Discovery of CREBBP Bromodomain Inhibitors by High-throughput Docking and Hit Optimization Guided by Molecular Dynamics

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Supplementary data

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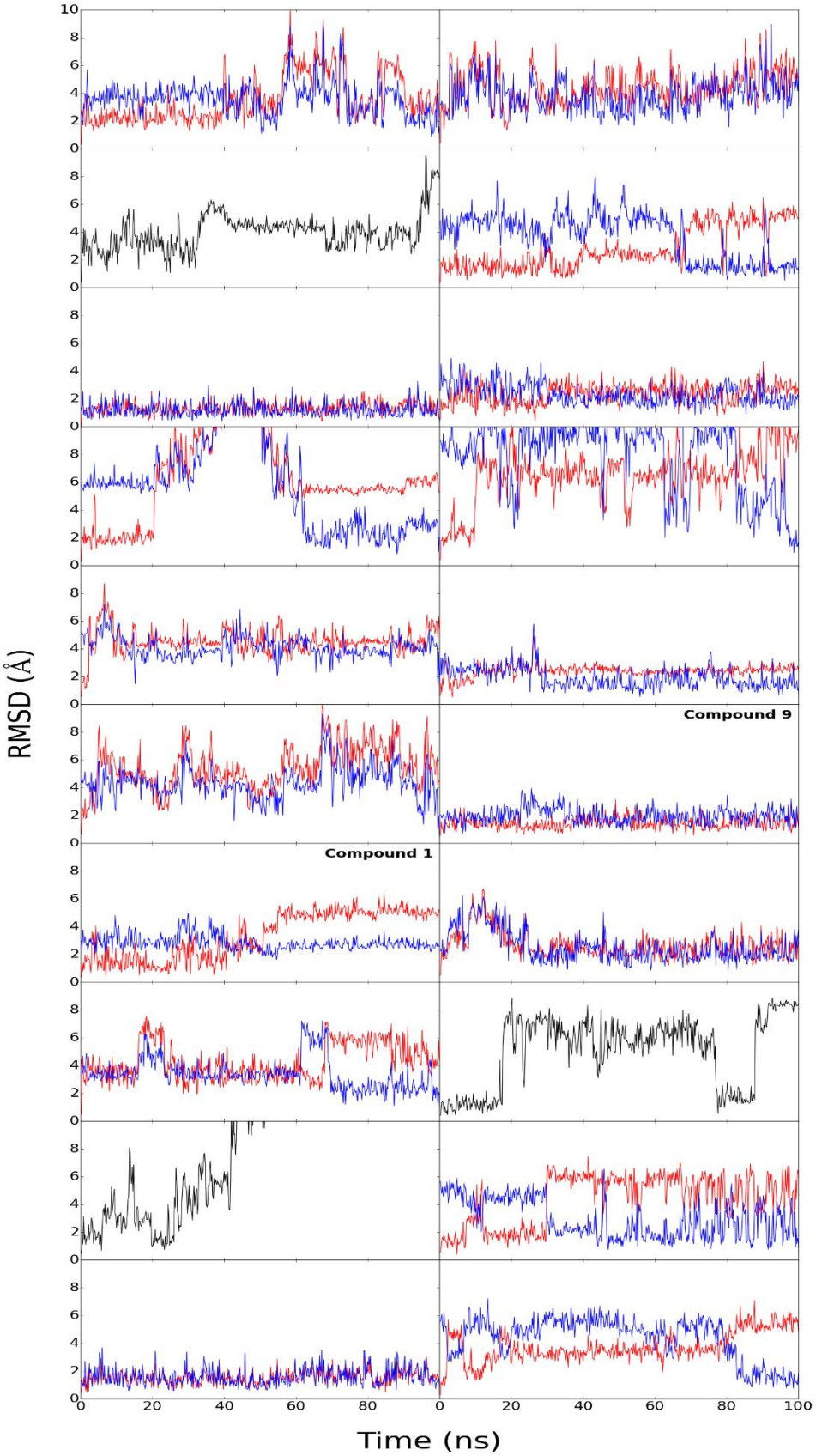


Figure S1. Time series of the RMSD of the heavy atoms of the compound from the starting structure, i.e., the docked pose (red) and from the final snapshot (blue) along the 100-ns MD runs of the top 20 compounds suggested by docking. The RMSD time-series were calculated after overlapping the rigid part of the protein. Full unbinding was observed for three compounds (black curves); these compounds were not purchased.

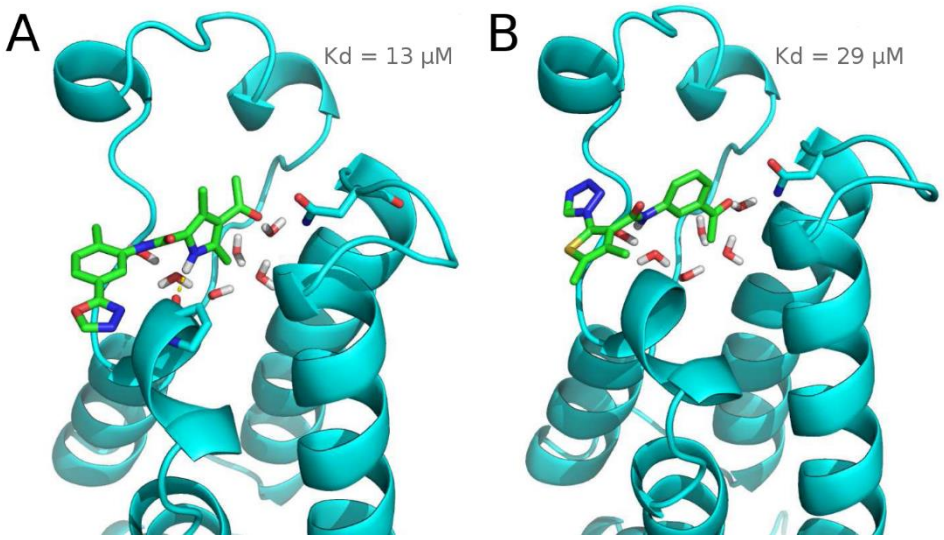


Figure S2. Docked pose of compound **1** in the 3P1C structure of CREBBP (panel A) and compound **9** in the 3SVH structure of CREBBP (panel B). The ligands are shown in sticks (carbon atoms in green) while the CREBBP bromodomain is represented by a ribbon model (cyan) with sticks showing the side chain of the conserved Asn1168 and the conserved water molecules. The K_d values were obtained by a competition binding assay (at DiscoveRx).

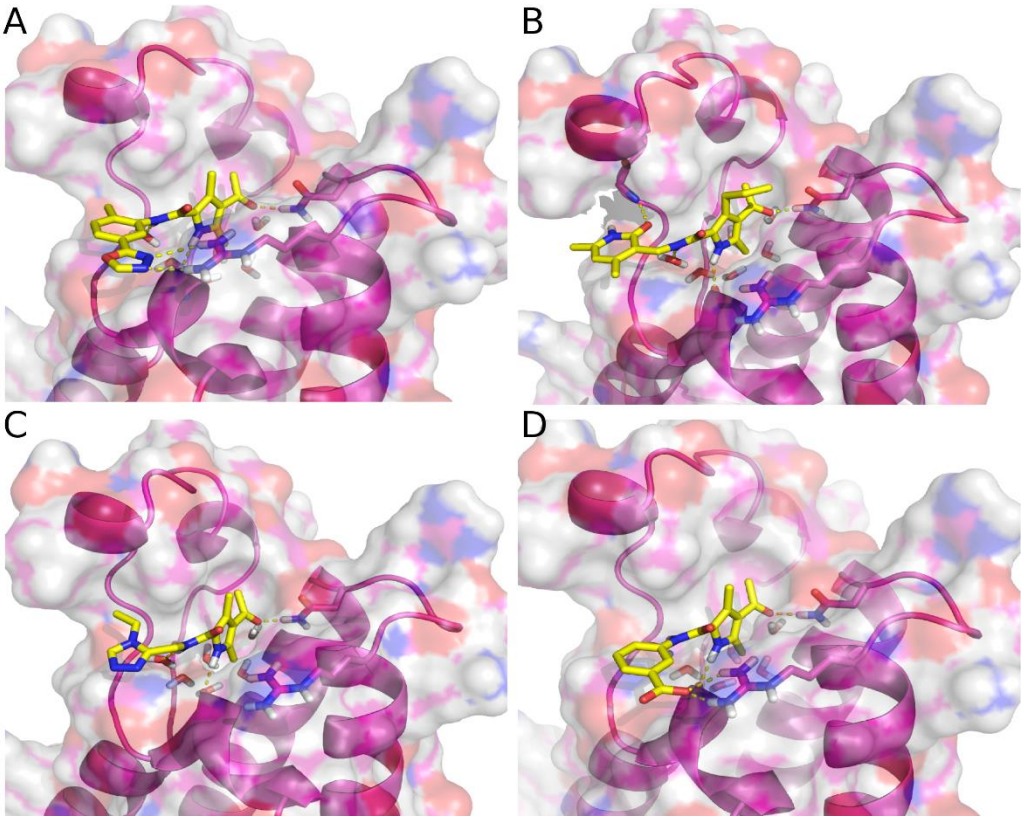


Figure S3. Binding pose of compounds **1** (panel A), **2** (B), **3** (C), and **6** (D) as predicted by docking. The protein backbone is shown by a ribbon model and transparent surface with sticks for the side chains of the conserved Asn1168 and Arg1173. Hydrogen bonds are shown by dashed lines.

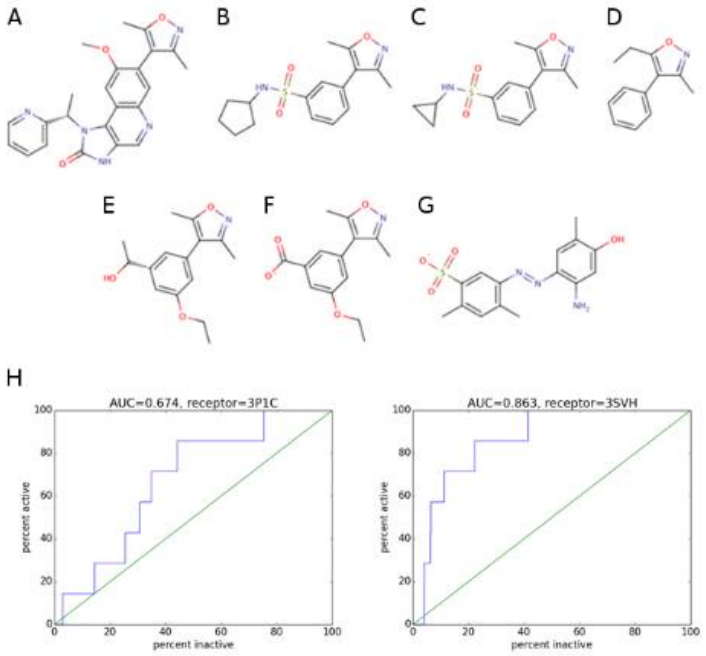


Figure S4. Seven known inhibitors of CREBBP (A-G) used as true positives for the ROC plots (H). (A) From PDB code 3ZYU,[1] (B) from PDB code 4A9N, (C) from PDB code 4A9M, (D) from PDB code 4A9O,[2] (E, F), [3] and (G) From PDB code 2L84.[4] (H) The two ROC plots were calculated using the docking results on the PDB structures 3P1C (left) and 3SVH (right).

Experimental section

TR-FRET assays

TR-FRET assays were carried out in duplicate at BPS Bioscience using a recombinant CBP bromodomain (BPS catalogue #31128) and the BET Ligand (BPS catalogue #33000) as provided in the CREBBP TR-FRET Assay Kit (BPS catalogue #32619). A 10 mM solution of the compound under investigation in DMSO was prepared and shipped to BPS Bioscience, where it was tested at 10 concentrations over the range of 0.001-10 µM (compounds **5** and **6**) or 0.01-100 µM (compound **1**) . Each compound solution was then diluted in water to obtain a 10% DMSO solution. 2 µL of this dilution were added to a 20 µL reaction mixture (12.5 nM CBP, 125 nM BET Ligand, including FRET dyes and the amount of compound needed to reach the required concentration). The resulting mixture was incubated for 2 hours at room temperature prior to reading the TR (time resolved)-FRET signal using a Tecan Infinite M1000 plate reader. The negative control consisted of the aforementioned mixture in which the buffer was added in place of compound. TR-FRET were recorded as the ratio of the fluorescence of the acceptor and the donor dyes (acceptor/donor).

The TR-FRET data was analyzed using Graphpad Prism software. The percent activity in the presence of each compound was calculated according to the following equation: % activity = [(F- Fb)/(Ft – Fb)]×100, where Ft is the TR-FRET signal in the absence of any compound (100 % activity), Fb the TR-FRET signal in the absence of the bromodomain (0 % activity) and F the TR-FRET signal in the presence of the compound. The percent inhibition was calculated according to the following equation: % inhibition = 100 - % activity. The values of % activity versus a series of compound concentrations were then

plotted using non-linear regression analysis of Sigmoidal dose-response curve generated with the equation $Y=B+(T-B)/1+10^{((LogIC50-X)\times Hill\ Slope)}$, where Y=percent activity, B=minimum percent activity, T=maximum percent activity, X= logarithm of compound and Hill Slope=slope factor or Hill coefficient. The IC_{50} value corresponds to the concentration causing a half-maximal percent activity.

BROMOscan assays

K_D determinations by means of BROMOscan technology was carried out at DiscoveRx. E. *coli* derived from BL21 strain was used as host to grow T7 phage strains displaying the bromodomains. E. *coli*, grown to log-phase, were infected with T7 phage (from a frozen stock, being the multiplicity of infection 0.4) and incubated while shaking at 32 °C for 90-150 minutes, until lysis. In order to remove cell debris, lysates were centrifuged at 5,000 x g and filtered (0.2 μm). Affinity resins were obtained by treating streptavidin-coated magnetic beads with biotinylated acetylated peptide ligands for 30 minutes at 25°C. Those beads were then blocked with excess of biotin and washed with blocking buffer (SeaBlock (Pierce), 1% bovine serum albumin, BSA, 0.05% Tween20, 1 mM dithiothreitol, (DTT) removing the unbound ligand and reducing non-specific phage binding.

During the experiment, the bromodomain, ligand-bound affinity beads and test compounds were combined in a buffer composed of 17% SeaBlock, 0.33x phosphate-buffered solution, PBS, 0.04% Tween20, 0.02% BSA, 0.004% sodium azide and 7.4 mM DTT. Test compounds were prepared as 50 mM in pure DMSO and diluted to 5 mM with monoethylene gycol, MEG (100× concentrated in respect to the top screening concentration, 50 μM). During the assay a DMSO and MEG final concentration of 0.1% and 0.9% respectively was used. The assays were carried out in polystyrene 96-well plates in a final volume of 0.135 mL. The assay plates were incubated at 25 °C with shaking for 1 hour and the affinity beads were washed with a buffer composed of 0.05% Tween 20 in PBS. The beads were then re-suspended in the elution buffer (1x PBS, 0.05% Tween 20, 2 μM non-biotinylated affinity ligand) and incubated at 25°C with shaking for 30 minutes. The bromodomain concentration in the eluates was measured by qPCR. Binding constants (K_d) were calculated with a standard dose-response curve using the Hill equation and curves were fitted using a non-linear least square fit with the Levenberg-Marquardt algorithm.

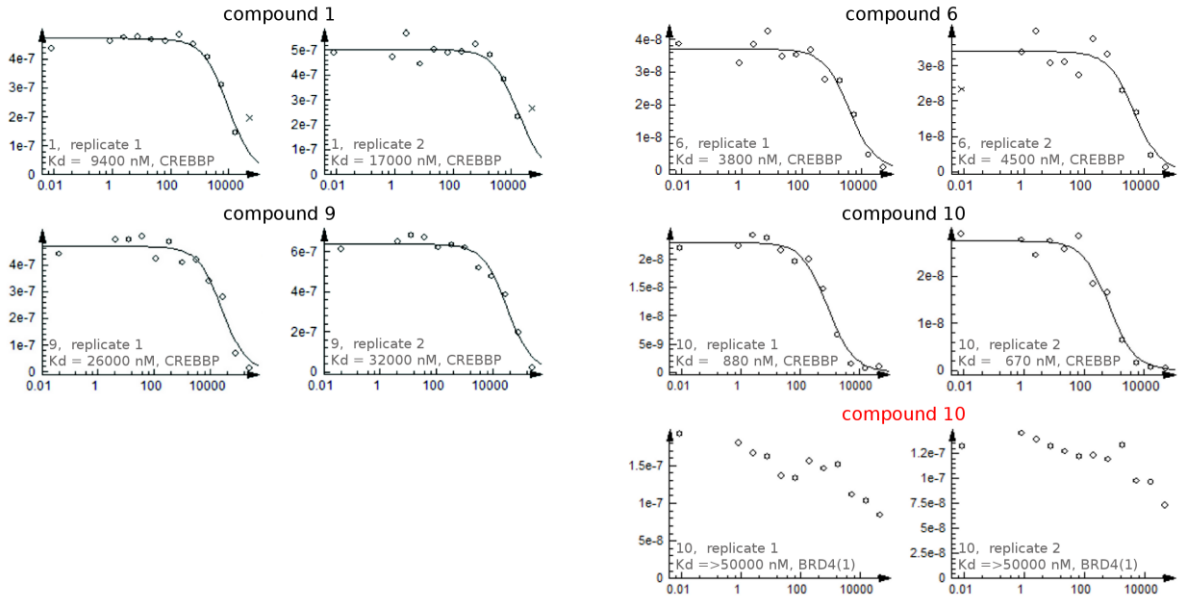


Figure S5. Dose-response curves for compounds **1**, **6**, **9,** and **10** obtained using the competition binding assay at DiscoveRx with CREBBP (black), and BRD4(1) only for compound **10** (red).

Crystallization, Data Collection, and Structure Determination

Crystals of the CREBBP bromodomain were grown at 4°C using the hanging drop vapor diffusion method. A 50 mM solution of compound **10** (in 100% DMSO) was added to a solution of CREBBP to reach a final DMSO concentration of 1% (v/v) and the mixture was incubated on ice for 1 hour before crystallization. Then equal volumes of protein (with compound **10**) and reservoir solutions (0.1 M MES pH 6.5, 0.10 MgCl2, 20 % PEG 6000, 10 % ethylene glycol) were mixed and crystals appeared after 1 to 2 days. The crystals were flash-frozen in liquid nitrogen with extra 10% ethylene glycol as cryoprotectant for measurements. Data sets were collected on a PILATUS 6MF detector at the Swiss Light Source beamline X06SA of the Paul Scherrer Institute (Villigen, Switzerland) and indexed, integrated and scaled with the XDS[5] and CCP4 programs.[6] The structures were solved by molecular replacement with PHASER[7] using the CREBBP structure (PDB entry 4NR5) as a search model and refined with PHENIX.[8] The atomic coordinates and structure factors of CREBBP in complex with inhibitor **10** have been deposited with the Protein Data Bank as entry 4TQN. Details are shown in Table S1.

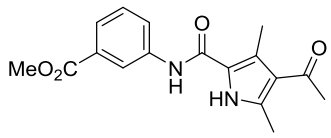
Table S1

	PDB code 4TQN
Space group	P1 21 1
Unit cell	
a (Å)	24.94
b (Å)	42.94
c (Å)	51.98
alpha	90.00
beta	97.24
gamma	90.00
Resolution range (Å)	42.94 -1.70
Unique reflections	12119(1768)
$\langle I/\sigma(I) \rangle$	15.6(5.3)
R merge	0.068(0.380)
Completeness (%)	99.9(99.2)
Multiplicity	6.5(6.2)
Refinement	
Resolution range (Å)	33.00-1.70
R factor/R free	0.1813/0.1990
Mean B factors (A2)	23.24
RMS bonds (Å)	0.006
RMS angles (°)	1.155

Chemistry

All reactions, unless otherwise stated, were carried out under a nitrogen atmosphere using standard Schlenk-techniques. All reagents were used as received unless otherwise noted. Solvents were purchased in the best quality available, degassed by purging thoroughly with nitrogen and dried over activated molecular sieves of appropriate size. Alternatively, they were purged with argon and passed through alumina columns in a solvent purification system (Innovative Technology). Reactions were monitored by thin layer chromatography (TLC) using Merck TLC silica gel 60 F_{254}. Flash column chromatography was performed over silica gel (230-400 mesh). NMR spectra were recorded on AV2 500 MHz Bruker spectrometers. Chemical shifts are given in ppm. The spectra are calibrated to the residual ^{1}H and ^{13}C signals of the solvents. Multiplicities are abbreviated as follows: singlet (s), doublet (d), triplet (t), quartet (q), doublet-doublet (dd), quintet (quint), septet (sept), multiplet (m), and broad (br). High-resolution electrospray ionization mass spectrometry was performed on a Finnigan MAT 900 (Thermo Finnigan, San Jose, CA, USA) double-focusing magnetic sector mass spectrometer. Ten spectra were acquired. A mass accuracy ≤2 ppm was obtained in the peak matching acquisition mode by using a solution containing 2 µL PEG200, 2 µL PPG450, and 1.5 mg NaOAc (all obtained from Sigma-Aldrich, Buchs, Switzerland) dissolved in 100 mL MeOH (HPLC Supra grade, Scharlau, E-Barcelona) as internal standard. The purity of all tested compounds was determined by HPLC on a Waters Acquity UPLC (Waters, Milford, MA) Top spectrometer using an Acquity BEH C18 HPLC column (1.7 µm, 1× 50 mm, Waters) with a mixture of H_2O + 0.1% HCOOH (A) and CH_3CN + 0.1% HCOOH (B) solvent (0.1 mL flow rate, linear gradient from 5% to 98% B within 4 min followed by flushing with 98% B for 1 min). Unless otherwise stated, all compounds showed ≥ 95 % purity.

Methyl 3-(4-acetyl-3,5-dimethyl-1*H*-pyrrole-2-carboxamido)benzoate (8)

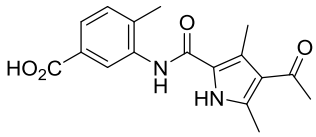


To a solution of 4-acetyl-3,5-dimethyl-1H-pyrrole-2-carboxylic acid (50 mg, 0.28 mmol) in DCM (0.5 mL) at 0 °C oxalyl chloride (24 µL, 0.28 mmol) and one drop of DMF were added. The reaction mixture was stirred at 0 °C for 30 min followed by stirring at 25 °C for 1 h. Methyl 3-aminobenzoate (35 mg, 0.23 mmol), Et_3N (77 µL, 0.55 mmol) and DMAP (4.0 mg, 0.033 mmol) were then added at 0 °C and the reaction was allowed to warm to 25 °C and stirred for 12 h. The reaction mixture was quenched with saturated NH_4Cl solution and extracted three times with EtOAc. The combined organic phases were dried over $MgSO_4$ and concentrated under reduced pressure. The residue was then purified by flash column chromatography (hexane/EtOAc, 1:1) affording the desired amide in pure form (38 mg, 53 %). Off white solid; mp 190-195 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ = 11.64 (s, 1H), 9.76 (s, 1H), 8.30 (s, 1H), 7.96 (d, *J* = 8.1 Hz, 1H), 7.66 (d, *J* = 7.7 Hz, 1H), 7.48 (t, *J* = 7.9 Hz, 1H), 3.87 (s, 3H), 2.49 (s, 3H), 2.49 (s, 3H), 2.38 (s, 3H); ^{13}C NMR (126 MHz, DMSO-d_6): δ = 194.3, 166.1, 159.8, 139.6, 136.9, 130.0, 129.2, 125.8, 124.1, 123.7, 122.2, 121.4, 120.1, 52.2, 31.1, 14.6, 12.4; IR (neat): ῡ = 3248, 2958, 2925, 1719, 1626, 1591, 1553, 1534, 1506, 1476, 1439, 1428, 1414, 1300, 1284, 1261, 1236, 1177, 1095, 1080, 1039, 948, 804, 782, 773, 749 cm^{-1}; HRMS (ESI), *m/z*: calcd for $C_{17}H_{18}N_2NaO_4^+$, 337.1159; found, 337.1158.

General procedure for ester hydrolysis

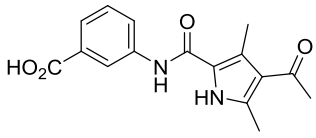
To a solution of the methyl ester (1 eq) in THF (0.1 M) 1M LiOH solution (5 eq) was added. The reaction mixture was stirred at 25 °C for 3-5 h. The reaction mixture was concentrated under reduced pressure and 1M HCl was added. The obtained precipitate was washed with hexanes, ether and cold DCM, affording the desired carboxylic acids in pure form.

3-(4-acetyl-3,5-dimethyl-1*H*-pyrrole-2-carboxamido)-4-methylbenzoic acid (5)



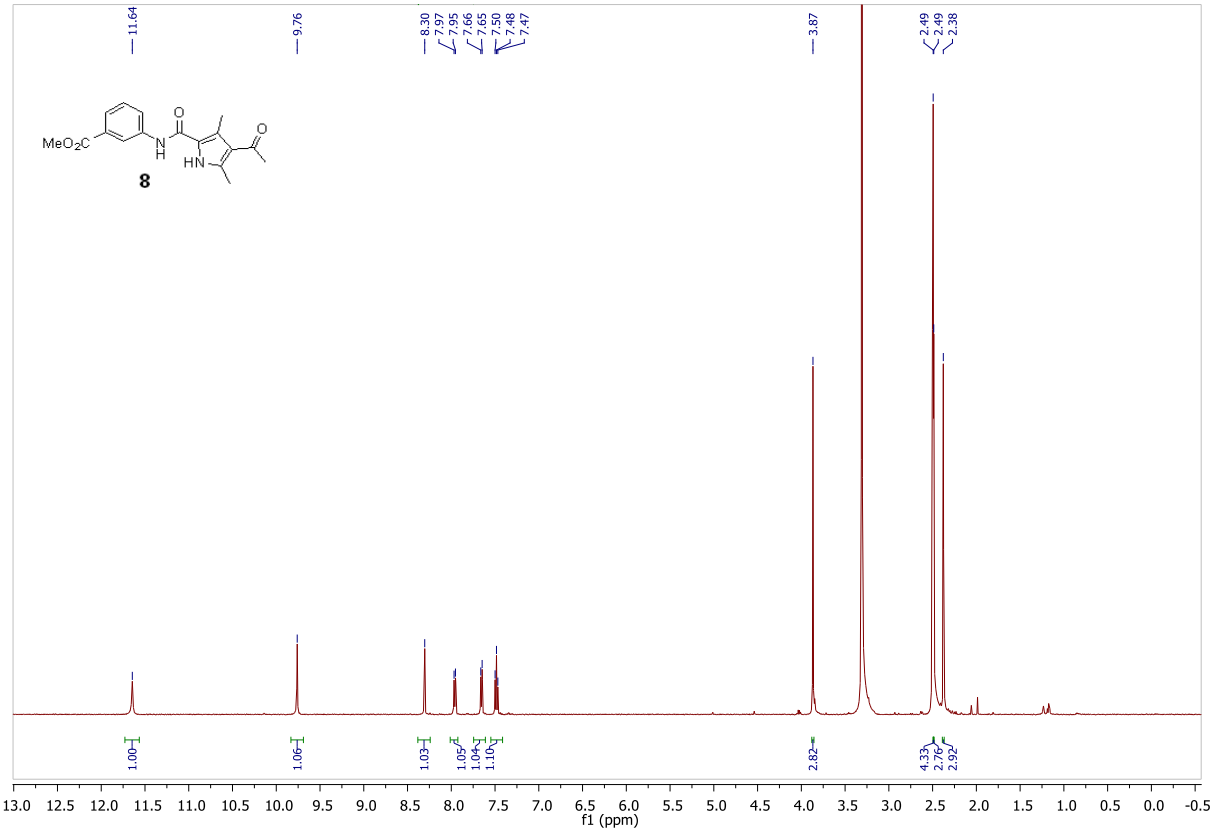
Off white solid; Yield: 59%; mp 267-270 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ = 12.88 (br, 1H), 11.74 (s, 1H), 9.17 (s, 1H), 8.05 (s, 1H), 7.69 (d, *J* = 7.7 Hz, 1H), 7.38 (d, *J* = 8.0 Hz, 1H), 2.52 (s, 3H), 2.48 (s, 3H), 2.48 (s, 3H), 2.30 (s, 3H); ^{13}C NMR (126 MHz, DMSO-d_6): δ = 194.5, 167.1, 160.0, 137.9, 137.0, 136.7, 130.7, 128.8, 126.1, 124.8, 122.1, 121.8, 31.2, 18.4, 14.6, 12.6, one C is missing due to overlapping; IR (neat): ῡ = 3451, 3257, 3018, 1719, 1617, 1580, 1540, 1480, 1457, 1412, 1367, 1312, 1280, 1254, 1224, 1189, 1175, 1075, 1040, 989, 919, 805, 765, 743 cm^{-1}; HRMS (ESI), *m/z*: calcd for $C_{17}H_{18}N_2NaO_4^+$, 337.1159; found, 337.1159.

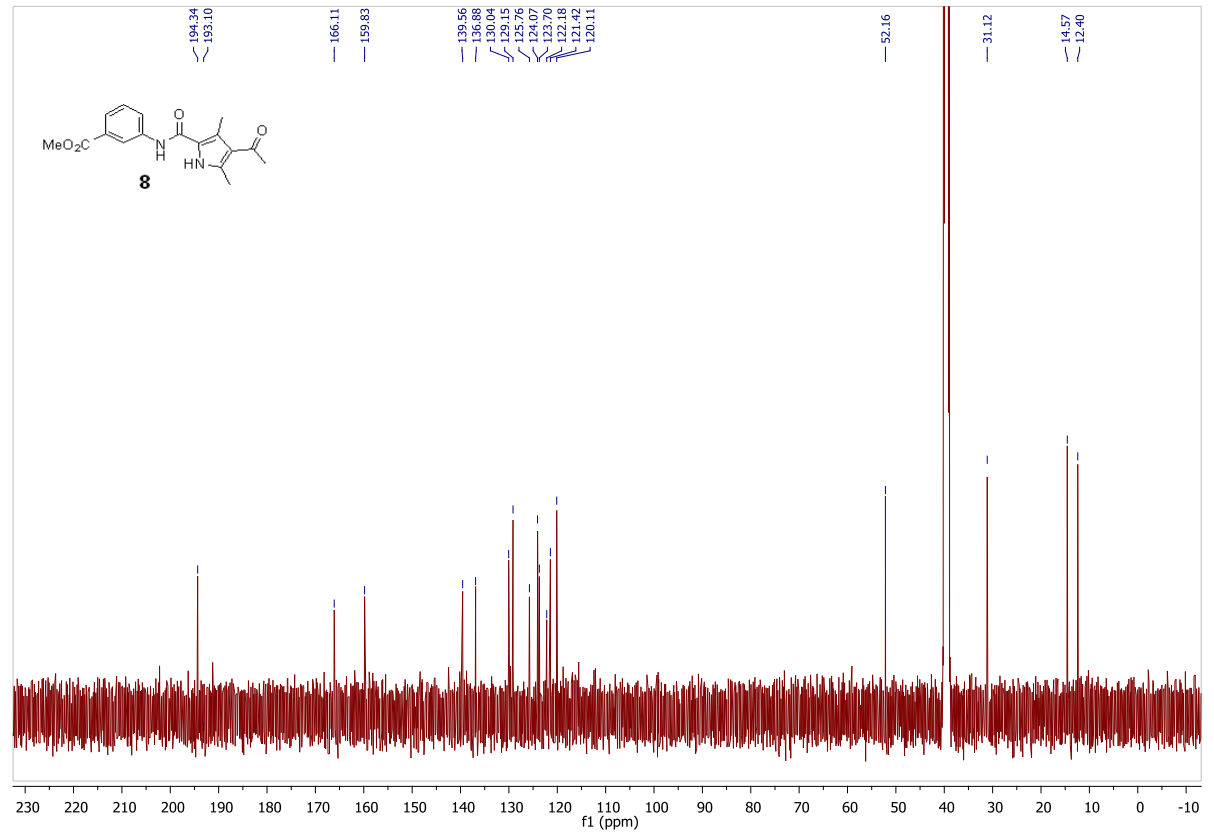
3-(4-acetyl-3,5-dimethyl-1*H*-pyrrole-2-carboxamido)benzoic acid (6)

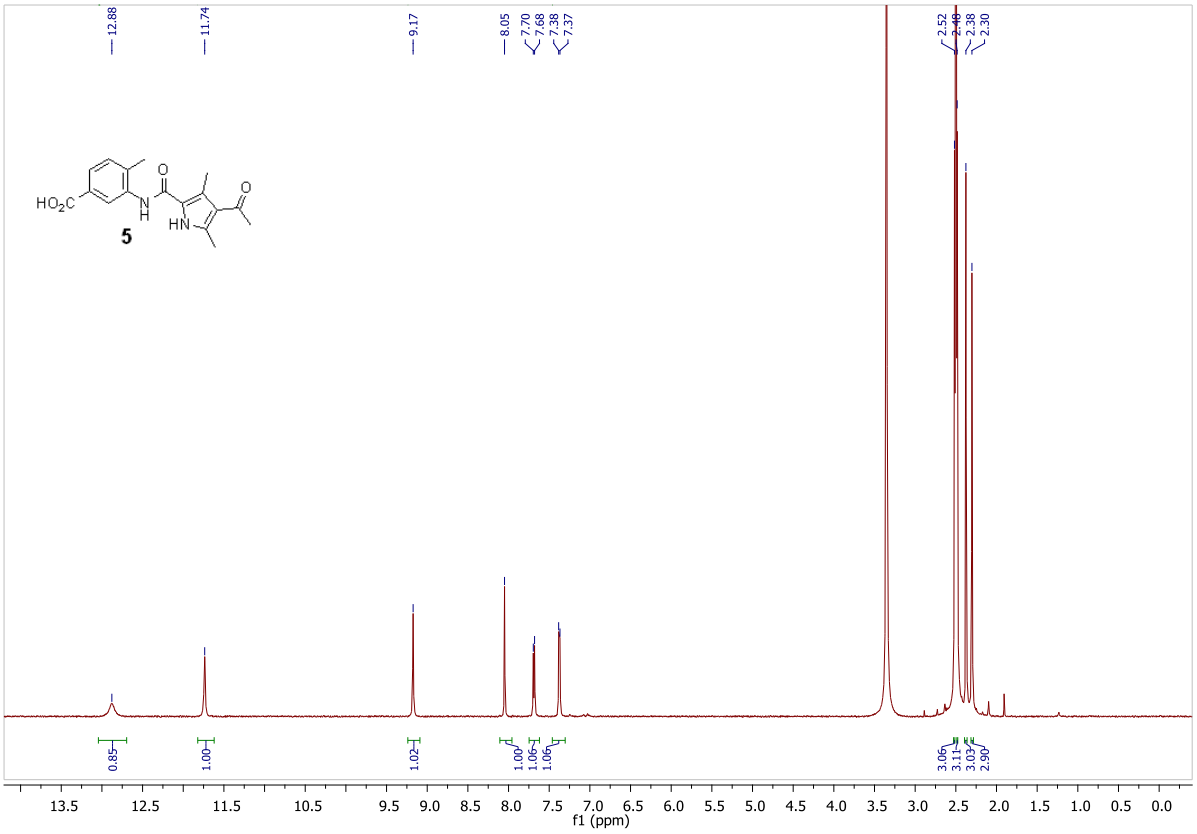


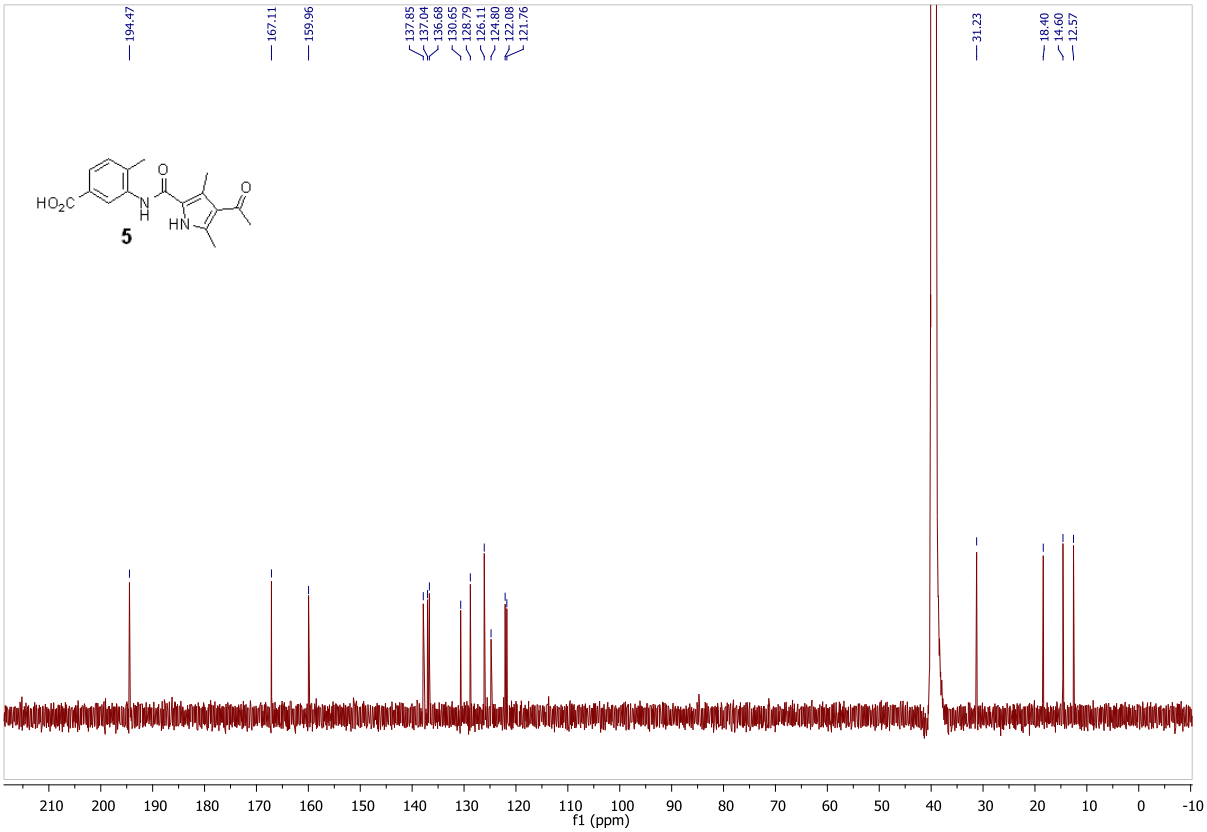
Pale brown solid; Yield: 65%; mp 249-253 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ = 10.03 (br, 1H), 8.28 (s, 1H), 7.99 (s, 1H), 7.62 (d, *J* = 7.5 Hz, 1H), 7.40 (t, *J* = 7.4 Hz, 1H), 2.37 (s, 3H), six H are missing due to overlapping with the solvent; ^{13}C NMR (126 MHz, DMSO-d_6): δ = 194.9, 160.3, 139.9, 137.6, 129.1, 126.6, 124.3, 122.6, 121.9, 121.0, 31.7, 15.2, 13.0, three C are missing due to overlapping; IR (neat): ῡ = 3441, 3182, 3109, 1704, 1626, 1591, 1542, 1483, 1439, 1413, 1363, 1294, 1250, 1186, 1069, 1038, 842, 806, 751 cm^{-1}; HRMS (ESI), *m/z*: calcd for $C_{16}H_{16}N_2NaO_4^+$, 323.1002; found, 323.0999.

^{1}H-NMR and ^{13}C-NMR spectra






12.88
11.74
9.17
8.05
7.70
7.68
7.38
7.37
2.52
2.48
2.38
2.30
HO2C
N
H
O
HN
O
5
0.85
1.00
1.02
1.00
1.06
1.06
3.06
3.11
3.03
2.90
13.5 12.5 11.5 10.5 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)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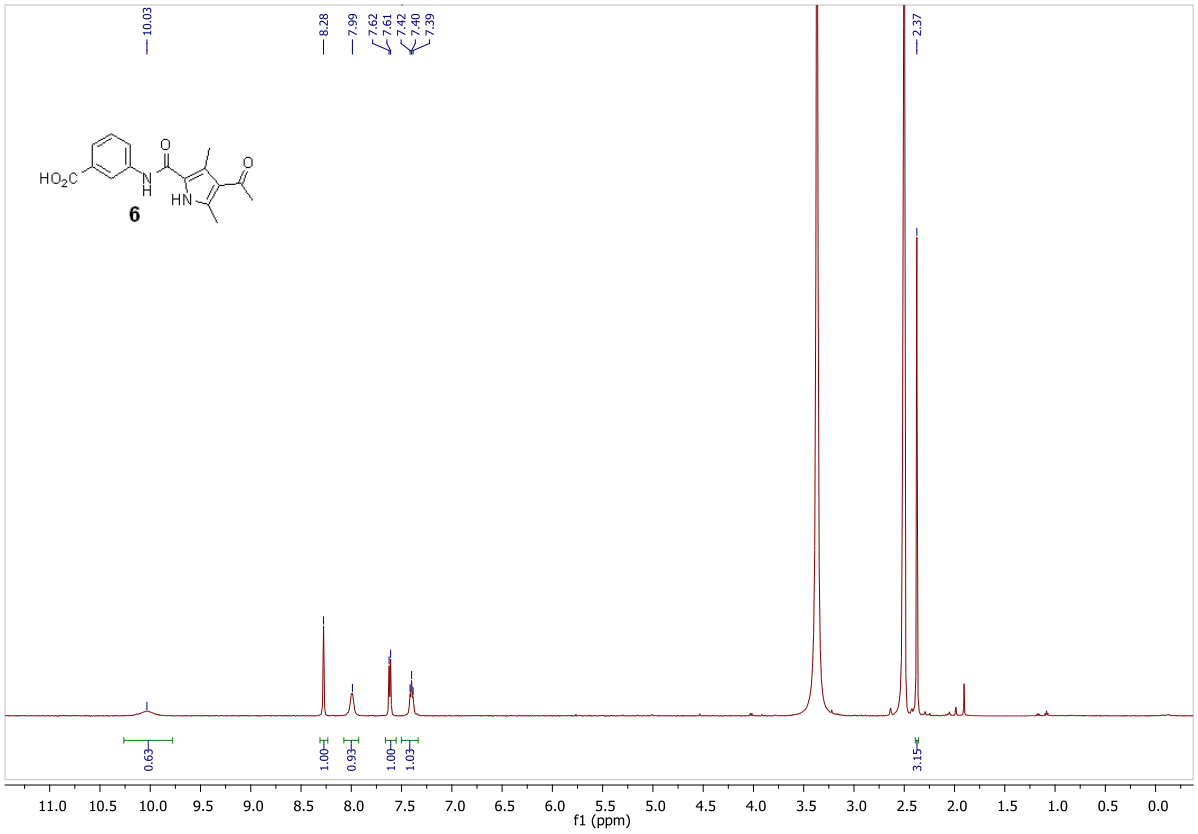
167.11
159.96
137.85
137.04
136.68
130.65
128.79
126.11
124.80
122.08
121.76
31.23
18.40
14.60
12.57
HO2C
N
H
O
HN
O
5
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)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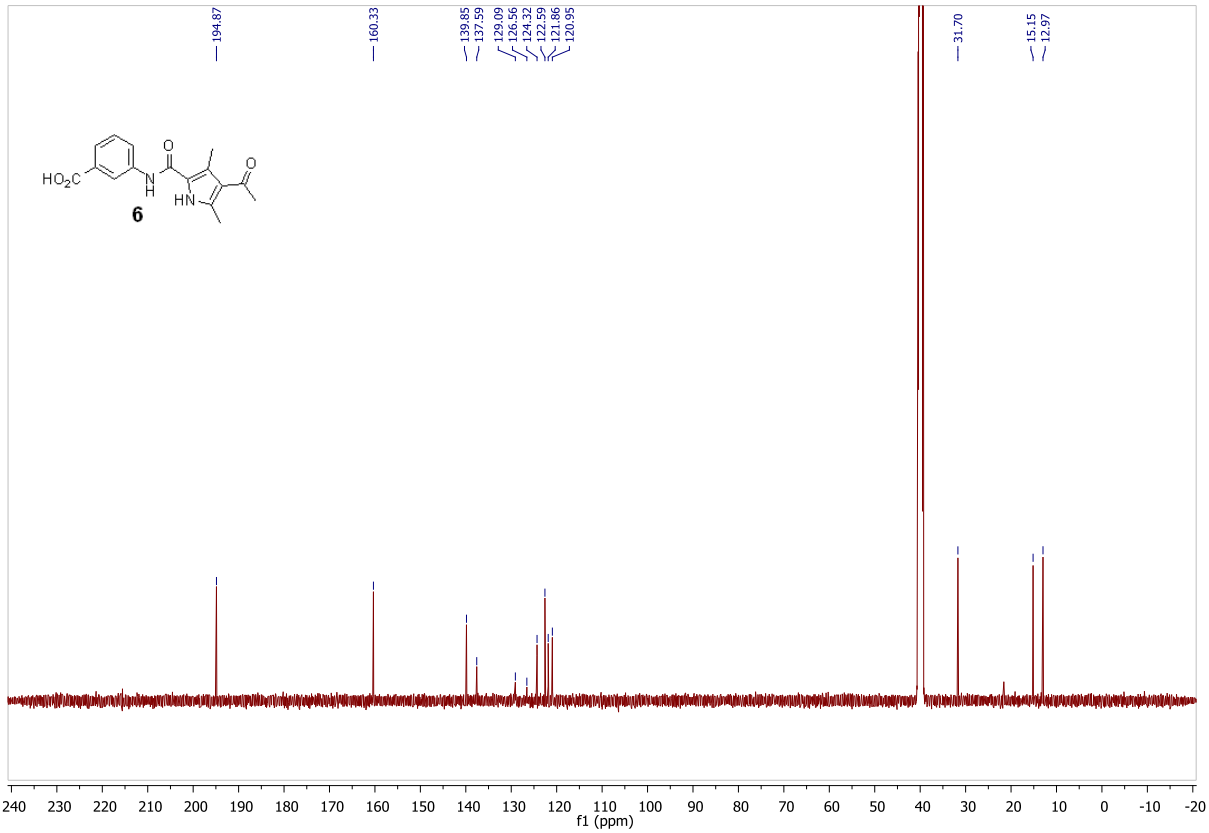
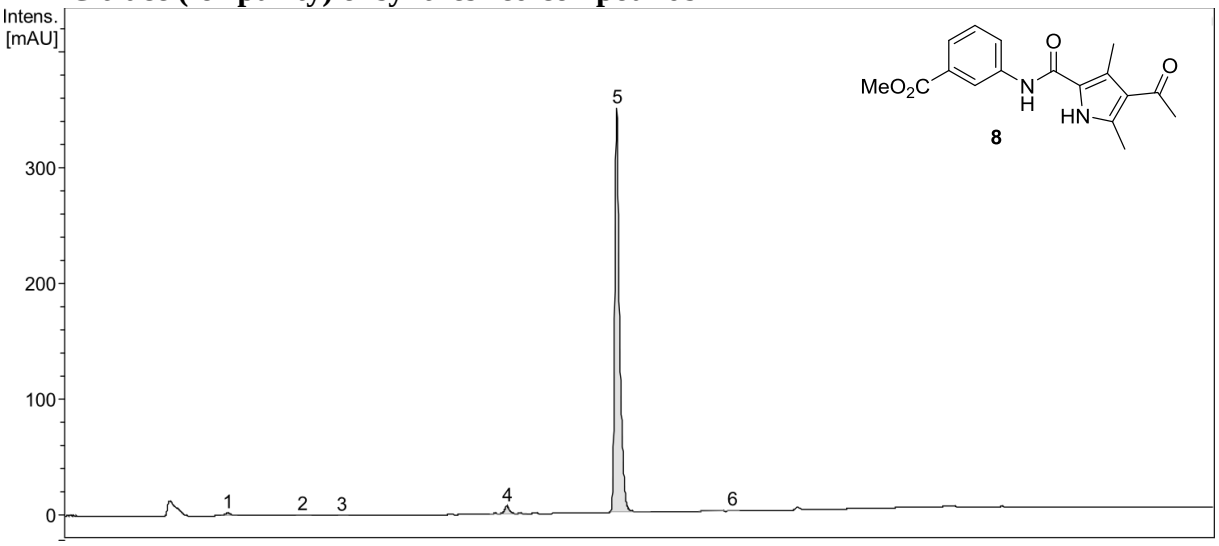
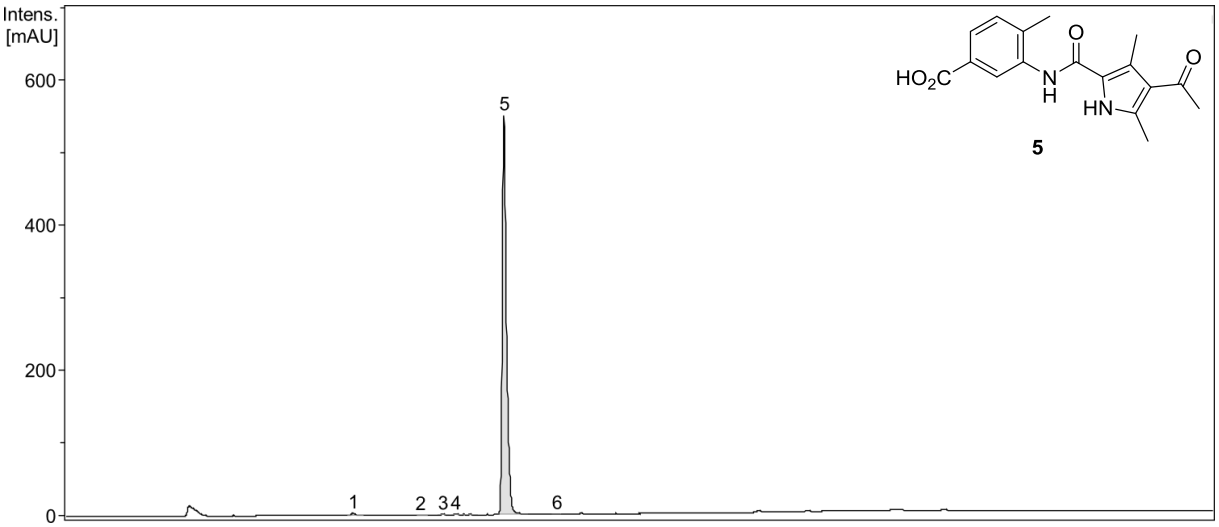
8.28
7.99
7.62
7.61
7.42
7.40
7.39
2.37
HO2C
N
H
O
HN
O
6
0.63
1.00
0.93
1.00
1.03
3.15
11.0 10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)


194.87
160.33
139.85
137.59
129.09
126.56
124.32
122.59
121.86
120.95
31.70
15.15
12.97
HO2C
N
H
O
HN
O
6
240 230 220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10 -20
f1 (ppm)

HPLC trace (for purity) of synthesized compounds



#	RT [min]	Area	Area Frac. %
1	0.9	3.4885	0.67
2	1.3	0.5209	0.10
3	1.4	0.3517	0.07
4	2.1	12.0190	2.3
5	2.6	504.3851	96.34
6	3.1	2.7686	0.53



#	RT [min]	Area	Area Frac. %
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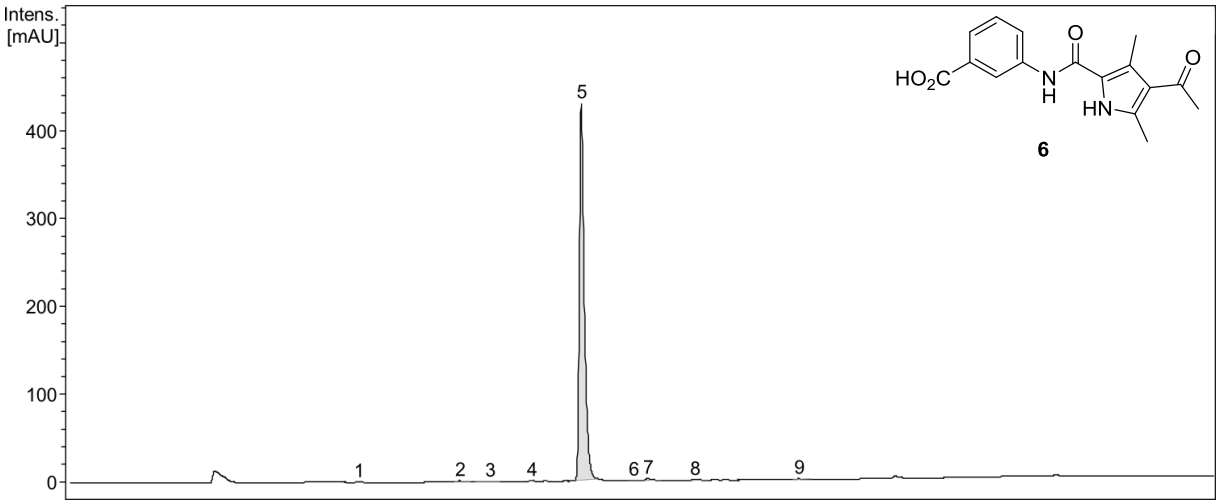
2	1.8	0.7413	0.10
3	1.9	1.6912	0.22
4	2.0	2.3257	0.30
5	2.2	753.8320	98.76
6	2.4	0.4699	0.06



#	RT [min]	Area	Area Frac. %
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2	1.7	1.4703	0.24
3	1.8	1.6665	0.28
4	1.9	2.1705	0.36
5	2.1	588.4345	97.92
6	2.3	0.6963	0.12
7	2.4	4.0236	0.67
8	2.6	1.0989	0.18
9	3.0	1.0456	0.17

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