound **A2H3**.

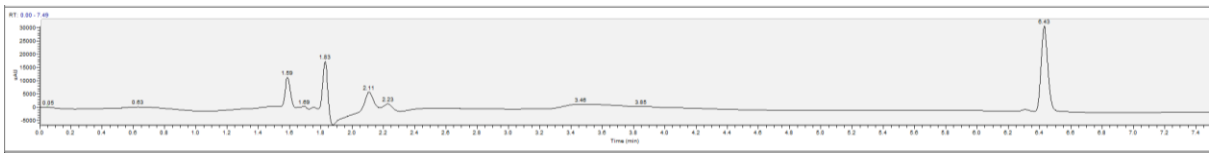


Figure S30. UV-chromatogram at 290 nm of compound **A2H3**, > 95% pure.

References

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