Electronic Supplementary Information

INVOLVEMENT OF COVALENT INTERACTIONS IN THE MODE OF ACTION OF PPARβ/δ ANTAGONISTS

Åsmund Kaupang,[a] Siri Hildonen,[a] Trine G. Halvorsen,[a] Magnus Mortén,[b] Anders Vik[a] and Trond Vidar Hansen[a*]

[a]Department of Pharmaceutical Chemistry, School of Pharmacy, University of Oslo, PO Box 1068, Blindern, 0316 Oslo, Norway

[b]Department of Chemistry, University of Oslo, PO Box 1033, Blindern, 0315 Oslo, Norway

[*]Corresponding Author; Tel.: +47 22 85 74 50; E-mail: t.v.hansen@farmasi.uio.no

Contents

GENERAL

All commercially available reagents and solvents were used as received. GSK0660 (≥98, HPLC), potassium phosphate buffer (Fluka, 0.050 M, pH 7.2), potassium dihydrogen phosphate, dipotassium hydrogen phosphate, ammonium hydrogen carbonate (bicarbonate) (ABC), 1,4-dithiothreitol (DTT) and iodoacetic acid (IAA), all of analytical grade and sequencing grade, L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin from bovine pancreas, were purchased from SIGMA-Aldrich, Inc. (St Louis, MO, USA). Acetonitrile Hypergrade for LC-MS (CH_3CN) and dimethyl sulfoxide (DMSO, dried p.a.) were purchased from Merck, Darmstadt, Germany. Human recombinant *N*-His_6-PPARδ was purchased from Cayman Chemical, Ann Arbor, MI, USA. Lanthascreen™ TR-FRET PPARδ Competitive Binding Assay (PV4893) was purchased from Life Technologies, Carlsbad, CA, USA.

MilliQ™ (mqH_2O) water was produced in-house using a MilliQ™-system (Merck Millipore, Billerica, MA, USA). GSK3787 (**1**),[1] CC618 (**2**)[2] and 5-H-CC618 (**7**)[2] were prepared as previously described. Potassium phosphate buffers (1.0 M, pH 7.2 – 8.1) were prepared by titration of stirred 1.0 M solutions of KH_2PO_4 in deionized water with 1.0 M solutions of K_2HPO_4 to the desired pH endpoint, as measured with a Metrohm 744 pH Meter, calibrated at pH 7.0 and pH 9.0, twice before use.

Stirring refers to magnetic stirring in all the procedures. The stated yields are based on isolated material. Chromatography was performed on silica gel (Merck 60, 40 - 63 µm) and thin layer chromatography (TLC) on aluminum-backed silica gel plates (Merck silica gel 60 F_{254}, 250 µm thickness). The plates were visualized with UV light (254 or 366 nm) or by staining with $KMnO_4$, K_2CO_3 and NaOH in water, or with $Ce(SO_4)_2$, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, H_2SO_4 in water.

HPLC analyses were performed on an Agilent Technologies 1200 Series instrument equipped with an Agilent Eclipse XDB-C18 reverse phase column and a UV detector (254 nm).

Nuclear magnetic resonance (NMR) spectroscopy was performed on Bruker DPX300/AVII400/DRX500/AV600 spectrometers operating at 300/400/500/600 MHz for ^1H and 75/101/125/151 MHz for ^{13}C. Chemical shifts (δ) are reported in parts per million and coupling constants (*J*) are reported in Hz. Chemical shifts are reported relative to the signal from the residual non-perdeuterated solvents, in accordance with the literature:[3] δ-^1H/δ-^{13}C (Solvent); 7.26/77.16 ($CDCl_3$), 2.50/39.52 (DMSO-d_6). The utilized DMSO-d_6 contains a 0.03% v/v TMS internal standard which, in the aqueous reactions mixtures followed by ^1H NMR, was used to align the spectra at 0.00 ppm for presentation. The obtained NMR data were processed and presented with MestReNova (ver. 8.0.1, MestreLab Research S.L.).

Mass spectrometry was carried out on a Fisons (Waters) VG ProSpec Q for electron impact ionization (EI) or a Micromass Q-TOF 2 for electrospray ionization (ESI), as indicated.

UV-spectroscopy was performed on a Biochrom Libra S32 PC, equipped with an 8-cell sample changer.

The TR-FRET assay was performed in black 96-well plates (Costar). The plates were read with a Victor X4 plate reader (PerkinElmer), equipped with the recommended filters and set up according to recommendations of the assay manufacturer.[4]

PROTEIN TREATMENT AND LIQUID CHROMATOGRAPHY - MASS SPECTROMETRY (LC-MS)

Ligand treatment and in-solution digestion of *N*-His$_6$-PPARβ/δ: 10 µg (1 mg/mL) of human recombinant *N*-His$_6$-PPARβ/δ, stored at -80 °C (CO_2(s)), was thawed in an ice/water bath, diluted to a concentration of 50 µg/mL using 190 µL of freshly made 50 mM ABC buffer, fractionated to 10 stock solutions of 20 µL and subsequently refrozen at -30 °C. Prior to ligand treatment and in-solution digestion, 10 µL of the above described 50 µg/mL stock solution of human recombinant *N*-His$_6$-PPARβ/δ was thawed on ice in a Protein LoBind Eppendorf tube (Eppendorf AG, Hamburg, Germany) and subsequently diluted with 10 µL of a freshly made solution of 375 µM of DTT in 50 mM ABC buffer. The solution was left on ice for 5 min and then added 10 µL of a 30 µM solution of compound **3, 4** or **7** in 50 mM ABC buffer (w/ 1% of CH_3CN), resulting in a final concentration of 125 µM of DTT[5] and 10 µM of ligand. The solution was incubated at ambient temperature for 15 min, diluted with 55 µL of 50 mM ABC buffer and added 5 µL of a solution containing 50 mM DTT in 50 mM ABC buffer. After incubation for 15 minutes at 95 °C and subsequent cooling to ambient temperature, 5 µL of a freshly prepared solution of 250 mM iodoacetic acid (IAA) in 50 mM ABC buffer was added and the solution was incubated in the dark at ambient temperature for 20 minutes. Following alkylation, 5 µL of a freshly prepared solution of containing 2.5 µg/mL bovine trypsin in 50 mM ABC buffer (trypsin:protein ratio, 1:40 (w/w)) was added and the sample was incubated at 37 °C overnight using a Thermomixer Comfort (Thermo Scientific) at 800 rpm. The final protein concentration was 5 µg/mL in all experiments.

LC-MS/MS analysis: Samples of 20 µL of the proteolytic peptide mixtures were injected into the Chromeleon Xpress-controlled Dionex HPLC system (Thermo Scientific, Bremen, Germany) and trapped on a C18, 5 mm x 300 µm i.d. Acclaim PepMap 100 (5 µm) enrichment column (Thermo Scientific). The loading mobile phase consisting of 3% CH_3CN, 0.1% FA and 97% mqH_2O, was delivered at 10 µL/min for 4 minutes. The analytes were transferred to a 150 × 0.075 mm i.d. Acclaim PepMap 100 (pore size 100 Å, particle diameter 3 µm; Thermo Scientific) at 300 nL/min. The mobile phases consisted of A: 5% CH_3CN, 0.1% FA and 95% mqH_2O, and B: 95% CH_3CN, 0.1% FA and 5% mqH_2O. A linear gradient was run from 0% to 50% B in 60 minutes. Subsequently, the elution strength was increased to 100% B, before the column was regenerated for at least 10 column volumes. Total analysis time per run was 87 minutes. The LC setup was connected to an Xcalibur 2.0.7-controlled LTQ Discovery Orbitrap MS equipped with a Nano-ESI ion source (Thermo Fischer). The nanospray ionization source was operated in the positive ionization mode (360 µm o.d. × 20 µm i.d. distal coated fused silica emitter, 10 µm i.d. tip (New Objective, Woburn, MA, USA)). The spray voltage was set at 2.2 kV. The heated capillary was kept at 275 °C. The capillary voltage was set at 45 V and the tube lens offset at 120 V. The mass spectrometer was operated in data-dependent positive ion mode. Survey MS scans were performed in the orbitrap analyzer at a resolution of 30000, over a mass range between m/z 250 - 2000 Da, with charge state disabled. The up to 6 most intense ions per scan were fragmented by collision induced dissociation (CID) at 35% relative collision energy, activation time of 30 ms, minimum signal required of 500 and analyzed in the linear ion trap. The wide band activation option was enabled and dynamic exclusion of a time window of 15 seconds was used to minimize the extent of repeat sequencing of the peptides.

Data interpretation: The MS raw files were processed with Proteome Discoverer 1.3 (Thermo Scientific), using the Sequest algorithm, searching against a FASTA file containing all verified human proteins in UNIPROT.[6] Enzyme specificity was set to trypsin. The initial parent and fragment ion maximum mass deviations were set to 20 ppm and 0.6 Da, respectively. The search included cysteine carboxymethylation and modification of cysteine, histidine, arginine or lysine, with masses corresponding to the possible chemical modifications disscussed in the text, as variable modifications. Peptides had to be fully tryptic, and up to two missed cleavages were allowed. A decoy database search was performed by searching against a database containing the reversed protein sequences with a strict target false discovery rate (FDR) of 0.01 and a relaxed FDR of 0.05.

Detected Cys249-containing peptides:

A representative MS/MS spectrum of the covalently modified Cys249-peptides obtained by treatment of PPARβ/δ with GSK3787 (**1**) or CC618 (**2**), has been reported previously.[2]

For comparison, we analyzed the tryptic peptides obtained from apo-PPARβ/δ (no added ligand). The Cys249-containing peptide, corresponding to the peptides shown for the ligand-treated protein solutions (Figure S2 – S4), is shown below (Figure S1).

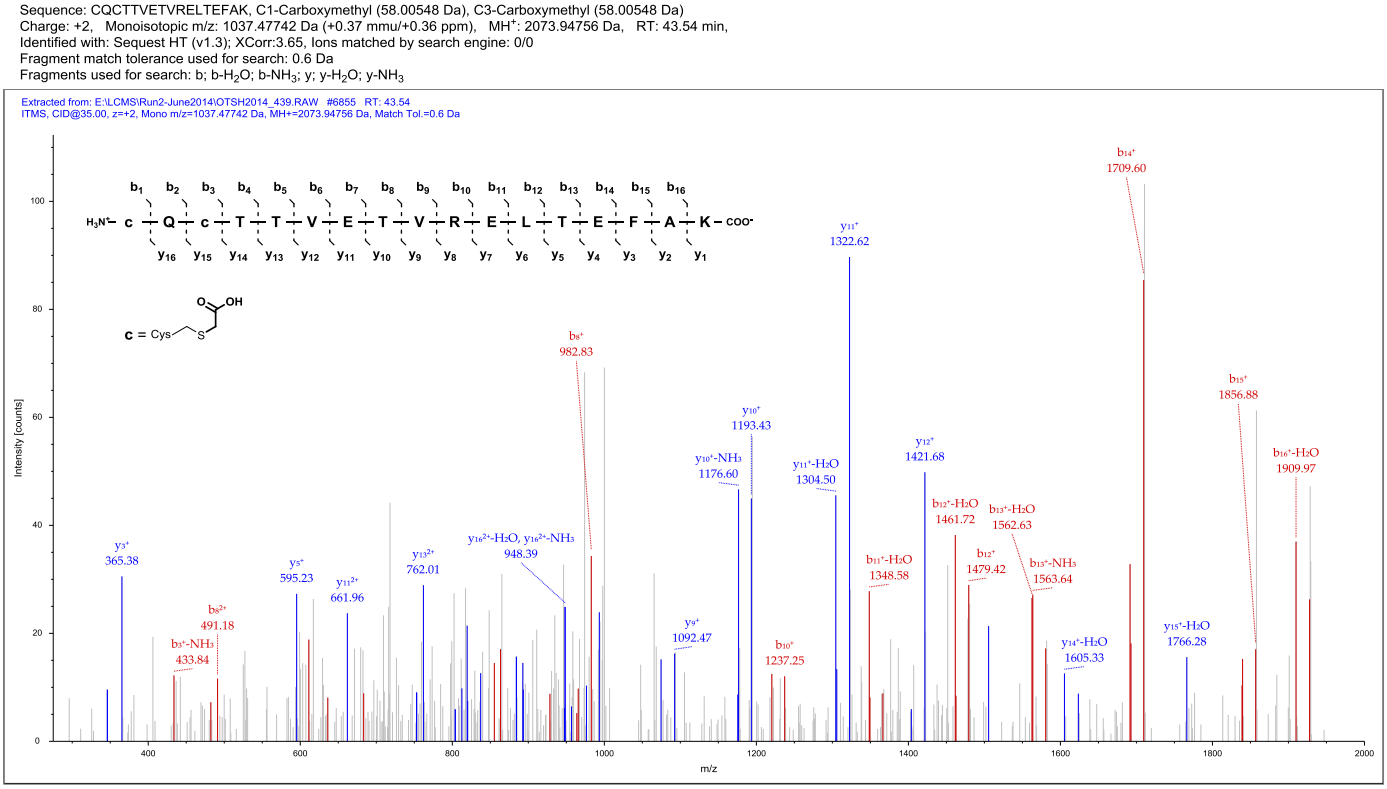


Figure S1 MS/MS spectrum of the Cys249-containing peptide CQCTTVETVRELTEFAK, obtained from the tryptic digestion of apo-PPARβ/δ, in which both Cys249 and Cys251 are found to be carboxymethylated. This spectrum is shown for comparison with Figures S2 – S4.

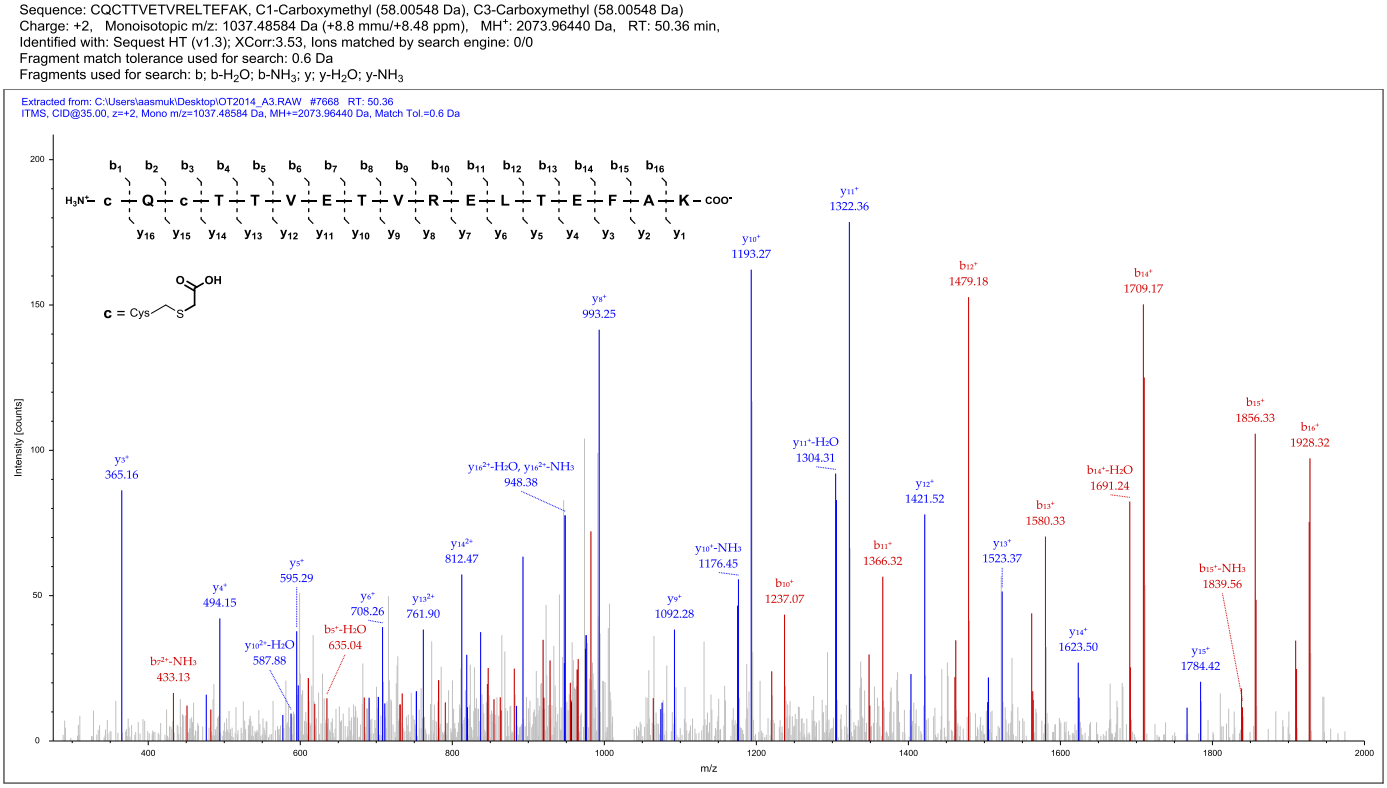


Figure S2 MS/MS spectrum of the Cys249-containing peptide CQCTTVETVRELTEFAK, obtained from the tryptic digestion of PPARβ/δ, treated with 10 μM of 5-H-CC618 (**7**), in which both Cys249 and Cys251 are found to be carboxymethylated. No modification of Cys249, corresponding to an S_NAr reaction with 5-H-CC618 (**7**), was observed.

Sequence: CQCTTVETVRELTEFAK, C1-Carboxymethyl (58.00548 Da), C3-Carboxymethyl (58.00548 Da)
Charge: +2, Monoisotopic m/z: 1037.48621 Da (+9.16 mmu/+8.83 ppm), MH^+: 2073.96514 Da, RT: 50.28 min,
Identified with: Sequest HT (v1.3); XCorr:2.95, Ions matched by search engine: 0/0
Fragment match tolerance used for search: 0.6 Da
Fragments used for search: b; b-H_2O; b-NH_3; y; y-H_2O; y-NH_3

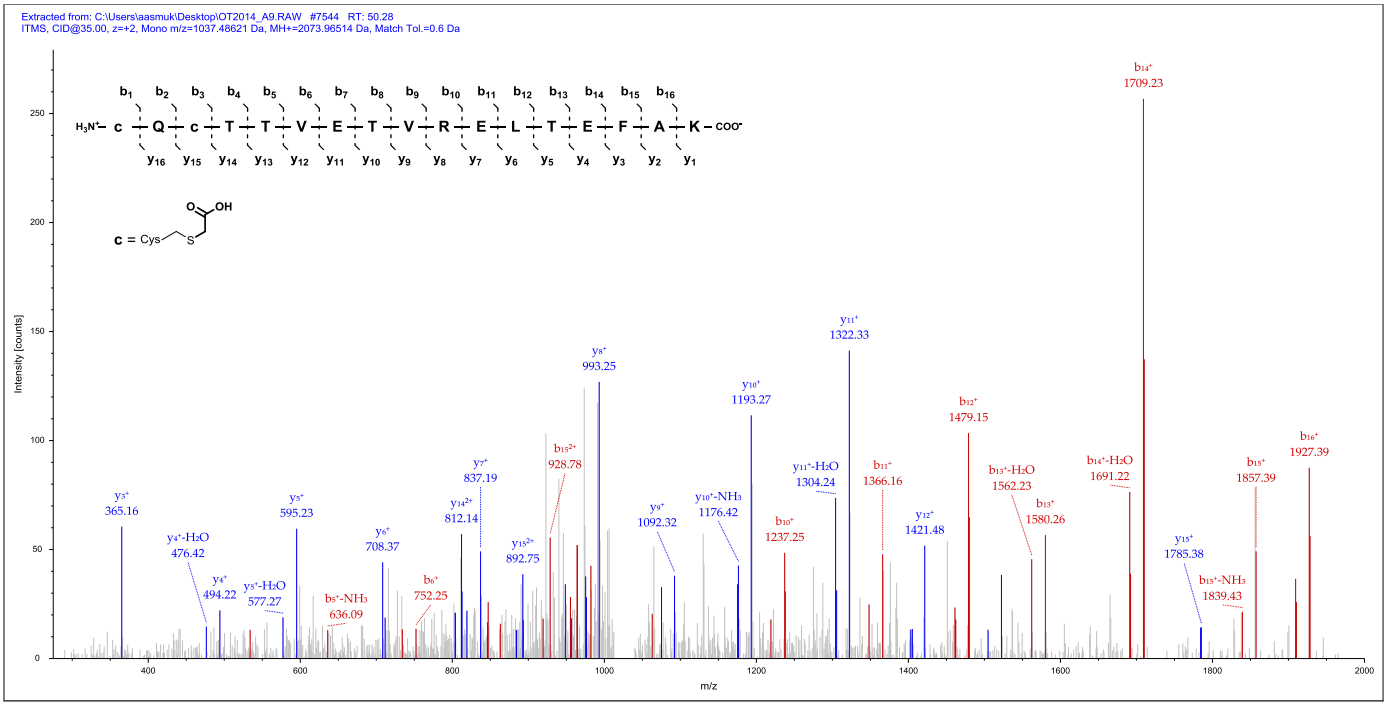


Figure S3 MS/MS spectrum of the Cys249-containing peptide CQCTTVETVRELTEFAK, obtained from the tryptic digestion of PPARβ/δ, treated with 10 μM of GSK0660 (**3**), in which both Cys249 and Cys251 are found to be carboxymethylated. No modification of Cys249, corresponding to an S_NAr reaction with GSK0660 (**3**), was observed.

Sequence: CQCTTVETVRELTEFAK, C1-Carboxymethyl (58.00548 Da), C3-Carboxymethyl (58.00548 Da)
Charge: +2, Monoisotopic m/z: 1037.48474 Da (+7.7 mmu/+7.42 ppm), MH^+: 2073.96221 Da, RT: 50.59 min,
Identified with: Sequest HT (v1.3); XCorr:3.02, Ions matched by search engine: 0/0
Fragment match tolerance used for search: 0.6 Da
Fragments used for search: b; b-H_2O; b-NH_3; y; y-H_2O; y-NH_3

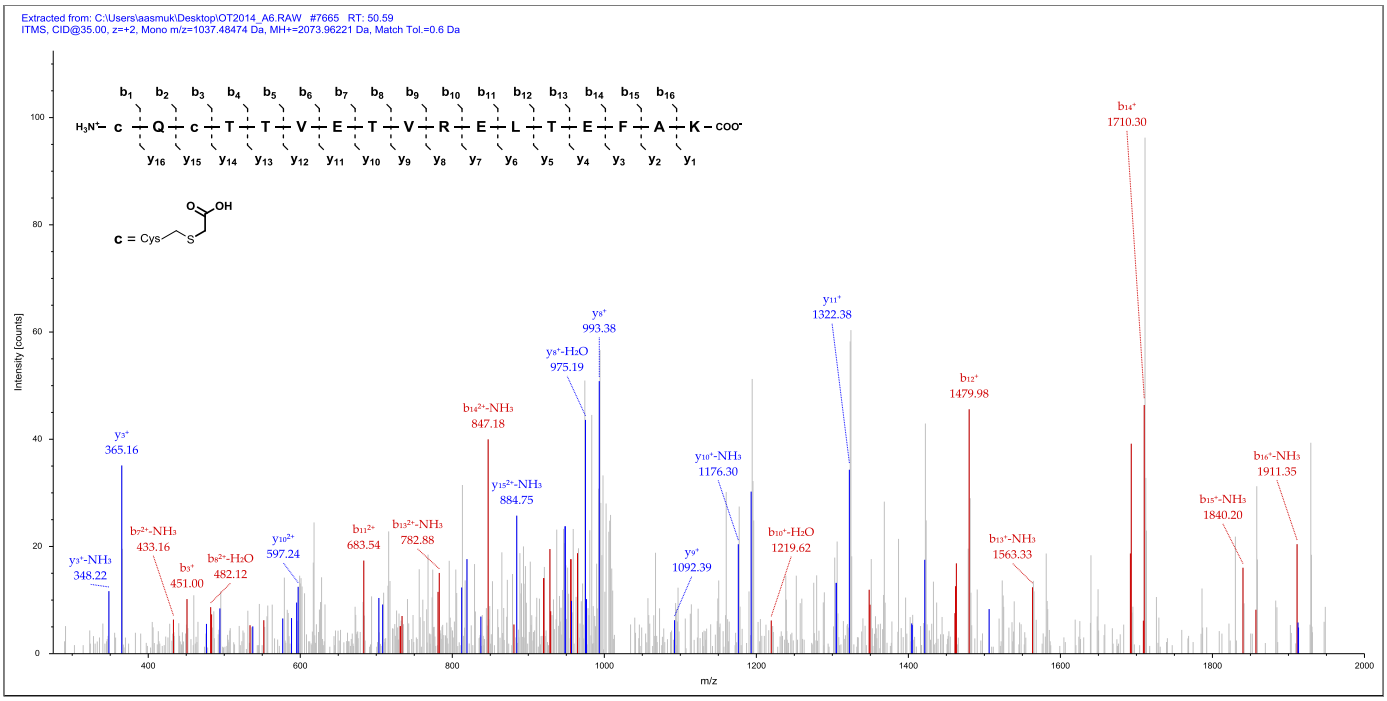


Figure S4 MS/MS spectrum of the Cys249-containing peptide CQCTTVETVRELTEFAK, obtained from the tryptic digestion of PPARβ/δ, treated with 10 μM of DG172 (**4**), in which both Cys249 and Cys251 are found to be carboxymethylated. No modification of Cys249, corresponding to a 1,4-conjugate addition reaction with DG172 (**4**), was observed.

ELECTROSPRAY IONIZATION – MASS SPECTROMETRY (ESI-MS)

To a 1 mL sample vial containing a CH_3OH solution with the indicated concentration of each compound and DMSO (see Table S1), was added a solution of the indicated amount of 2-ME in potassium phosphate buffer (PB) (0.050 M, pH 7.2) to a CH_3OH:PB-ratio of 6:1 (total volumes ranged from 200 to 500 µL). The vial was purged with argon, capped and vigorously shaken. It was then kept in the dark, at ambient temperature, for 96 hours, before performing the mass spectrometrical analysis, using electrospray ionization (ESI) and a quadrupole time-of-flight (Q-TOF) detector, in positive and negative ion modes. HCOOH and triethylamine were used to aid ionization in positive and negative ion mode, respectively. The recorded ions (≥ 2% of the base peak) are listed for the reactions with each compound below and assigned where possible.

	Compound		2-ME					
Entry	Name	Conc. (mM)	Conc. (mM)	Amount (eq)	Ratio CH_3OH:PB	PB pH[a]	PB (M)	DMSO %v/v
1	GSK3787 (**1**)[1]	6.00	60.0	10	6:1	7.2	0.050	2.4
2	CC618 (**2**)[2]	6.00	60.0	10	6:1	7.2	0.050	2.4
3	GSK0660 (**3**)[7,8]	4.0	400.0	100	6:1	7.2	0.050	5.3
4	GSK0660 (**3**)	3.0	30	10	6:1	7.2	1.0	0
5	GSK0660 (**3**)	3.0	30	10	6:1	7.8	1.0	0
6	GSK0660 (**3**)	3.0	300	100	6:1	7.8	1.0	0
7	GSK0660 (**3**)	3.0	0	0	6:1	7.8	1.0	0
8	DG172 (**4**)[9]	6.0	150.0	25	6:1	7.2	0.050	2.4

[a]The initial pH of the buffer.

Table S1 Experimental details from the reactions analyzed by ESI-MS.

Entry 1: GSK3787 ($C_{15}H_{12}ClF_3N_2O_3S$) + 2-ME (10 eq), in CH_3OH/PB (0.050 M, pH 7.2):

TOF+

***m/z* (%)**	**Assignment**
206.0 (24)	[2-((5-(trifluoromethyl)pyridin-2-yl)thio)ethan-1-ol – H_2O + H]$^+$
224.0 (100)	[2-((5-(trifluoromethyl)pyridin-2-yl)thio)ethan-1-ol + H]$^+$
270.0/272.0 (12/4)	[2-(4-chlorobenzamido)ethane-1-sulfinic acid + Na]$^+$
285.9/287.9 (6/2)	[2-(4-chlorobenzamido)ethane-1-sulfinic acid + K]$^+$ *or* [2-(4-chlorobenzamido)ethane-1-sulfonic acid + Na]$^+$
431.0/433.0 (17/6)	[GSK3787 + K]$^+$

TOF-

***m/z* (%)**	**Assignment**
154.0/156.0 (12/4)	[4-chlorobenzimidate]$^-$
246.0/248.0 (100/34)	[2-(4-chlorobenzamido)ethane-1-sulfinate]$^-$
262.0/264.0 (13/5)	[2-(4-chlorobenzamido)ethane-1-sulfonate]$^-$

Entry 2: CC618 ($C_{20}H_{15}F_6N_3O_3S_2$) + 2-ME (10 eq), in CH_3OH/PB (0.050 M, pH 7.2):

TOF+

***m/z* (%)**	**Assignment**
206.0 (100)	[2-((5-(trifluoromethyl)pyridin-2-yl)thio)ethan-1-ol – H_2O + H]$^+$
224.0 (9)	[2-((5-(trifluoromethyl)pyridin-2-yl)thio)ethan-1-ol + H]$^+$
379.0 (71)	[2-(4-methyl-2-(4-(trifluoromethyl)phenyl)thiazole-5-carboxamido)ethane-1-sulfinic acid + H]$^+$
401.0 (27)	[2-(4-methyl-2-(4-(trifluoromethyl)phenyl)thiazole-5-carboxamido)ethane-1-sulfinic acid + Na]$^+$
417.0 (10)	[2-(4-methyl-2-(4-(trifluoromethyl)phenyl)thiazole-5-carboxamido)ethane-1-sulfinic acid + K]$^+$ *or* [2-(4-methyl-2-(4-(trifluoromethyl)phenyl)thiazole-5-carboxamido)ethane-1-sulfonic acid + Na]$^+$
501.0 (18)	Not assigned
524.0 (11)	[CC618 + H]$^+$,
562.0 (9)	[CC618 + K]$^+$

TOF-

***m/z* (%)**	**Assignment**
377.0 (100)	[2-(4-methyl-2-(4-(trifluoromethyl)phenyl)thiazole-5-carboxamido)ethane-1-sulfinate]$^-$
393.0 (10)	[2-(4-methyl-2-(4-(trifluoromethyl)phenyl)thiazole-5-carboxamido)ethane-1-sulfonate]$^-$

Entry 3: GSK0660 ($C_{19}H_{18}N_2O_5S_2$) + 2-ME (100 eq), in CH_3OH/PB (0.050 M, pH 7.2):

TOF+

***m/z* (%)**	**Assignment**
214.1 (6)	[3-methoxy-N^1-phenylbenzene-1,4-diamine]$^+$ **HRMS** calcd. for $C_{13}H_{14}N_2O$: 214.1106; found: 214.1116 (+ 4.6 ppm)
229.0 (15)	[2-(methoxycarbonyl)thiophene-3-sulfinic acid + Na]$^+$ **HRMS** calcd. for $C_6H_6O_4S_2Na$: 228.9605; found: 228.9613 (+ 3.38 ppm)
244.9 (4)	[2-(methoxycarbonyl)thiophene-3-sulfinic acid + K]$^+$ **HRMS** calcd. for $C_6H_6O_4S_2K$: 244.9344; found: 244.9353 (+ 3.42 ppm)
441.1 (41)	[GSK0660 + Na]$^+$
457.0 (100)	[GSK0660 + K]$^+$
859.1 (24)	[2 GSK0660 + Na]$^+$
875.1 (33)	[2 GSK0660 + K]$^+$

TOF-

***m/z* (%)**	**Assignment**
417.0 (100)	[GSK0660 – H]$^-$
453.0 (3)	[GSK0660 + Cl]$^-$

Entry 4: GSK0660 ($C_{19}H_{18}N_2O_5S_2$) + 2-ME (10 eq), in CH_3OH/PB (1.0 M, pH 7.2):

TOF+

***m/z* (%)**	**Assignment**
244.9 (3)	[2-(methoxycarbonyl)thiophene-3-sulfinic acid + K]$^+$
457.0 (100)	[GSK0660 + K]$^+$
495.0 (14)	[GSK0660 –H + 2 K]$^+$
875.1 (2)	[2 GSK0660 + K]$^+$

TOF-

***m/z* (%)**	**Assignment**
359.1 (12)	[GSK0660 – H – (CO_2CH_3 + H)]$^-$
417.1 (100)	[GSK0660 – H]$^-$

Entry 5: GSK0660 ($C_{19}H_{18}N_2O_5S_2$) + 2-ME (10 eq), in CH_3OH/PB (1.0 M, pH 7.8):

TOF+

***m/z* (%)**	**Assignment**
244.9 (2)	[2-(methoxycarbonyl)thiophene-3-sulfinic acid + K]$^+$
457.0 (100)	[GSK0660 + K]$^+$
495.0 (2)	[GSK0660 –H + 2 K]$^+$

TOF-

***m/z* (%)**	**Assignment**
359.1 (28)	[GSK0660 – H – (CO_2CH_3 + H)]$^-$
417.1 (100)	[GSK0660 – H]$^-$

Entry 6: GSK0660 ($C_{19}H_{18}N_2O_5S_2$) + 2-ME (100 eq), in CH_3OH/PB (1.0 M, pH 7.8):

TOF+

***m/z* (%)**	**Assignment**
244.9 (4)	[2-(methoxycarbonyl)thiophene-3-sulfinic acid + K]$^+$
386.7 (5)	Not assigned
457.0 (100)	[GSK0660 + K]$^+$
495.0 (49)	[GSK0660 –H + 2 K]$^+$

TOF-

***m/z* (%)**	**Assignment**
359.1 (9)	[GSK0660 – H – (CO_2CH_3 + H)]$^-$
417.1 (100)	[GSK0660 – H]$^-$

Entry 7: GSK0660 ($C_{19}H_{18}N_2O_5S_2$) in CH_3OH/PB (1.0 M, pH 7.8):

TOF+

***m/z* (%)**	**Assignment**
244.9 (3)	[2-(methoxycarbonyl)thiophene-3-sulfinic acid + K]$^+$
386.8 (5)	Not assigned
457.0 (100)	[GSK0660 + K]$^+$
495.0 (17)	[GSK0660 –H + 2 K]$^+$

TOF-

***m/z* (%)**	**Assignment**
189.1 (8)	Not assigned
205.1 (4)	Not assigned
243.1 (3)	Not assigned
359.1 (43)	[GSK0660 – H – (CO_2CH_3 + H)]$^-$ **HRMS** calcd. for $C_{17}H_{15}N_3O_3S_2$: 359.0524; found: 359.0529 (+1.35 ppm)
417.1 (100)	[GSK0660 – H]$^-$

Entry 8: DG172 ($C_{20}H_{20}BrN_3$) + 2-ME (25 eq), in CH_3OH/PB (0.050 M, pH 7.2):

TOF+

***m/z* (%)**	**Assignment**
382.1/384.1 (56/54)	$[DG172 + H]^+$
420.1/422.1 (6/7)	$[DG172 + K]^+$
460.1/462.1 (98/100)	$[DG172\text{-}2\text{-}ME + H]^+$
482.1/484.1 (3/3)	$[DG172\text{-}2\text{-}ME + Na]^+$
498.1/500.1 (17/19)	$[DG172\text{-}2\text{-}ME + K]^+$

NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY

To an NMR tube was added DMSO-d_6 (500 µL), followed by a DMSO-d_6 solution (20 µL) containing the indicated amount of each compound (see Table S2). The solution was vortexed to homogeneity before adding the indicated amount of 2-ME in potassium phosphate buffer (PB) (0.050 M, pH 7.2, 100 µL). The NMR tube was then vigorously shaken prior to insertion into the NMR magnet (Bruker DRX500). Subsequently, a presaturation water suppression pulse sequence (lc1pnf2) was employed to record water-suppressed ^{1}H NMR spectra at fixed intervals, instantiated by the multi_zgvd script in the Bruker TopSpin (ver. 1.3) software. The resulting NMR data were processed with MestReNova (ver. 8.0.1, MestReLabs Inc.). The absolute integrals of comparable signals from the starting material and eventual identifiable products, spanning the entire time series, were exported to Microsoft Excel, normalized and Origin (ver. 9.1.0, OriginLab Corporation) was then used to produce the graphs seen in figures S8, S10 and S12.

Compound		2-ME		Buffer		NMR experimental details	
Name	Amount (µmol)	Amount (µmol)	Amount (eq)	Concentration (M)	pH[a]	Recording interval (minutes)	Recording time (hours)
GSK3787 (**1**)[1]	5.00	50.25	10.05	0.050	7.2	15	24
CC618 (**2**)[2]	5.00	50.25	10.05	0.050	7.2	15	24
5-H-CC618 (**7**)[2]	5.00	50.25	10.05	0.050	7.2	15	24
GSK0660 (**3**)[7,8]	2.25	56.25	25.00	0.050	7.2	15	24
GSK0660 (**3**)	2.25	225.6	100.3	0.050	7.2	15	24
DG172 (**4**)[9]	7.50	7.50	10.00	0.050	7.2	10	24
DG172 (**4**)	7.50	187.5	25.00	0.050	7.2	10	24
DG172 (**4**)	7.50	750.0	100.0	0.050	7.2	10	24
DG172·HCl (**4·HCl**)	5.00	50.00	10.00	1.0	7.2	(0, 24 h)	24
DG172·HCl (**4·HCl**)	5.00	50.00	10.00	1.0	7.5	(0, 24 h)	24
DG172·HCl (**4·HCl**)	5.00	50.00	10.00	1.0	7.8	(0, 24 h)	24
DG172·HCl (**4·HCl**)	5.00	50.00	10.00	1.0	8.1	(0, 24 h)	24

[a]The initial pH of the buffer.

Table S2 Experimental details from the reactions monitored by ^{1}H NMR.

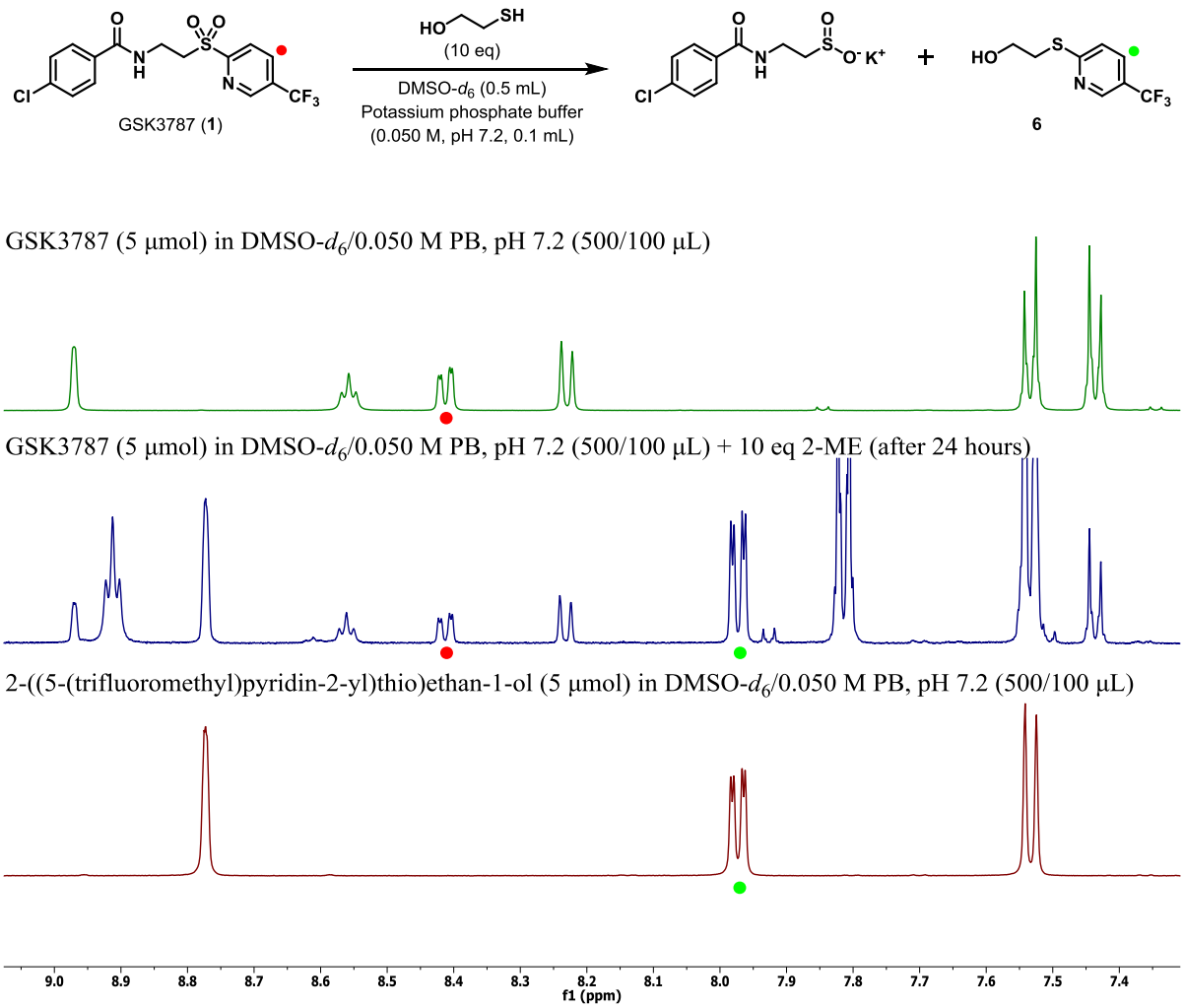


Figure S5 ^{1}H NMR monitoring (partial spectrum) of GSK3787 (**1**) treated with 2-ME (10 eq). The integrals of the signals marked with red and green spheres were followed for 24 hours (see Figure S8) The spectrum of the independently synthesized sulfide 2-((5-(trifluoromethyl)pyridin-2-yl)thio)ethan-1-ol (**6**) is shown at the bottom for comparison.

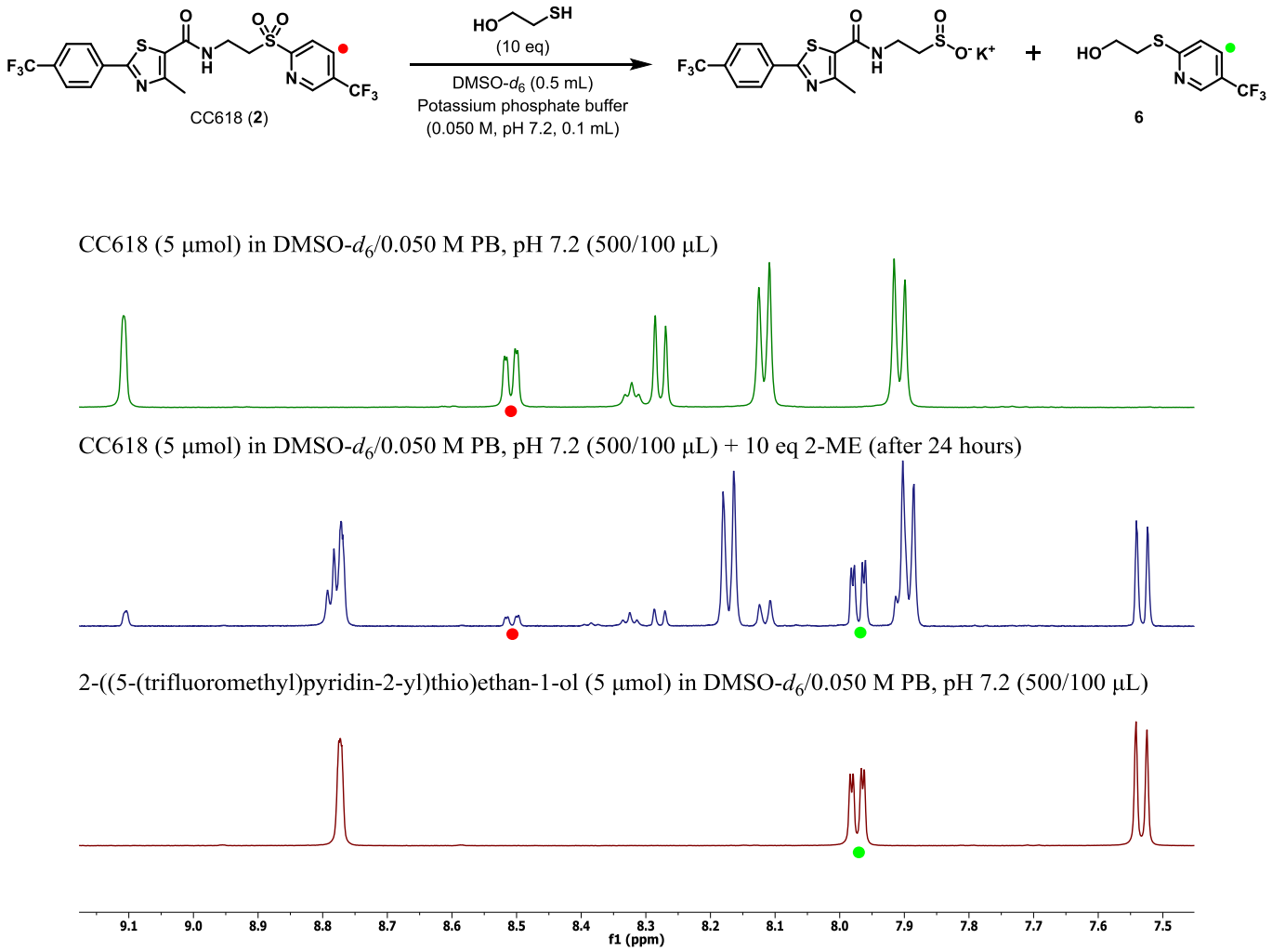


Figure S6 ^{1}H NMR monitoring (partial spectrum) of CC618 (**2**) treated with 2-ME (10 eq). The integrals of the signals marked with red and green spheres were followed for 24 hours (see Figure S8). The spectrum of the independently synthesized sulfide 2-((5-(trifluoromethyl)pyridin-2-yl)thio)ethan-1-ol (**6**) is shown at the bottom for comparison.

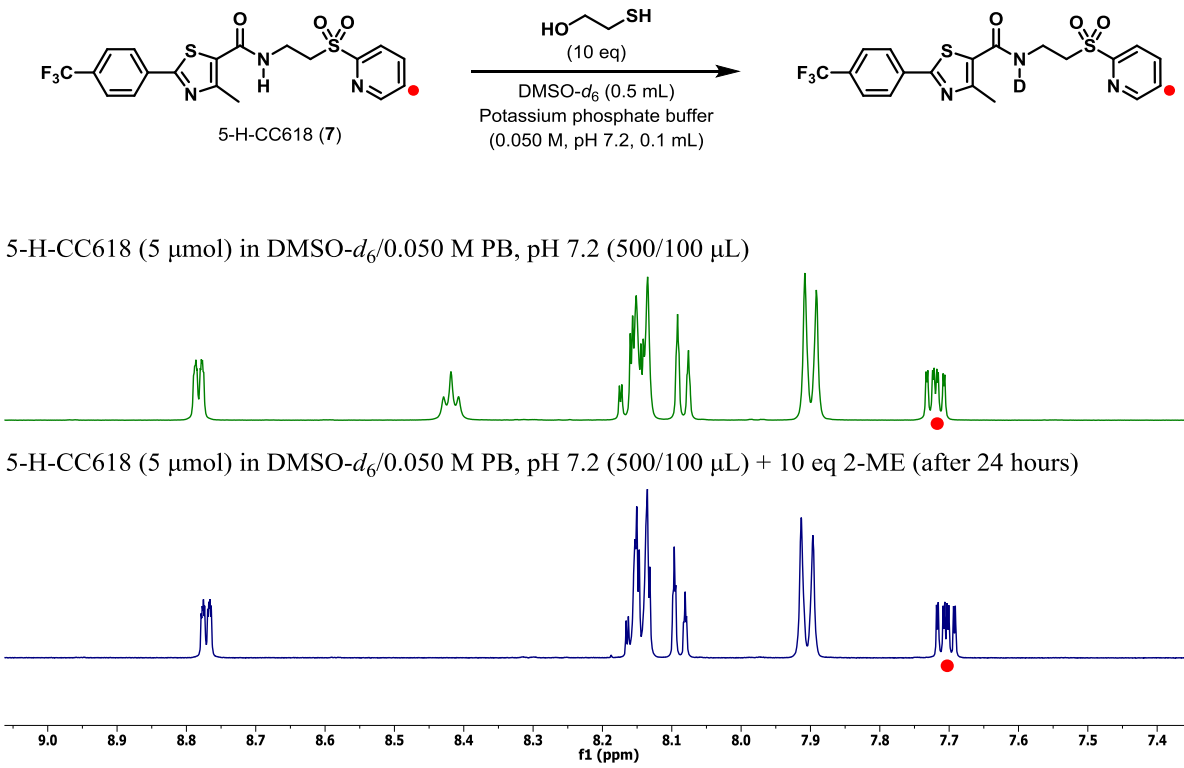


Figure S7 ^{1}H NMR monitoring (partial spectrum) of 5-H-CC618 (**7**) treated with 2-ME (10 eq). The integrals of the signal marked with a red sphere were followed for 24 hours (see Figure S8).

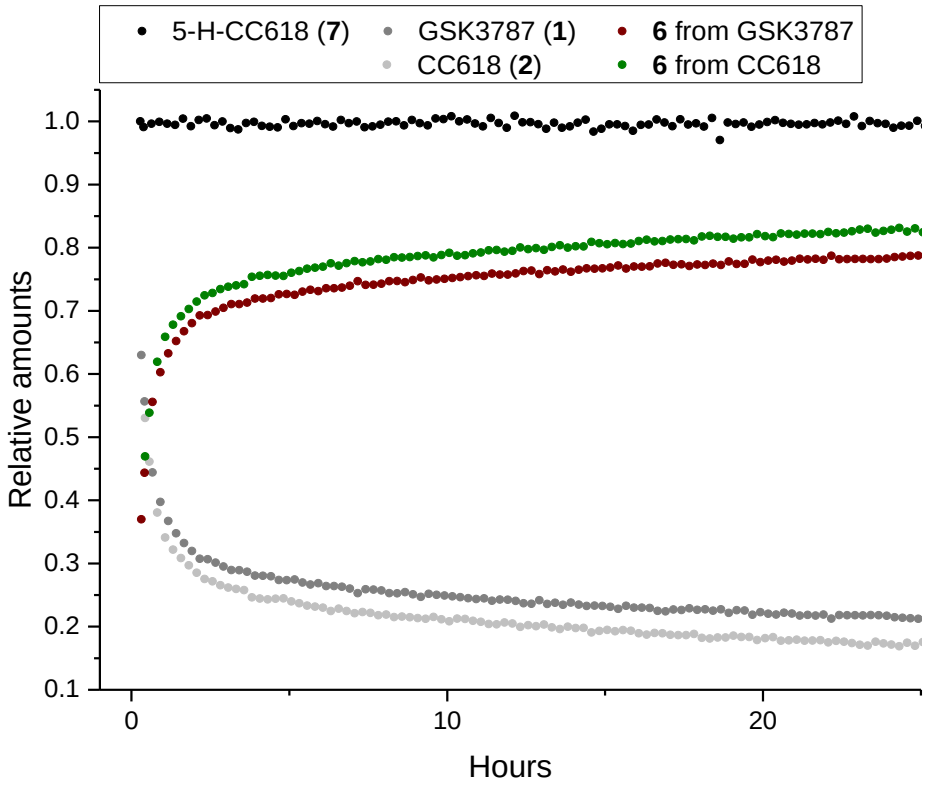


Figure S8 Starting materials (**1**, **2** or **7**) and the produced sulfide 2-((5-(trifluoromethyl)pyridin-2-yl)thio)ethan-1-ol (**6**), as observed by ^{1}H NMR spectroscopy, upon treatment of GSK3787 (**1**), CC618 (**2**) or 5-H-CC618 (**7**) with 2-ME (10 eq) in DMSO-d_6/potassium phosphate buffer (0.050 M, pH 7.2).

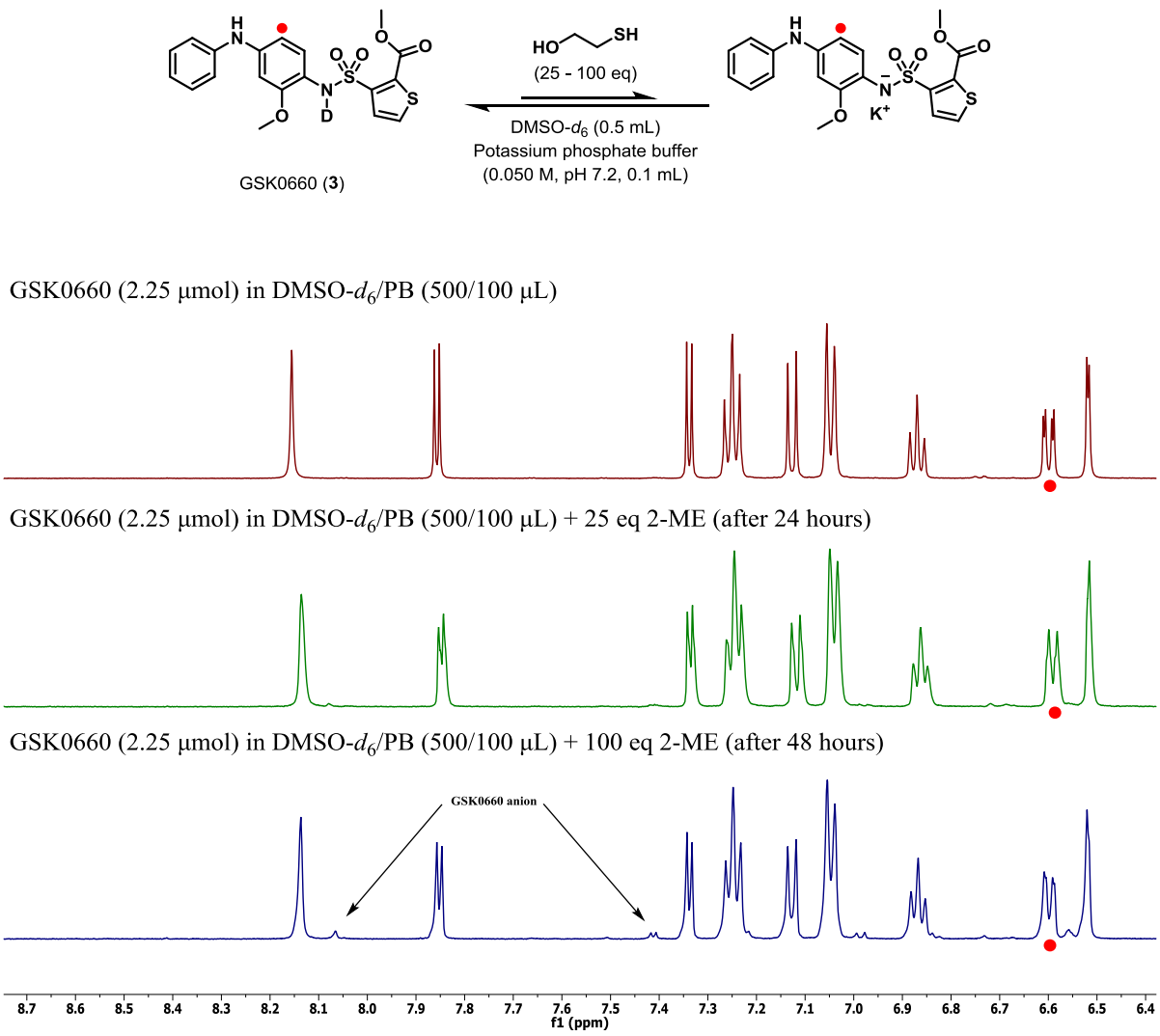


Figure S9 ^{1}H NMR monitoring (partial spectrum) of GSK0660 (**3**) treated with 2-ME (25 or 100 eq). The integrals of the signal marked with a red sphere were followed for 24 or 48 hours (see Figure S10).

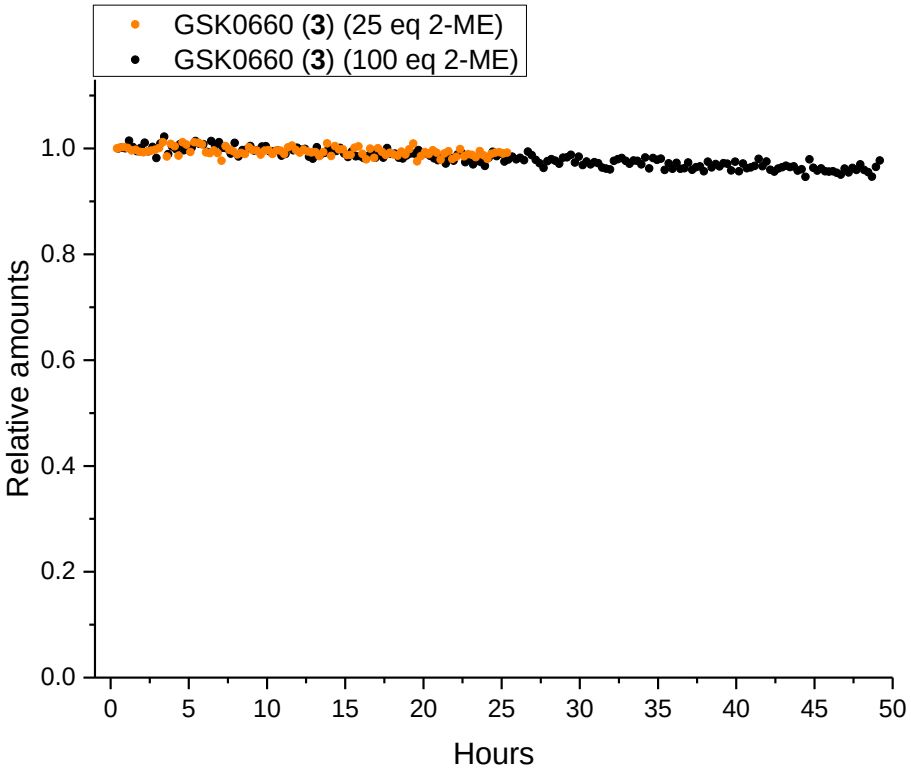


Figure S10 GSK0660 (**3**), as observed by ^{1}H NMR spectroscopy, upon treatment with 2-ME (25 or 100 eq) in DMSO-d_6/0.050 M potassium phosphate buffer. The integrals of the signals from the protons marked with red spheres in Figure S9 were followed for 24 – 48 hours.

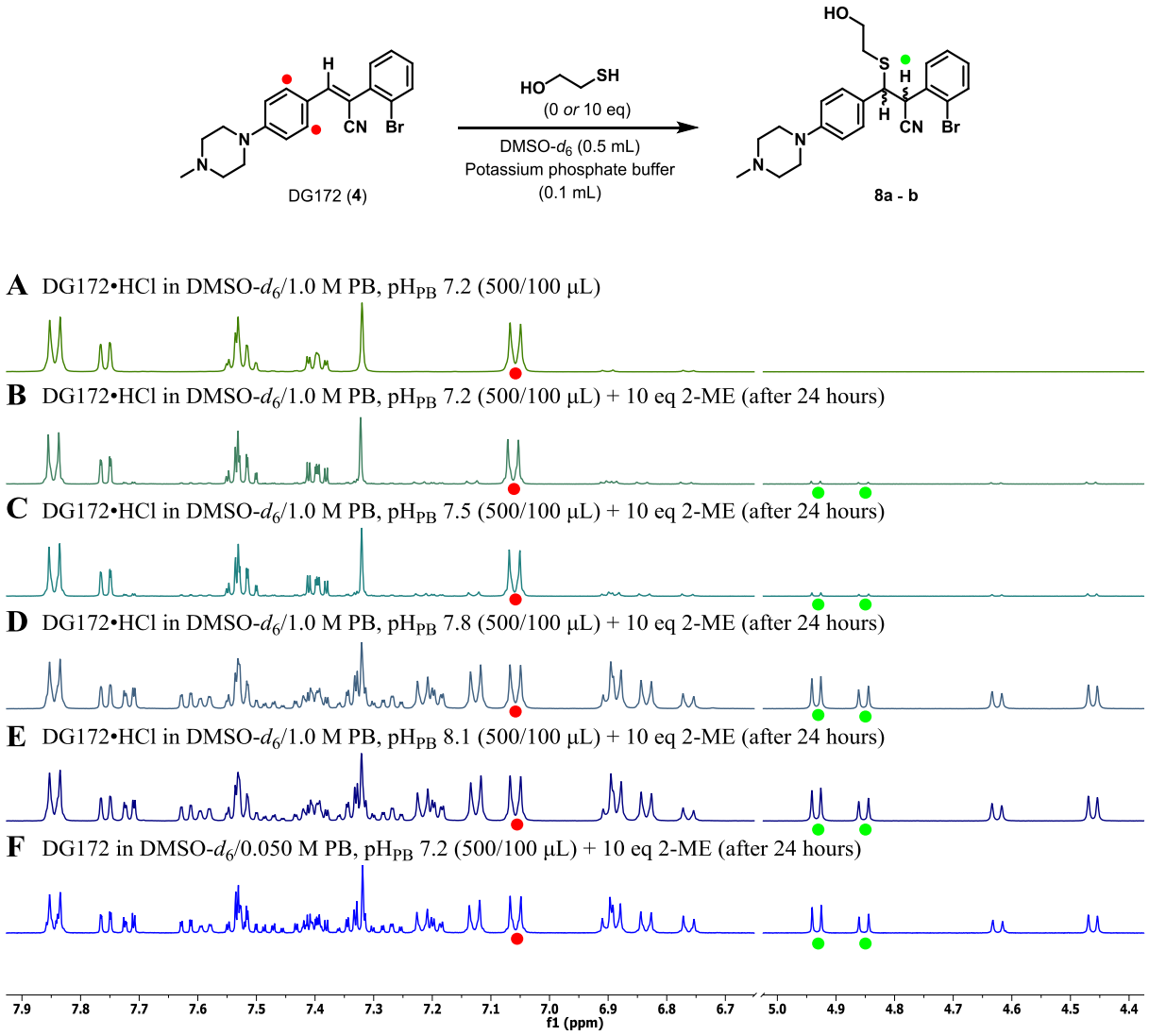


Figure S11 ^{1}H NMR monitoring (split, partial spectrum) of DG172·HCl (**4·HCl**) alone (**A**), treated with 2-ME (10 eq) in DMSO-d_6/1.0 M potassium phosphate buffer, at increasing pH (7.2 – 8.1, **B** – **E**) and DG172 (**4**) treated with 2-ME (10 eq) in DMSO-d_6/0.050 M potassium phosphate buffer (pH 7.2) (**F**), shown for comparison. The integrals of the signals from the protons marked with red and green spheres in **B** – **E** (after 24 hours) were used to create Table S3.

Buffer pH (1.0 M)	DG172 (**4**)	DG172-2-ME adducts (**8a-b**)
7.2	0.903	0.097
7.5	0.879	0.121
7.8	0.403	0.597
8.1	0.400	0.600

Table S3 Extent of formation of DG172-2-ME adducts (**8a – b**) upon treatment of DG172·HCl (**4·HCl**) with 2-ME (10 eq) for 24 hours at pH 7.2, 7.5, 7.8 and 8.1, as described in Figure S11. The values were obtained from single experiments and were normalized from the absolute integrals of comparable peaks in starting material and product (red and green spheres in **B** – **E** in Figure S11), here given as their ratio.

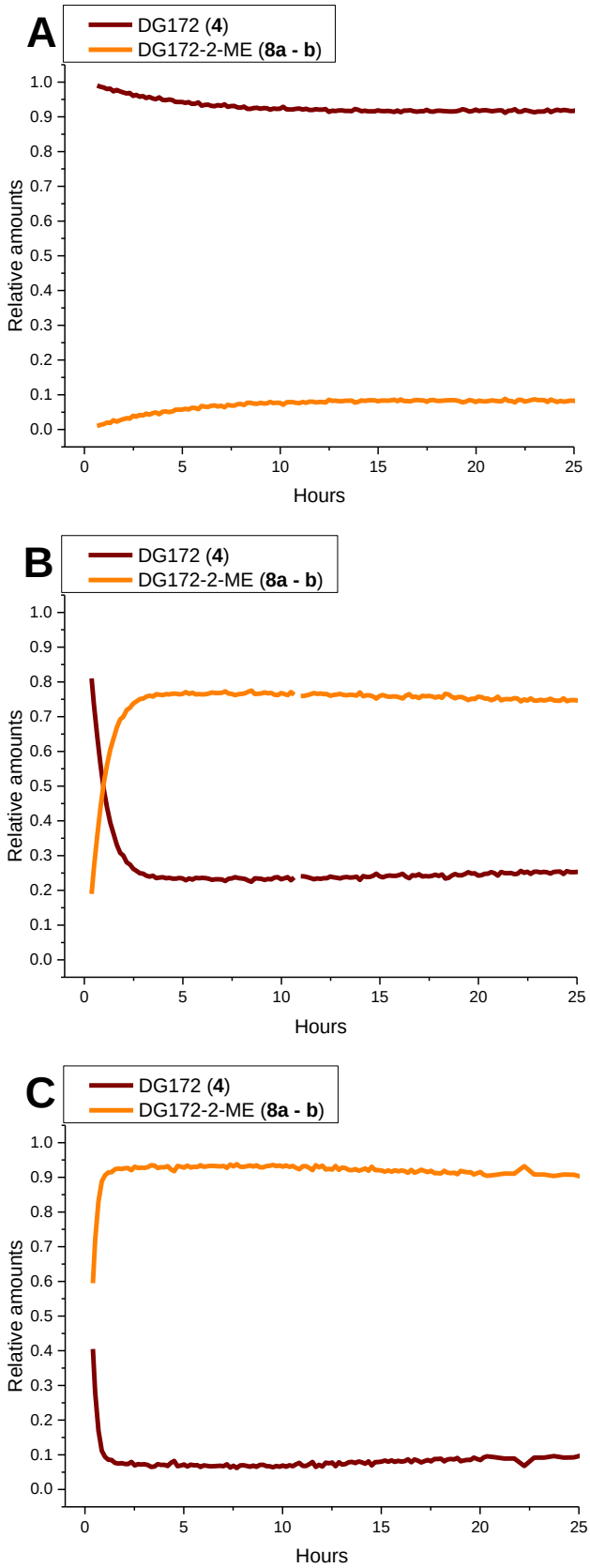


Figure S12 DG172 (**4**) and the DG172-2-ME adducts (**8a – b**), as observed by ^{1}H NMR spectroscopy, upon treatment with 2-ME in DMSO-d_6/0.050 M potassium phosphate buffer. **A**: 2-ME (1 eq), **B**: 2-ME (25 eq), **C**: 2-ME (100 eq). The integrals of the signals from the protons marked with red and green spheres in Figure S11 were followed for 24 hours.

UV-SPECTROSCOPY AND pK_A-CALCULATIONS

The possibility for reversal of the 1,4-conjugate addition reaction to DG172 (**4**) was investigated. Although other factors may be involved in facilitating the reverse reaction,[10] the intrinsic reversibility of the 1,4-conjugate addition can manifest itself when the stability of the intermediate α-carbanion (reflected in the acidity of the α-proton of the adduct) is sufficient for the α-proton to be lost to solution (here an aqueous solution at pH 7.2). Subsequently, the α,β-beta-unsaturated double bond may reform, with elimination of the nucleophile (here, a thiolate).[10–13] Taking cues from an elegant UV-study on the reversibility of the addition of 2-ME to a series of RSK2-inhibitors, containing highly activated conjugate acceptor motifs,[14] we monitored the addition of 2-ME to DG172 (**4**) in potassium phosphate buffer. We observed a significant decrease in the acrylonitrile absorption peak upon addition of 2-ME (1024 eq) to DG172 (150 µM) in 0.050 M potassium phosphate buffer (3.0 mL). Following tenfold dilution, the reappearance of the acrylonitrile absorption was monitored at fixed intervals during the course of 72 hours. The obtained data indicate that the addition of 2-ME to DG172 (**4**) is slowly reversible under these conditions (see Figure S13). The 2-ME adduct of DG172 is, however, isolable (see synthesis and isolation below), in contrast to the adducts of other highly activated conjugate acceptors, sought isolated in previous work.[10] In the work on RSK2 kinase inhibitors,[14] the mode of action the ligands was established as a synergistic combination of non-covalent interactions and reversible, covalent binding. Their UV-study of the 1,4-conjugate additions of 2-ME in PBS to these structures, demonstrated that the adducts reverted to the starting materials within seconds of dilution, as observed by the reappearance of the acrylonitrile UV-absorption peak. Consequently, their putative RSK2-adducts could not be detected by protein LC-MS.

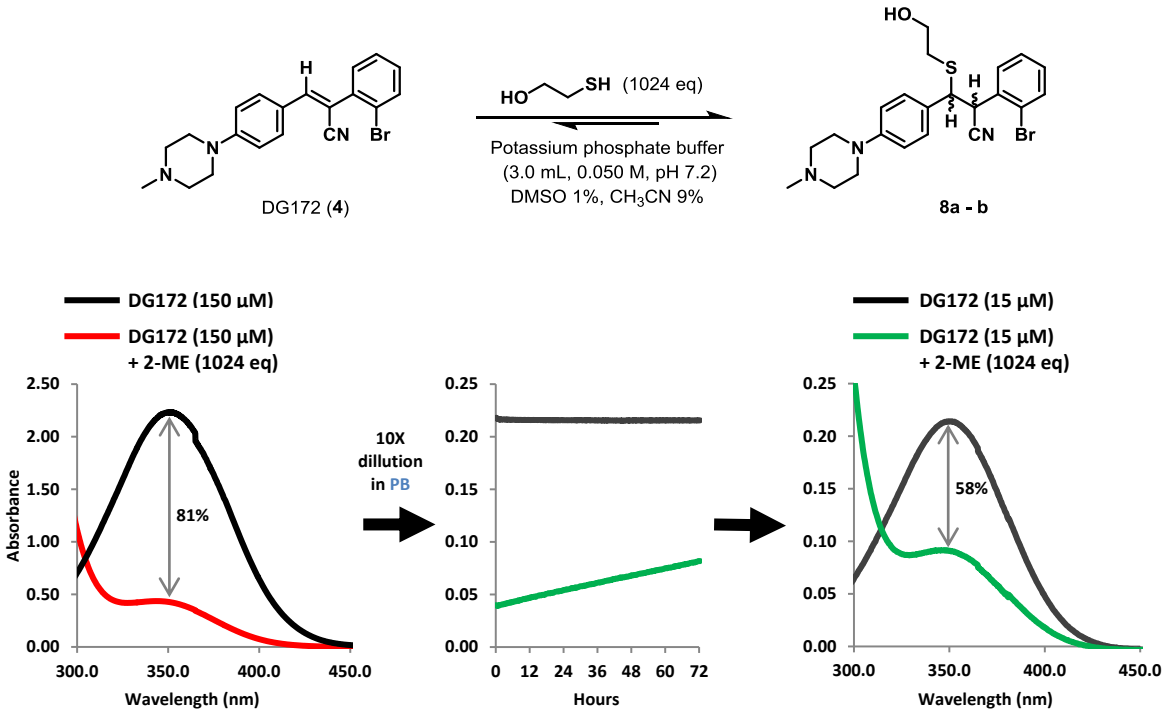


Figure S13 Dilution of a solution of DG172 (**4**), treated with 2-ME (1024 eq), results in the slow reversal of the 1,4-conjugate addition and reformation of the acrylonitrile moiety, over the course of 72 hours. The percentages shown between the curves refer to the extent of reduction of the acrylonitrile absorption peak of the treated solution, relative to the absorption peak of the untreated solution.

Interestingly, a series of less activated α,β-unsaturated analogues did produce observable adducts, strongly suggesting that, given the structural similarity of these RSK2-inhibitors, the reversibility of the conjugate addition

could account for the lack of observable adducts.[14] In order to evaluate the addition of 2-ME to DG172 (**4**) in this context, we calculated the pK_a's of the 2-ME adducts of the reported RSK2 inhibitors, as well as that of the DG172-2-ME adducts (**8a – b**). As can be seen from Table S3, the calculated pK_a of the DG172-2-ME adducts (**8a – b**) lies closer to those calculated for the adducts of compound "14" and compound "15", which did not produce RSK2-adducts observable by LC-MS.

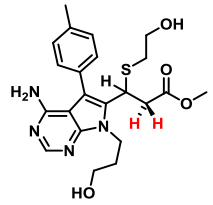
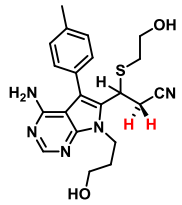
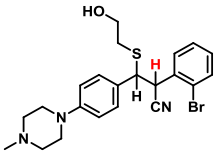
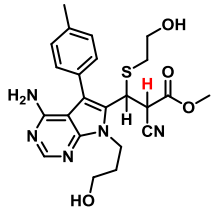
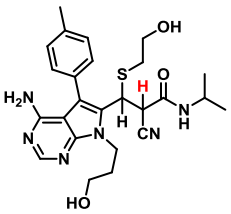
Compound		**pK_a**[15]	**Protein adduct found by LC-MS**
Compound "12"-2-ME adduct[14]		24.90	**Yes**
Compound "13"-2-ME adduct[14]		24.87	**Yes**
DG172-2-ME adduct (**8a – b**)		13.35	**No**
Compound "14"-2-ME adduct[14]		8.65	**No**
"Compound 15"-2-ME adduct[14]		7.72	**No**

Table S4 Calculated pK_a's of 2-ME adducts of reported RSK2-inhibitors[14] compared with that of the DG172-2-ME adducts (**8a – b**).

OFF-RATE MEASUREMENTS WITH TR-FRET

The competitive ligand binding assay kit was reconfigured for use in off-rate measurements as previously described for work involving kinases.[15–17]

Tb-anti-GST and GST-PPARβ/δ were thawed on ice and added to the supplied assay buffer (20 µL) to a (2X) concentration of 500 nM and 200 nM, respectively. The solution was mixed by gentle inversion. 2 µL of this solution was added to each assay well. Ligand solutions (2X concentration) were prepared in the supplied assay buffer and 2 µL of each of these solutions were added into the drop of protein solution in each assay well. The wells were sealed and incubated for 1 hour in the dark, prior to 50 times dilution with a solution containing an excess of the tracer ligand (100 nM). The final volume was 200 µL in all wells. The plate was then read at multiple time-points, to monitor the development of an eventual FRET signal. The final concentrations of Tb-anti-GST, GST-PPARβ/δ, ligands and DMSO were as noted in Table S5. A high concentration (10 µM) of GW501516 (**5**) was used as a positive control (Entry 1, Table S5) and DMSO (final concentration 2%v/v) as the negative control.

		During protein treatment		After dillution			
Entry	**Compound**	**Ligand conc. (nM)**	**Ligand conc. vs. IC_{50} (eq)[a]**	**GST-PPARβ/δ conc. (nM)**	**Tb-anti-GST conc. (nM)**	**Ligand conc. (nM)**	**DMSO %v/v**
1	GW501516 (**5**)[18]	10000	6667[b]	2	5	200	2
2	GW501516 (**5**)	100	67[b]	2	5	2	2
3	DG172 (**4**)[9]	2000	67[c]	2	5	40	2
4	DG172 (**4**)	300	10[c]	2	5	6	2
5	GSK3787 (**1**)[1]	6500	10[d]	2	5	130	2

[a]The equivalents shown are rounded off to the nearest integer for ease of interpretation.

[b]The IC_{50} value of GW501516 (1.5 nM) in the competitive ligand binding configuration of this assay is previously reported.[4]

[c]The IC_{50} value of DG172 (26.9 nM) in the competitive ligand binding configuration of this assay is previously reported.[9] We obtained a similar value (32.7 nM, data not shown) and the average of these values (29.8 nM) was used in the calculation in Table S4.

[d]The IC_{50} of GSK3787 (648 nM) in the competitive ligand binding configuration of this assay was calculated from measurements taken after 1 hour of incubation (data not shown).

Table S5 Experimental details from the off-rate measurements monitored by TR-FRET.

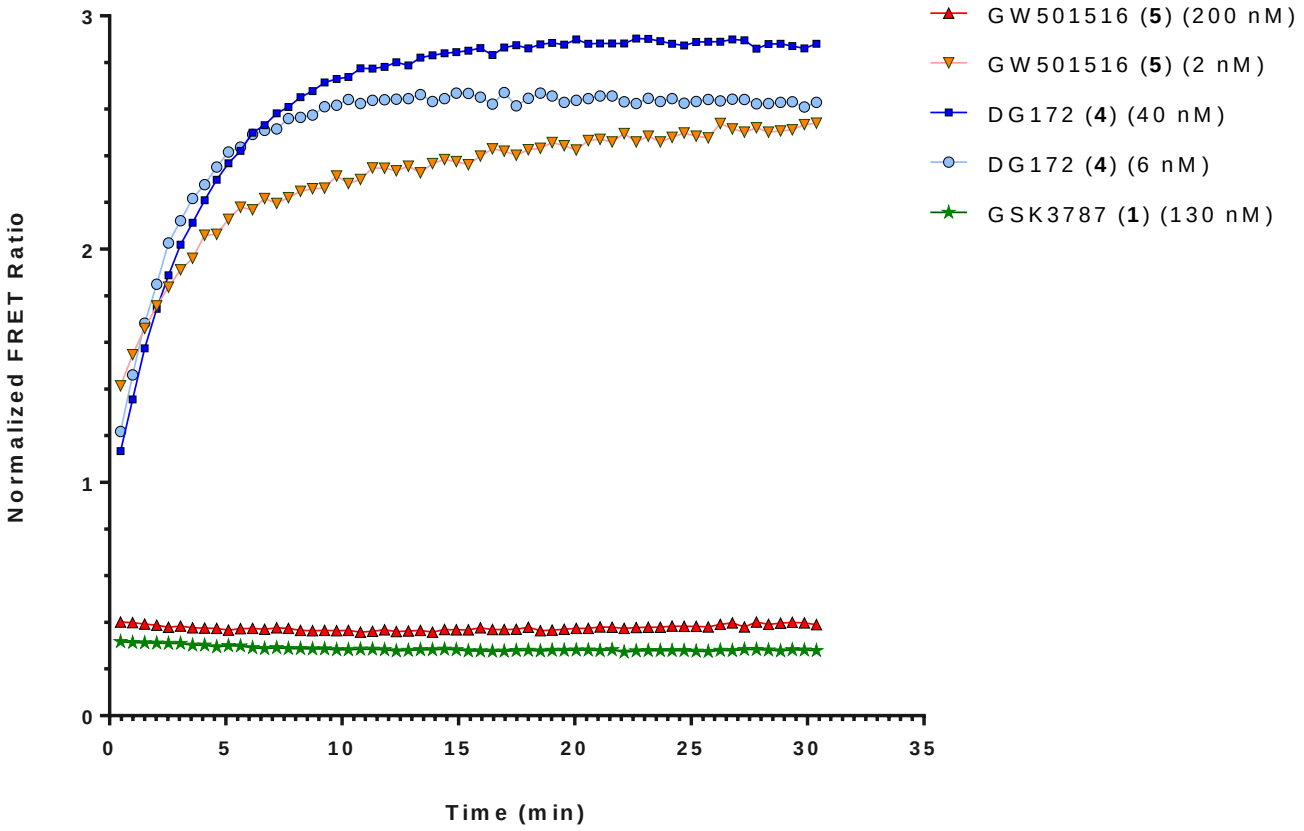
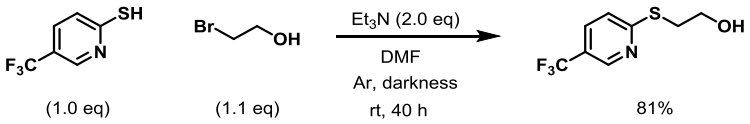


Figure S14 Displacement of ligands **1**, **4** and **5** from the LBP of PPARβ/δ by the fluorescent tracer ligand Fluormone Pan PPAR Green (100 nM) as a function of time. The normalized FRET ratio represents values from single wells, normalized based on control wells (DMSO, n = 2). The concentrations in parentheses refer to the final concentrations of the ligands after dilution.

SYNTHETIC CHEMISTRY

Synthesis of identified products:

2-((5-(trifluoromethyl)pyridin-2-yl)thio)ethan-1-ol (6)[19]



(1.0 eq) (1.1 eq) 81%

To a stirred solution of 5-(trifluoromethyl)-pyridine-2-thiol (97%, 0.092 g, 0.50 mmol, 1.0 eq) in DMF (5 mL), at ambient temperature under an argon atmosphere, was added 2-bromoethanol (95%, 41 µL, 0.55 mmol, 1.1 eq), followed by Et_3N (≥99%, 139 µL, 1.00 mmol, 2.0 eq). The flask was capped with a septum, flushed with argon and the reaction was stirred, in the dark, for 40 hours. The mixture was then diluted with H_2O (10 mL), extracted with EtOAc (3 x 20 mL) and the combined organic phases were washed with saturated aqueous NaCl (5 x 10 mL). The organic phase was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure, at or below 40 °C, leaving a pale yellow oil (0.107 g). A fritted, 3 cm x 3 cm ∅ column of silica gel was packed in heptane, the crude product was dissolved in a minimal amount of CH_2Cl_2 and loaded onto the column. The CH_2Cl_2 was evaporated under a stream of argon, followed by elution of the column with heptane (50 mL) and EtOAc:heptane/5:95 (400 mL). Finally, the product was eluted with EtOAc:heptane/10:90 (250 mL) and the solvents evaporated under reduced pressure, at or below 40 °C, leaving a clear oil (0.093 g, 81%).

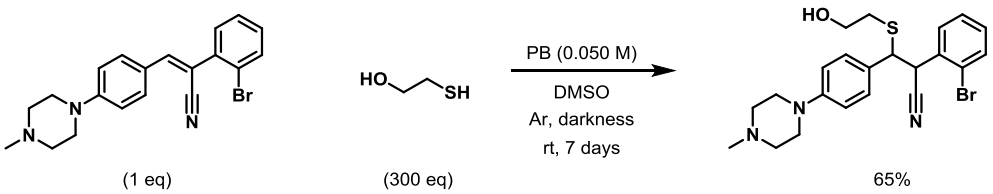
^{1}H NMR (300 MHz, $CDCl_3$) δ 8.68 – 8.60 (m, 1H), 7.76 – 7.66 (m, 1H), 7.42 – 7.32 (m, 1H), 3.95 (dd, *J* = 5.9, 5.2 Hz, 2H), 3.66 (s, 1H), 3.42 (dd, *J* = 5.9, 5.2 Hz, 2H).

^{13}C NMR (75 MHz, $CDCl_3$) δ 164.0 (q, *J* = 1.4 Hz), 145.9 (q, *J* = 4.3 Hz), 133.3 (q, *J* = 3.4 Hz), 123.6 (q, *J* = 271.8 Hz), 123.0 (q, *J* = 33.5 Hz), 122.5, 62.8, 33.8.

MS (EI) *m/z* (%) 223 (11) $[M]^{+\cdot}$, 204 (90), 192 (68), 179 (100), 160 (5), 146 (47), 135 (41), 126 (14), 75 (6), 69 (10), 45 (9).

HRMS (EI) calcd. for $C_8H_8F_3NOS$: 223.027871; found: 223.027281(+ 2.6 ppm).

2-(2-bromophenyl)-3-((2-hydroxyethyl)thio)-3-(4-(4-methylpiperazin-1-yl)phenyl)propanenitrile (8a - b)



To a stirred solution of (*Z*)-2-(2-bromophenyl)-3-(4-(4-methylpiperazin-1-yl)phenyl)acrylonitrile (DG172, **4**)[9] (0.038 g, 0.10 mmol, 1.0 eq) in DMSO (6.5 mL), at ambient temperature under an argon atmosphere, was added 2-mercaptoethanol (2-ME) (2.1 mL, 30.0 mmol, 300 eq), followed by potassium phosphate buffer (PB) (0.050 M, pH 7.2, 1.5 mL). The flask was capped with a septum, flushed with argon and the reaction was stirred at ambient temperature, in the dark, for 7 days. The mixture was then diluted with H_2O (20 mL) and EtOAc (20 mL) and transferred to a separatory funnel, where the organic phase was separated and the aqueous phase further extracted with EtOAc (2 x 20 mL). The combined organic phases were washed with a 1:3 mixture of $NaHCO_3$ (aq. sat.) and H_2O (3 x 20 mL) and then with brine (7 x 20 mL), before drying over anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure, at or below 40 °C, and the crude material redissolved in a minimal amount of CH_2Cl_2, before loading it onto a fritted, 4.5 cm x 3 cm ∅ column of silica gel, packed in CH_2Cl_2. The column was eluted with CH_2Cl_2 (70 mL), CH_3OH:CH_2Cl_2/1:99 (200 mL), CH_3OH:CH_2Cl_2/2:98 (500 mL) and finally with CH_3OH:CH_2Cl_2/5:95 (450 mL) to elute the product fractions containing both diastereomers. Evaporation of the solvents under reduced pressure, at or below 40 °C, afforded a colourless foam (0.030 g, 65%).

Major diastereomer:

^{1}H NMR (600 MHz, $CDCl_3$) δ 7.60 – 7.55 (m, 1H), 7.18 – 7.12 (m, 1H), 7.14 – 7.09 (m, 1H), 7.06 (d, *J* = 8.5 Hz, 1H), 6.92 (dd, *J* = 7.5, 1.9 Hz, 1H), 6.77 (d, *J* = 8.6 Hz, 1H), 4.86 (d, *J* = 5.5 Hz, 1H), 4.23 (d, *J* = 5.5 Hz, 1H), 3.66 (td, *J* = 5.9, 2.4 Hz, 2H), 3.19 (t, *J* = 5.1 Hz, 4H), 2.74 (dt, *J* = 13.9, 6.0 Hz, 1H), 2.68 (dt, *J* = 13.7, 5.9 Hz, 1H), 2.57 (q, *J* = 5.0 Hz, 6H), 2.34 (s, 3H).

^{13}C NMR (151 MHz, $CDCl_3$) δ 151.2, 133.4, 133.1, 130.7, 130.2, 129.7, 127.9, 126.5, 123.3, 118.3, 115.5, 60.8, 55.1, 50.5, 48.6, 46.2, 44.9, 35.2.

Minor diastereomer:

^{1}H NMR (600 MHz, $CDCl_3$) δ 7.69 (dd, *J* = 7.8, 1.7 Hz, 1H), 7.58 – 7.54 (m, 1H), 7.40 (d, *J* = 8.6 Hz, 2H), 7.40 – 7.34 (m, 1H), 7.21 (td, *J* = 7.7, 1.6 Hz, 1H), 6.87 (d, *J* = 8.7 Hz, 2H), 4.69 (d, *J* = 5.0 Hz, 1H), 4.25 (d, *J* = 5.1 Hz, 1H), 3.51 – 3.44 (m, 1H), 3.47 – 3.40 (m, 1H), 3.23 – 3.21 (m, 4H), 2.59 – 2.54 (m, 10H), 2.49 – 2.44 (m, 1H), 2.44 – 2.38 (m, 1H), 2.35 (s, 4H).

^{13}C NMR (151 MHz, $CDCl_3$) δ 151.1, 133.6, 133.0, 131.2, 130.4, 129.4, 128.8, 128.0, 123.2, 118.4, 115.9, 60.7, 55.1, 51.6, 48.6, 46.2, 45.8, 35.6.

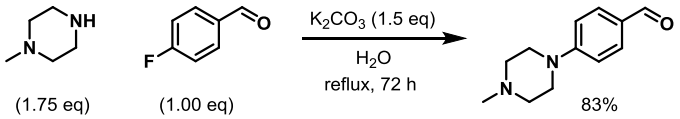
Mixture:

MS (EI) *m/z* (%) 461/459 (1/1) $[M]^{+\cdot}$, 384/383/382/381 (13/23/14/22), 265 (100), 203 (11), 71/70 (11/11), 43 (16).

HRMS (EI) calcd. for $C_{22}H_{26}BrN_3OS$: 459.097996; found: 459.096948 (+ 2.3 ppm).

Synthesis of starting materials:

4-(4-methylpiperazin-1-yl)benzaldehyde (9)[20,21]



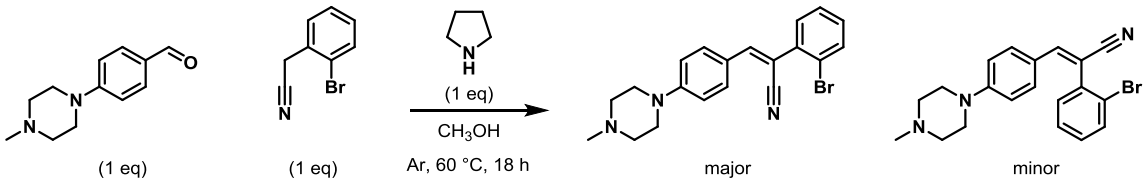
(1.75 eq) (1.00 eq) 83%

4-Fluorobenzaldehyde (98%, 0.55 mL, 5.03 mmol, 1.00 eq) was added dropwise to a stirred solution of 1-methylpiperazine (0.97 mL, 8.73 mmol, 1.75 eq) and K_2CO_3 (1.044 g, 7.55 mmol, 1.50 eq) in H_2O (5 mL), at ambient temperature and the flask was fitted a reflux condenser. The flask was placed in a glycerol bath which was heated to 102 °C. The reaction was stirred for 72 hours, allowed to cool to ambient temperature and then partitioned between H_2O (20 mL) and CH_2Cl_2 (20 mL). The aqueous phase was extracted with CH_2Cl_2 (2 x 20 mL) and the combined organic phases washed with H_2O (2 x 20 mL), brine (20 mL) and then dried over anhydrous Na_2SO_4. Evaporation of the solvent under reduced pressure, at or below 35 °C, afforded a pale yellow solid. This material was redissolved in CH_2Cl_2, added silica gel (1 tbsp) and the solvent evaporated under reduced pressure, at or below 35 °C. The resulting material was loaded onto a short silica gel column (1 tbsp, 3 cm ∅), packed in CH_2Cl_2, and the column was eluted with CH_2Cl_2 (100 mL), followed by CH_3OH:CH_2Cl_2/10:90 to elute the product. Evaporation of the solvents under reduced pressure, at or below 35 °C, furnished the product as a pale yellow solid (0.851 g, 83%), which was reasonably pure by TLC and NMR-analyses. The spectral data were in accordance with the reported.[20]

^{1}H NMR (400 MHz, $CDCl_3$) δ 9.77 (bs, 1H), 7.74 (app d, *J* = 8.9 Hz, 2H), 6.90 (app d, *J* = 8.9 Hz, 2H), 3.44 – 3.36 (m, 4H), 2.56 – 2.49 (m, 4H), 2.34 (bs, 3H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 190.5, 155.1, 131.9, 127.2, 113.6, 54.8, 47.2, 46.2.

(Z)-2-(2-bromophenyl)-3-(4-(4-methylpiperazin-1-yl)phenyl)acrylonitrile (DG172, 4)[9]

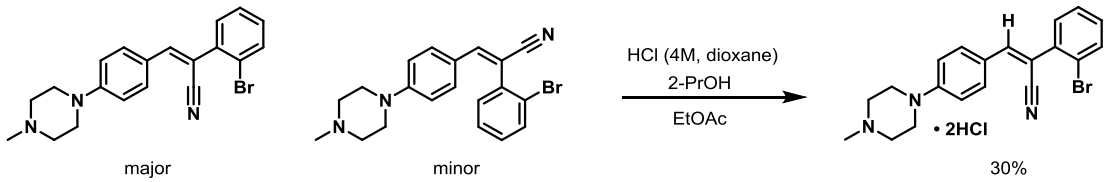


To a solution of 4-(4-methylpiperazin-1-yl)benzaldehyde (0.500 g, 2.45 mmol, 1.0 eq) in CH_3OH (4 mL), at ambient temperature in an air atmosphere, was added pyrrolidine (0.20 mL, 2.45 mmol, 1.0 eq). The resulting orange solution was stirred for 5 minutes before adding 2-bromophenylacetonitrile (97%, 328 µL, 2.45 mmol, 1.0 eq). The flask was fitted with a reflux cooler capped with a septum and the system was evacuated with argon. The flask was then lowered into a glycerol bath at 60 °C and stirred for 18 hours. TLC analysis at this point indicated that the reaction had proceeded to approximately 50% completion. Nevertheless, ¼ of the reaction mixture (1 mL) was removed and evaporated onto silica gel (3 g), under reduced pressure, at or below 30 °C. The resulting dry material was loaded onto a 13 cm x 4 cm ∅ column of silica gel (80 g), which had been packed dry and equilibrated with CH_2Cl_2. The column was then eluted with CH_3OH:CH_2Cl_2/0:100 - 2:98 (30 min) and then CH_3OH:CH_2Cl_2/2:98 (50 min) to apparently elute a single yellow band. The fractions were combined and the solvents removed under reduced pressure, at or below 30 °C, to leave a yellow oil. TLC analysis (5 elutions in CH_3OH:CH_2Cl_2/5:95) of this material, indicated that the reaction had proceeded with near complete selectivity for the *Z*-isomer (the *E*-isomer is seen as a close-running spot of slightly higher R_f).

MS(EI) *m/z* (%) 384/383/382/381 (22/98/30/100), 339 (9), 313/312/311/310 (7/16/9/16), 298/296 (10/10), 203 (47), 71 (40), 70 (31), 43 (42).

HRMS (EI) calcd. for $C_{20}H_{20}BrN_3$: 381.084059; found: 381.083817 (+ 0.6 ppm).

(Z)-2-(2-bromophenyl)-3-(4-(4-methylpiperazin-1-yl)phenyl)acrylonitrile dihydrochloride (DG172·2HCl, 4·2HCl)[9]



major minor 30%

To obtain the *Z*-isomer exclusively, the procedure for making the dihydrochloride salt of DG172 (**4·2HCl**) was followed, essentially as reported:[9] To a stirred solution of EtOAc (30 mL) was added HCl in dioxane (5 mL, 4M). To the resulting solution, was added dropwise, the material isolated as described above, to give a clear solution. After 5 min, a white precipitate formed. The suspension was added 2-propanol (3 mL) and stirred for an additional 5 min, before filtering the suspension on a Büchner funnel with suction. The filter cake was washed repeatedly with aliquots of a solution of HCl in dioxane (1 mL, 4M) in EtOAc (10 mL). The filter paper, with the filter cake, was then transferred to a vial and the remaining solvents removed under reduced pressure, at or below 30 °C, to leave a nearly white powder (85 mg, 30% based on ¼ of reaction above) which was stored at 4 °C under an argon atmosphere.

^{1}H NMR (600 MHz, DMSO, 25 °C) δ 11.47 (s, 1H), 7.88 (app d, *J* = 8.9 Hz, 2H), 7.75 (dd, *J* = 8.0, 1.2 Hz, 1H), 7.55 (dd, *J* = 7.7, 1.7 Hz, 1H), 7.50 (td, *J* = 7.5, 1.2 Hz, 1H), 7.40 (s, 1H), 7.38 (partial td, *J* = 7.5, 1.7 Hz, 1H), 7.15 (app d, *J* = 9.0 Hz, 1H), 5.68 (bs, 1H + H_2O), 4.06 (app bd, *J* = 13.9 Hz, 2H), 3.47 (app bd, *J* = 11.9 Hz, 2H), 3.34 – 3.28 (m, 2H), 3.15 – 3.08 (m, 2H), 2.78 (d, *J* = 4.6 Hz, 3H).

^{13}C NMR (151 MHz, DMSO, 25 °C) δ 151.1, 148.0, 136.4, 133.2, 131.6, 130.85, 130.79, 128.5, 123.6, 122.2, 118.0, 114.8, 104.8, 51.6, 44.1, 41.9.

MS (ESI) *m*/*z* (%) 385/384/383/382 (15/95/19/100).

HPLC (inj. vol. 5 µL (1 mg/mL, H_2O), eluent: 10% CH_3CN, 60% CH_3OH, 30% H_2O/HCOOH (10 mM)) Retention time (%): 5.97 min (3 %, *E*-isomer), 10.07 min (97 %, *Z*-isomer).

Notes:

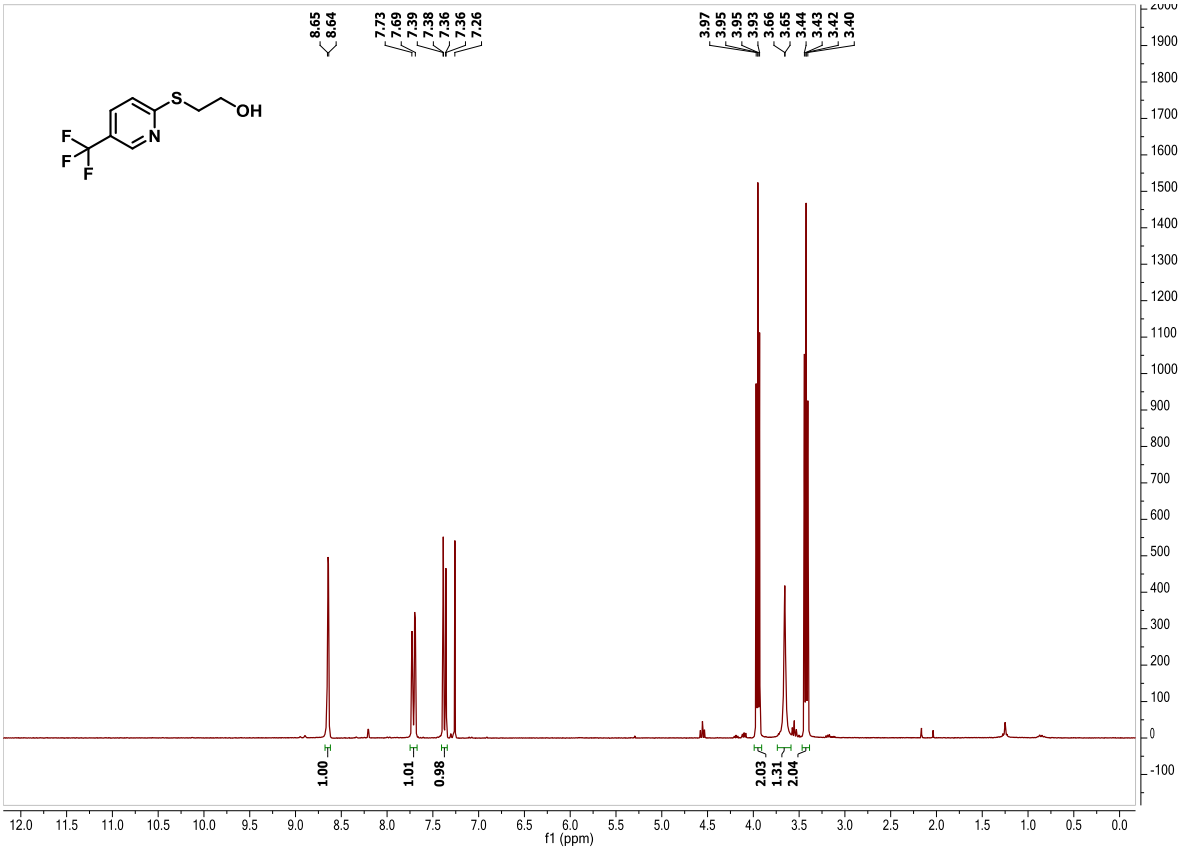
1) If left in air, the colour of the above obtained powder was observed to change to pale yellow, likely indicating the loss of HCl from the aniline-nitrogen to air-borne water and the subsequent formation of the mono-hydrochloride salt. The loss of HCl to the solvent is also apparent in the ESI-MS analysis of the dihydrochloride salt dissolved in CH_3OH, in which only the singly protonated species is observed.

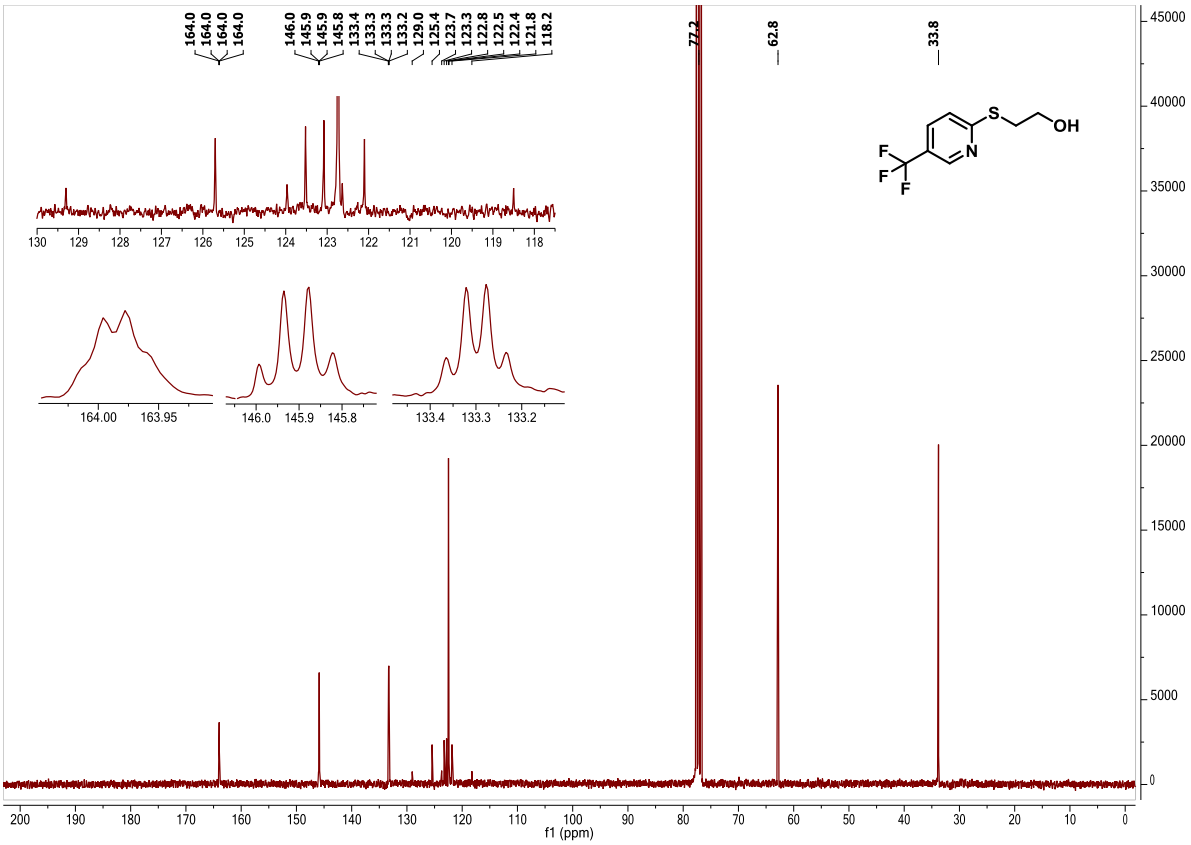
2) Chromatographical separation of the *Z*-isomer from the *E*-isomer proved difficult in our hands, but the *E*-isomer is obtainable by preparative TLC on a 25 x 25 cm aluminum-backed sheet of silica gel (0.250 µm) of approximately 50 mg of material, using CH_3CN as the eluent (multiple elutions). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.73 – 7.68 (m, 1H), 7.44 – 7.24 (m, 3H), 7.29 (s, 1H), 3.29 – 3.23 (m, 4H), 2.53 – 2.48 (m, 4H), 2.33 (s, 3H).

3) A DMSO solution of the free amines (*E*- and *Z*-isomer) obtained by chromatography, as described above, was heated to 90 °C, in a glycerol bath, for a period of 72 hours. Subsequent ^{1}H NMR analysis of the solution showed an increased amount of the *E*-isomer. Thus, DG172 (**4**), as the free amine, might be unstable towards isomerization at elevated temperatures.

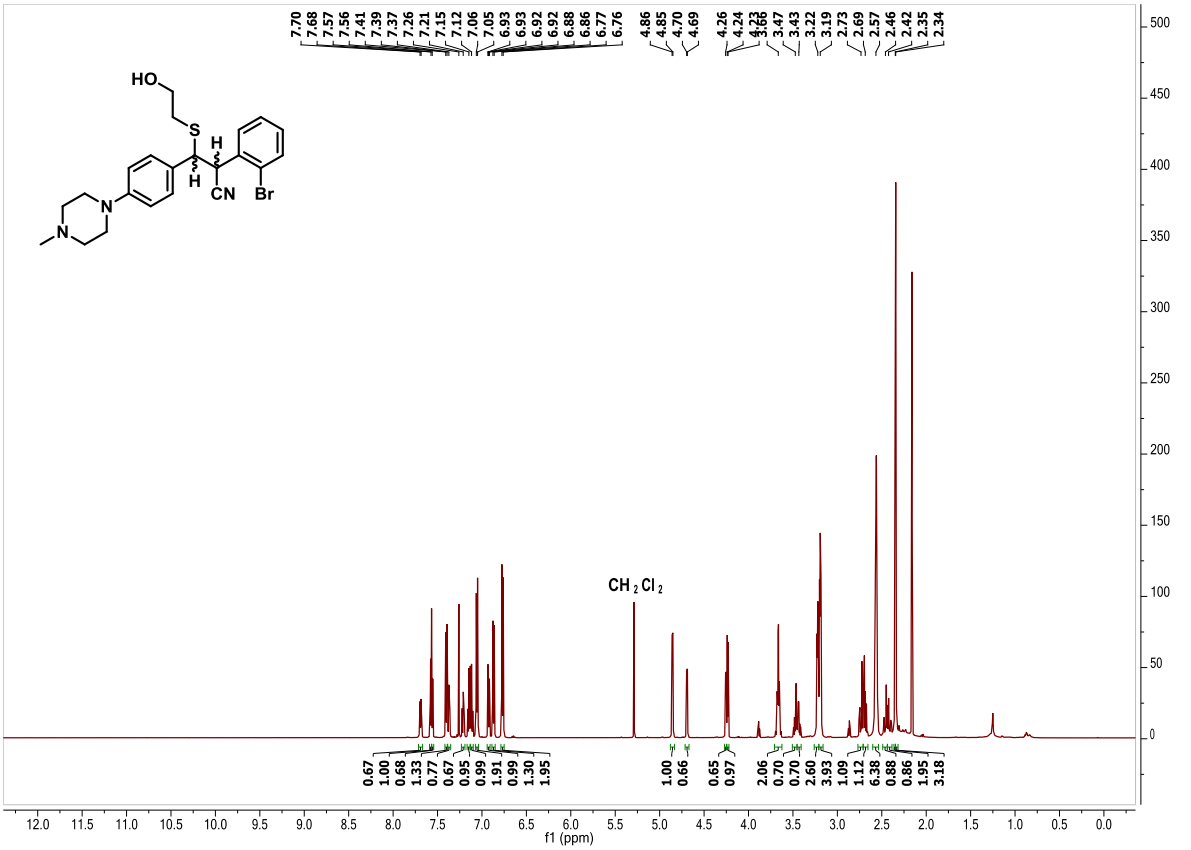
NMR SPECTRA

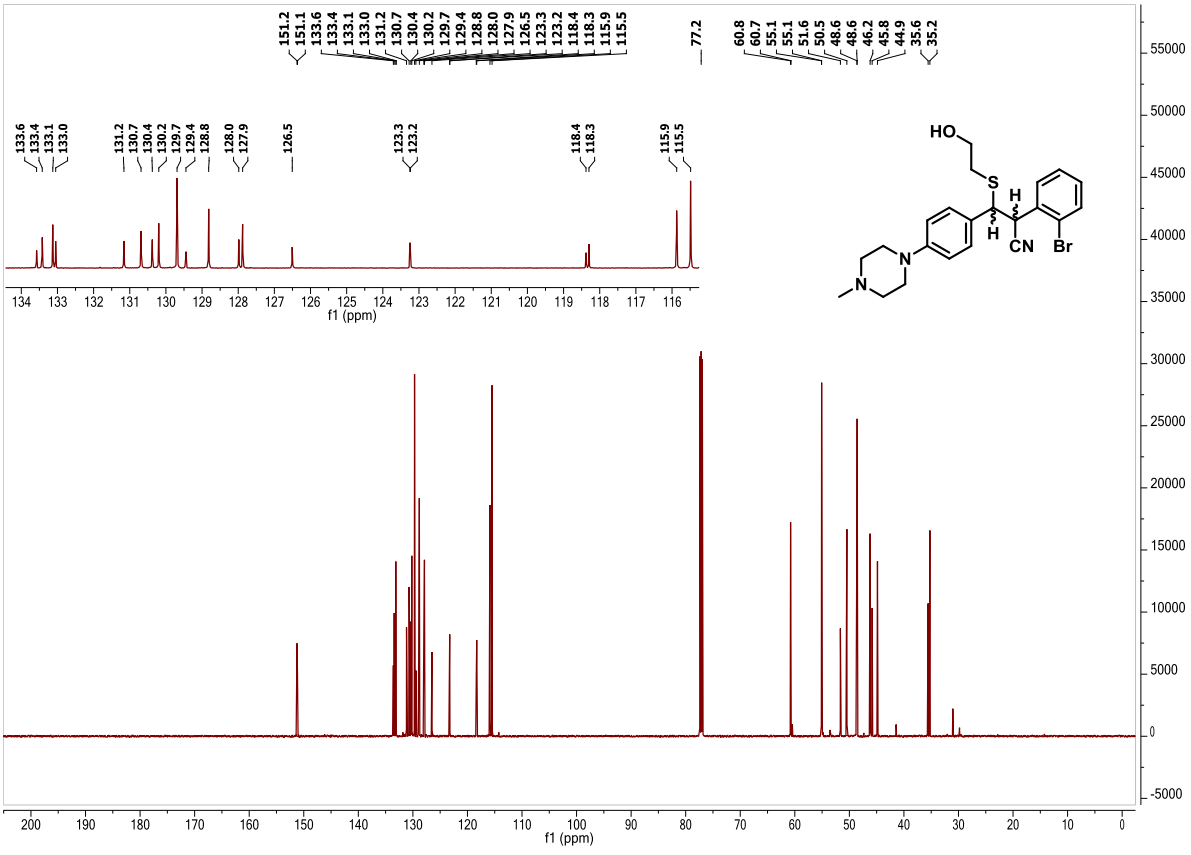
2-((5-(trifluoromethyl)pyridin-2-yl)thio)ethan-1-ol (6)[19]



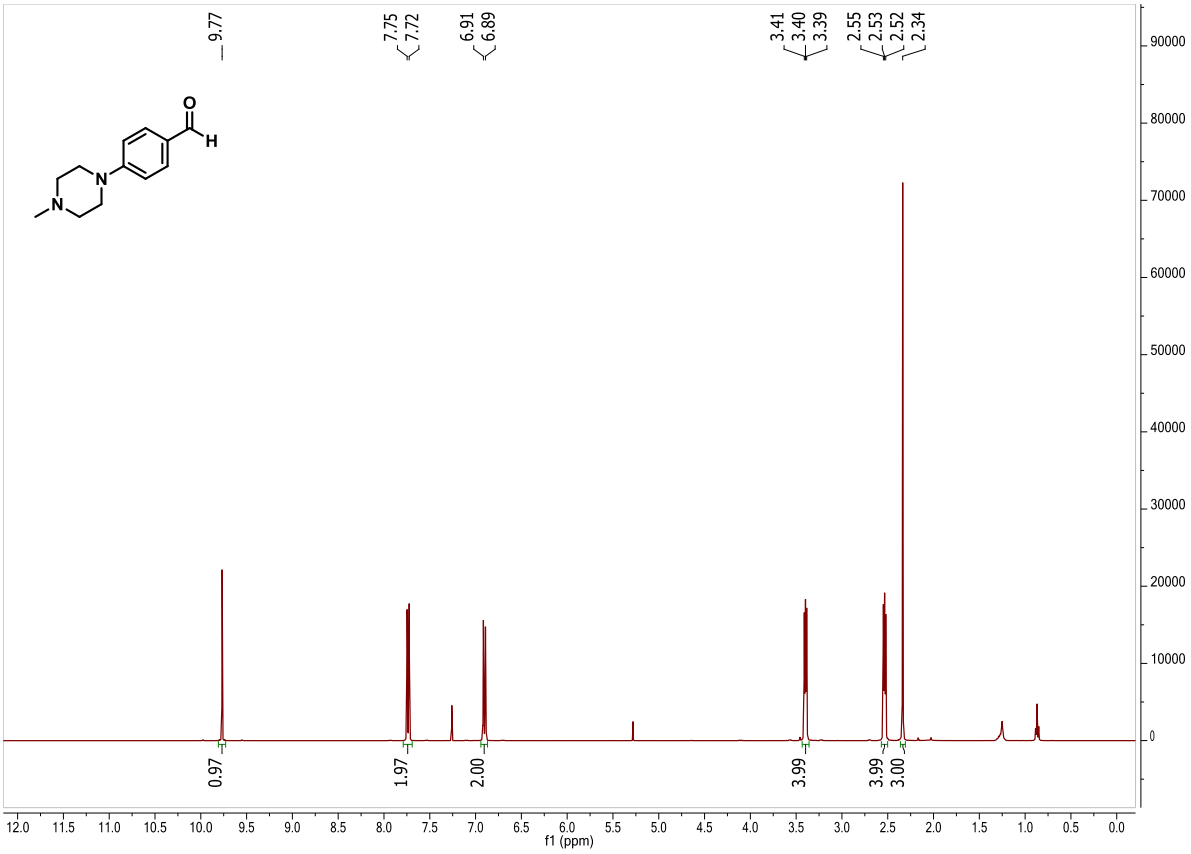


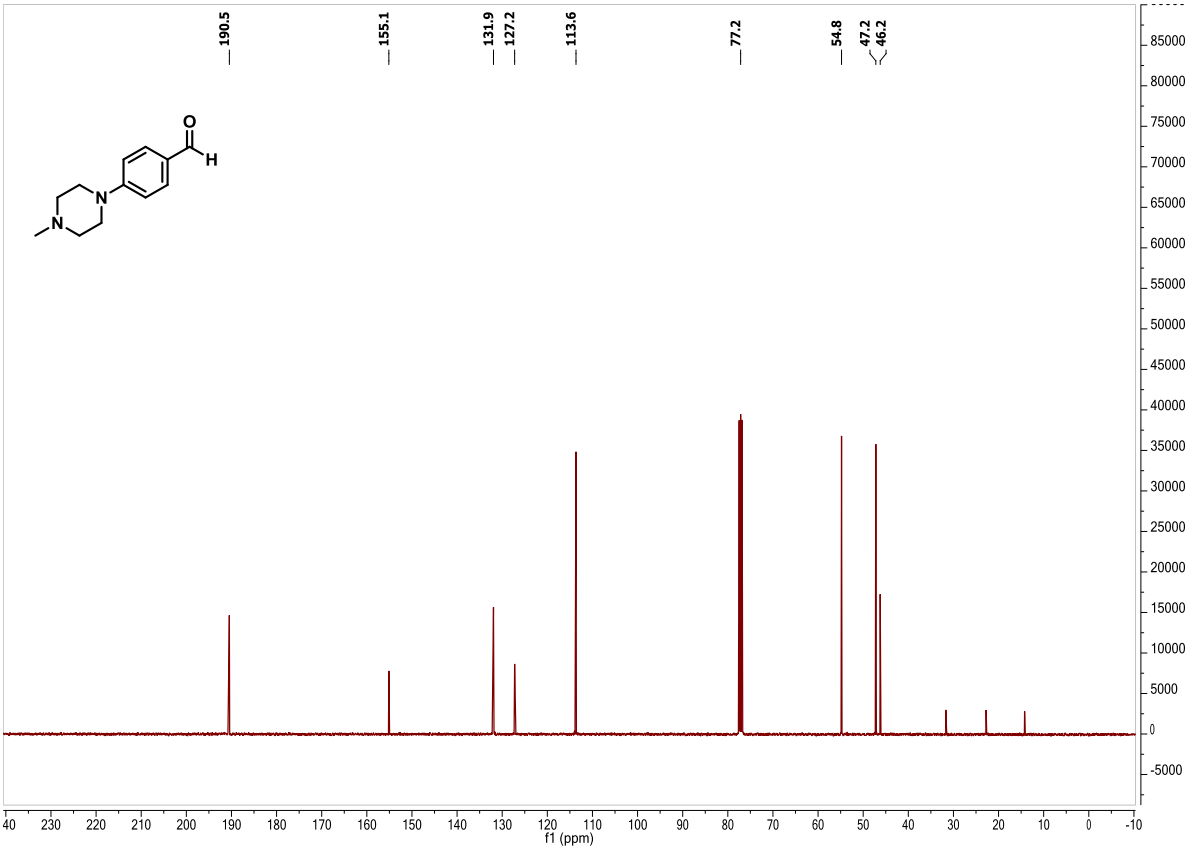
2-(2-bromophenyl)-3-((2-hydroxyethyl)thio)-3-(4-(4-methylpiperazin-1-yl)phenyl)propanenitrile (8a - b)



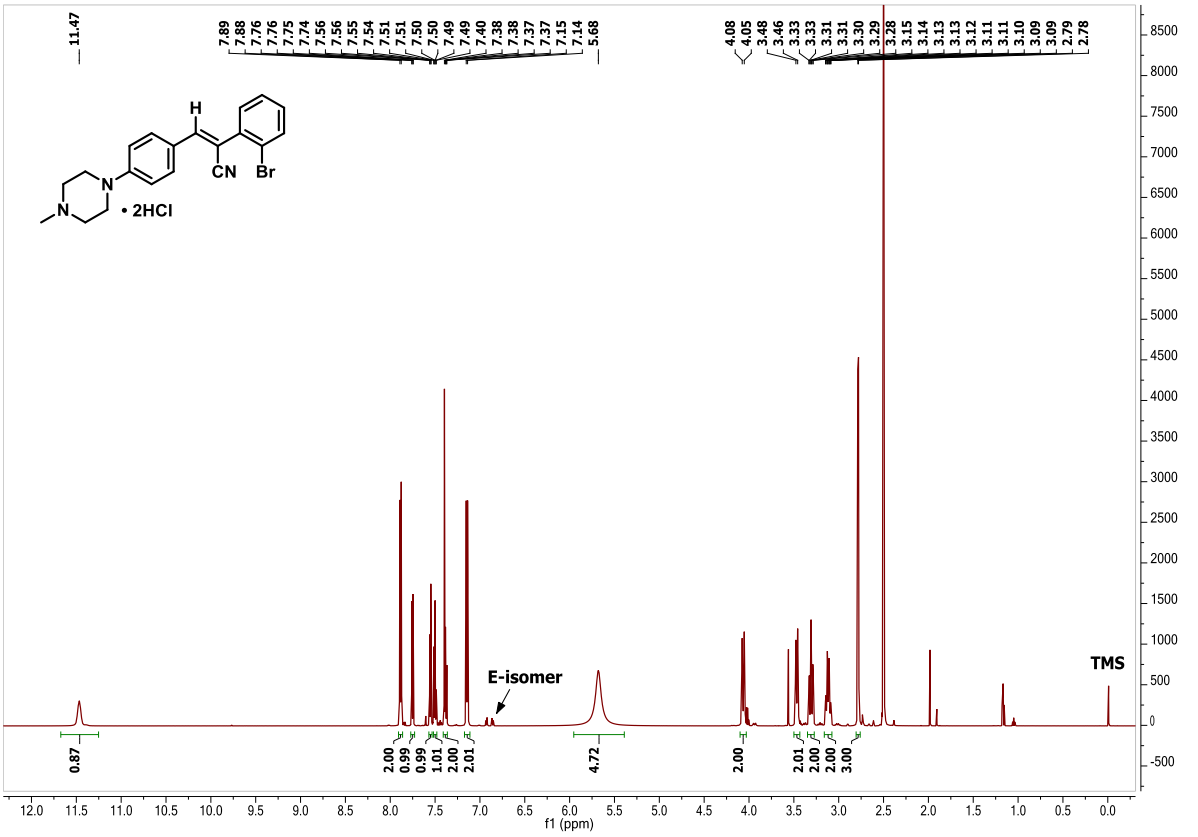


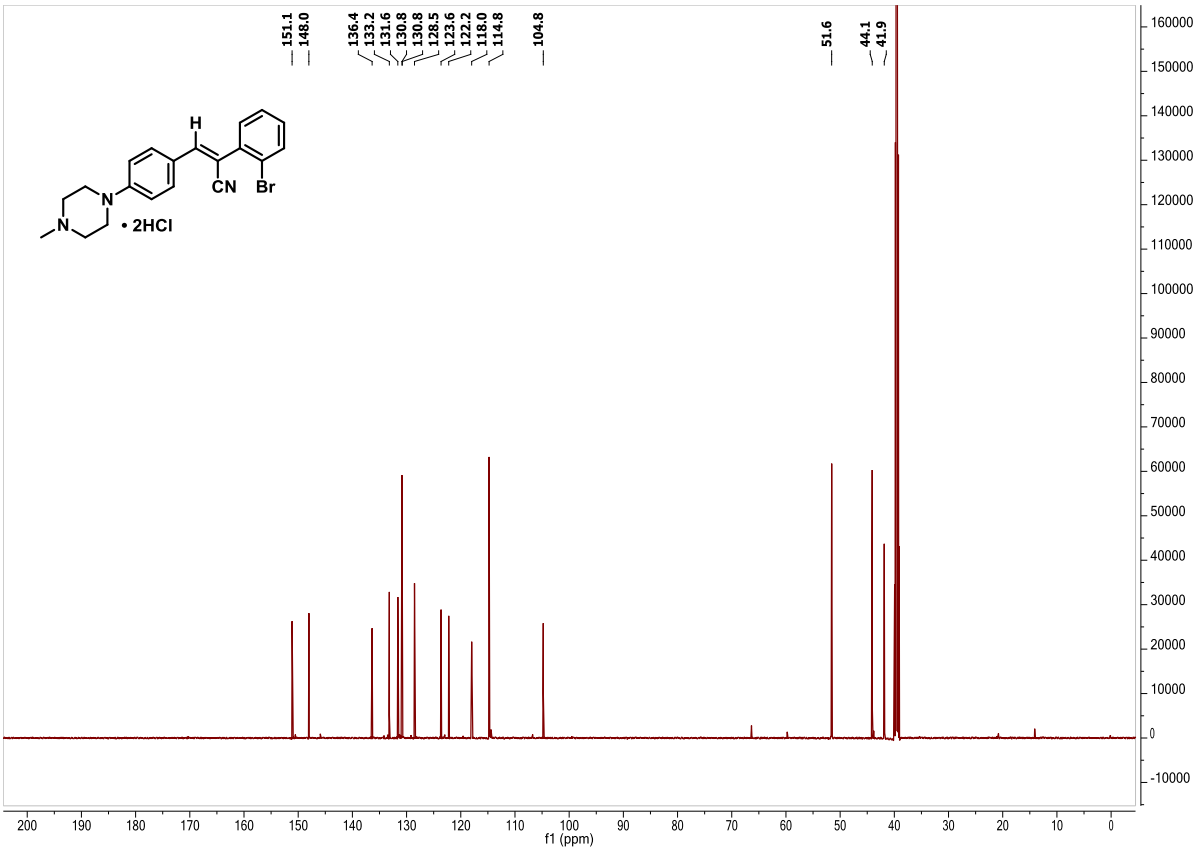
4-(4-methylpiperazin-1-yl)benzaldehyde (9)[20]





(Z)-2-(2-bromophenyl)-3-(4-(4-methylpiperazin-1-yl)phenyl)acrylonitrile dihydrochloride(DG172·2HCl, 4·2HCl)[9]





HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) CHROMATOGRAMS

(Z)-2-(2-bromophenyl)-3-(4-(4-methylpiperazin-1-yl)phenyl)acrylonitrile dihydrochloride (DG172·2HCl, 4·2HCl)[9]

```
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Sample Name: DG1722HCl-2-OC18                                                1
================================================================================
Injection Date  : Fri, 17. Apr. 2015          Seq Line   :            0
Sample Name     : DG1722HCl-2-OC18            Location   :       Vial 2
Acq Operator    : Åsmund                      Inj. No.   :            0
                                              Inj. Vol.  :         5 µl

Acq. Method     : C18 ISOKRATISK.M
Analysis Method : C:\CHEM32\1\DATA\JORN\C18 ISOKRATISK.M
Sample Info:      10% MeCN, 60% MeOH, 30% H2O/HCOOH (10mM)              ->
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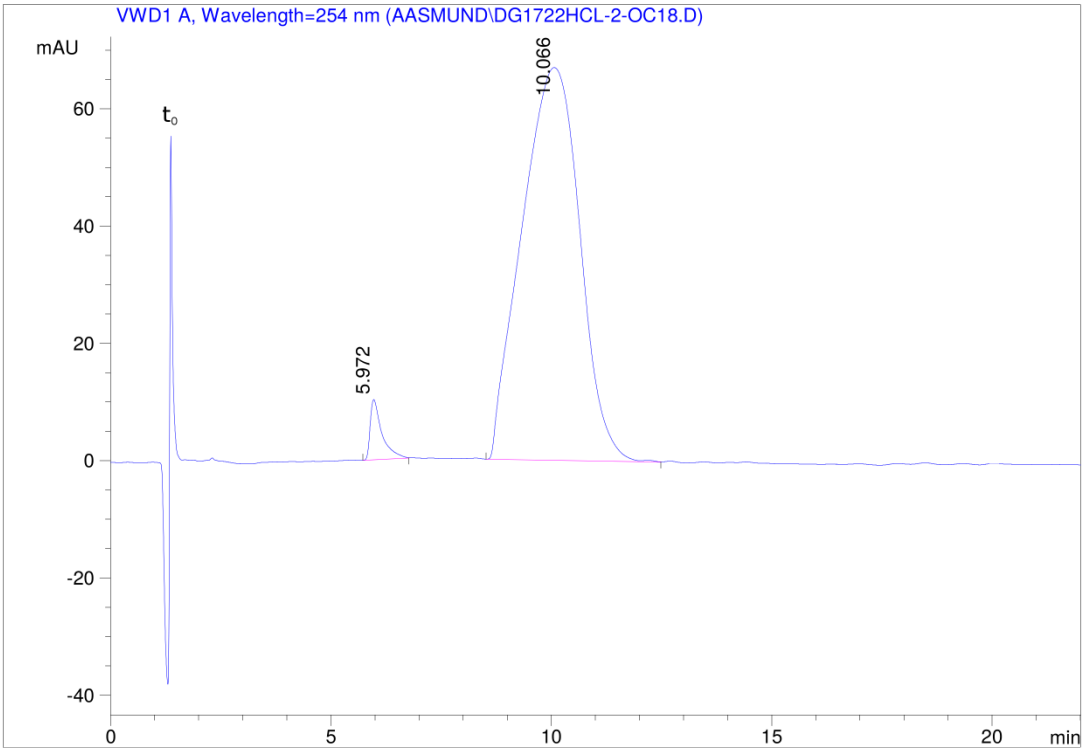


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================================================================================
                          Area Percent Report
================================================================================
Sorted By Signal
Multiplier            :  1.000000
Dilution              :  1.000000

Signal  1: VWD1 A, Wavelength=254 nm

 Peak    RetTime   Type     Width       Area         Height       Area %
  #       [min]              [min]      [mAU*s]       [mAU]
-----|----------|-----|----------|------------|------------|----------
    1|     5.972|BB   |    0.2673|  1.89320e2 |    10.27770|    3.0025
    2|    10.066|BV   |    1.4673|  6.11620e3 |    66.97217|   96.9975
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                          *** End of Report ***
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