Macrophage migration inhibitory factor is a drug target at the intersection of herpes simplex virus 1 replication and AD-relevant cellular pathology

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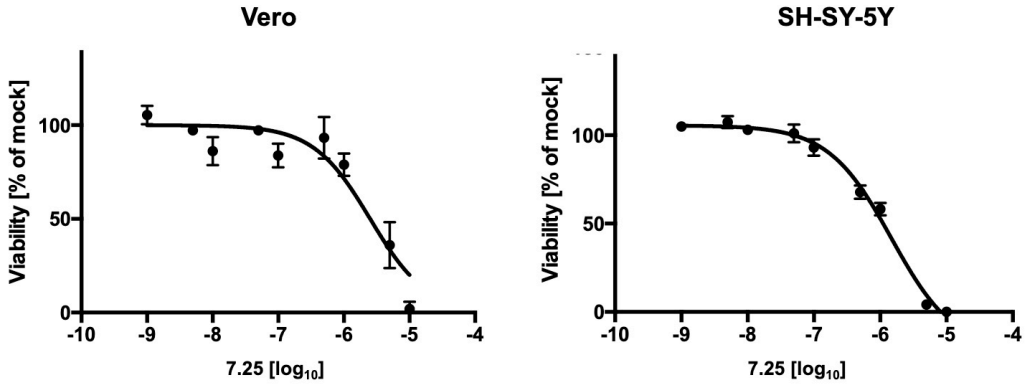
Supplementary Figures and Chemical Methods

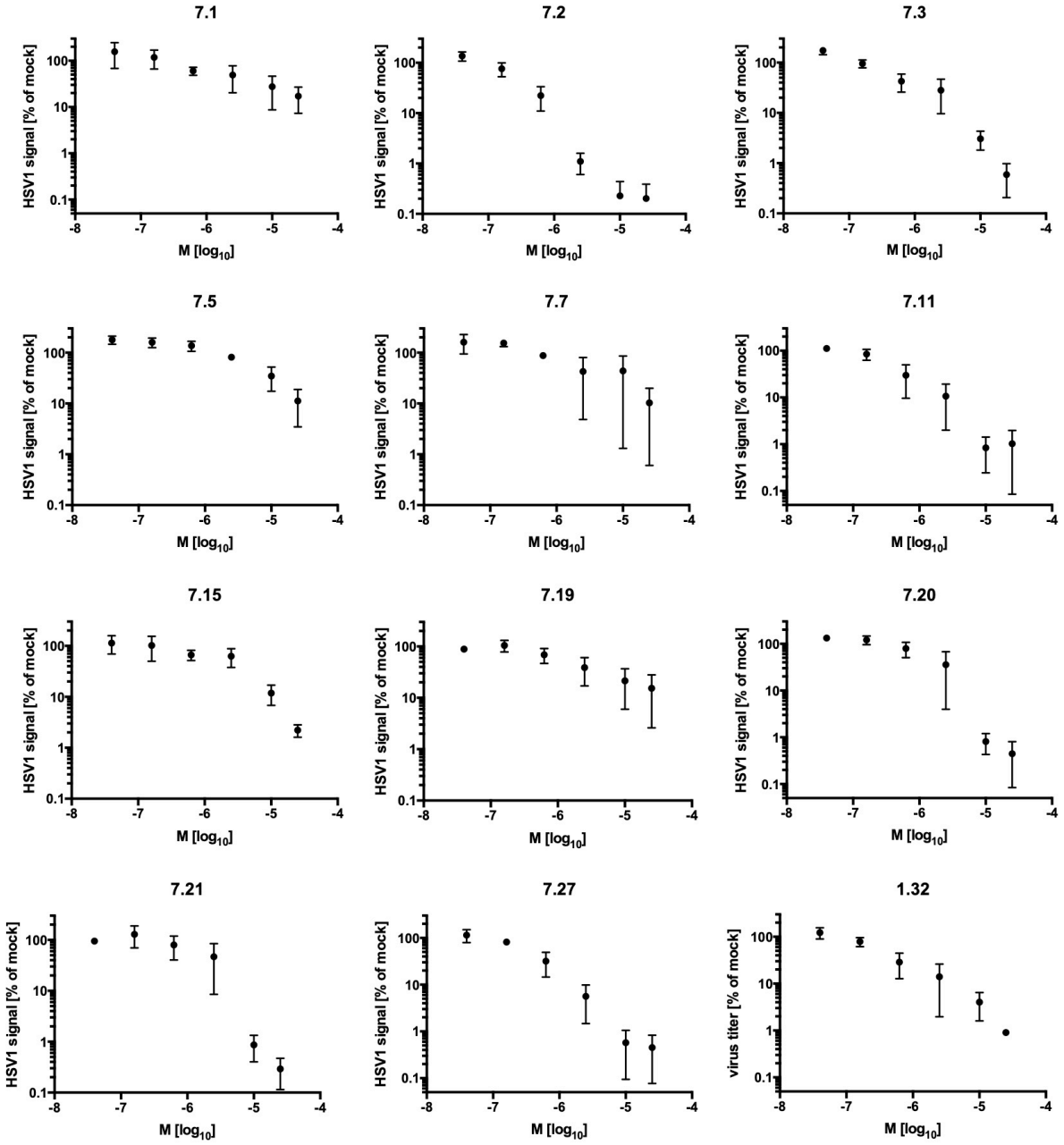
Supplementary Figure 1



Supplementary Figure 1 (related to Figure 1)

MTT assay of Vero (left) or SH-SY5Y (right)cells treated with increasing concentrations of 7.25 for 24h (Vero) or 48h (SH-SY5Y) resulted in a LD_{50} of 1,12 µM (Vero) or 1,48 µM (SH-SY5Y). Each data point displays the mean +/- SEM of three independent experiments (n=3).

Supplementary Figure 2

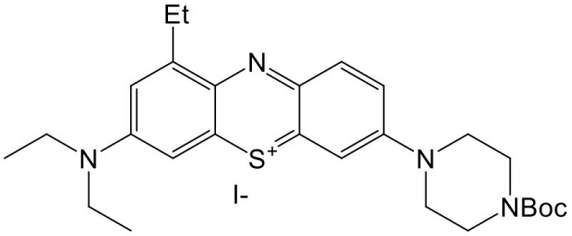


Supplementary Figure 2 (related to Table 1)

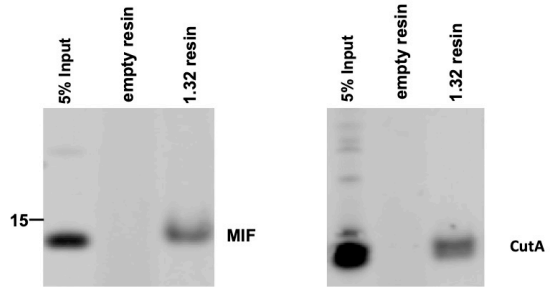
The IC_{50} of analogs of 7.25 were determined by in-Cell ELISA after Vero cells were treated with increasing concentrations of the compounds and infected with HSV-1 (moi=1) for 20h. Each data point displays the mean +/- SEM of three independent experiments (n=3).

Supplementary Figure 3

A



B

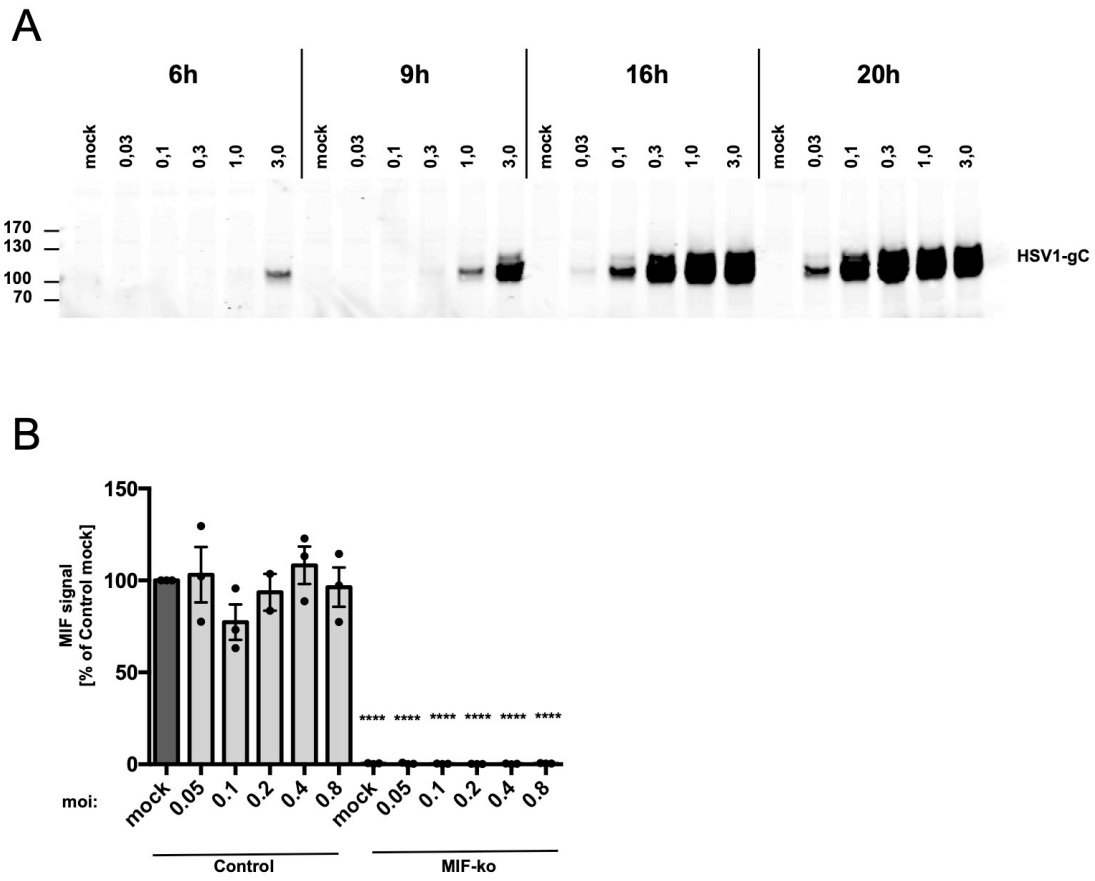


Supplementary Figure 3 (related to Table 2)

A Structure of Boc-modified 1.32 allowing coupling to resin via a piperazine linker.

B DRAC assay with cell lysates (500 µg) derived from SH-SY5Y-tau P301S cells that were applied either on 20 µl empty control resin or resin presenting 1.32. MIF (left) as well as cutA (right) were specifically eluted from drug resins by urea. On the left 5% of input material is shown.

Supplementary Figure 4

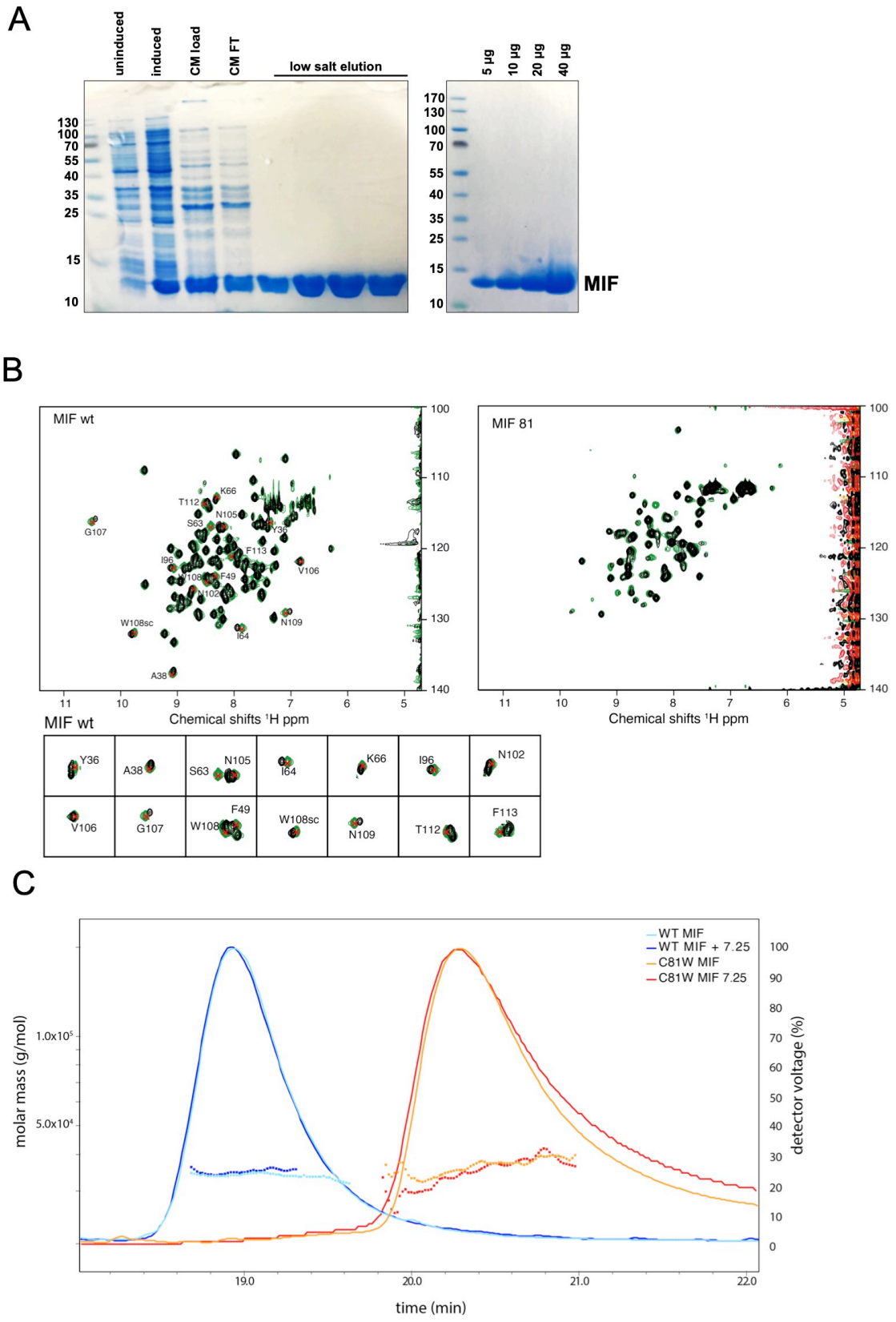


Supplementary Figure 4 (related to Figure 2)

A SH-SY5Y-tau P301S cells could efficiently be infected with HSV-1. The cells were infected with increasing mois (0,03 to 3,0) and then lysed after 6h, 9h, 16h or 24h. The lysates were analyzed by Western Blot using an antibody against the glycoprotein C (gC) of HSV-1.

B Expression of MIF was not modulated upon infection with HSV-1. The diagram displays the quantification of MIF normalized to GAPDH shown in Figure 2C. Data are presented as mean +/- SEM. One-way ANOVA (Dunnet´s post-hoc) ****p<0.0001.

Supplementary Figure 5



Supplementary Figure 5 (related to Figure 3)

A Recombinant expression and purification of human wildtype MIF in *E.coli*. Expression of tag-free MIF was induced in BL21 bacteria and MIF was then purified by ion exchange chromatography. The purification steps are shown in the SDS-PAGE on the left. The right image shows the purity (>95%) of MIF after loading up to 40 µg on a SDS gel.

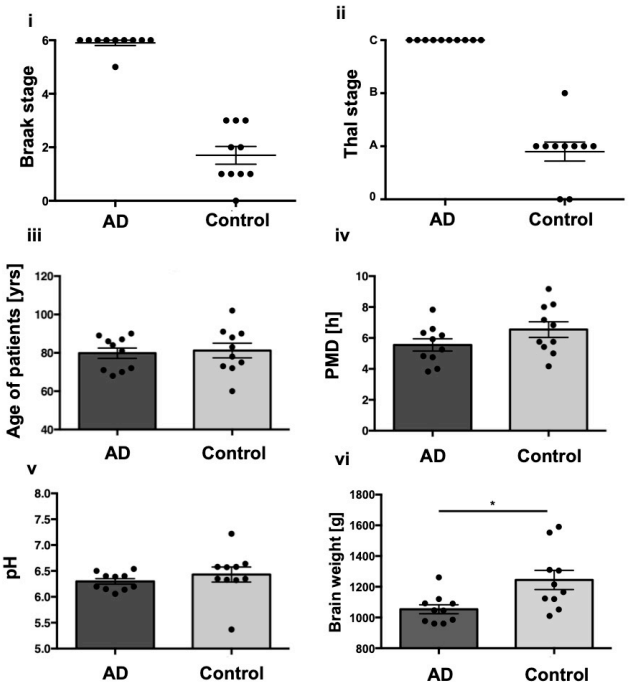
B HSQC spectra of the apo-MIF (green) and of the MIF in the presence of 7.25 (black). The resonances showing the highest perturbations are zoomed in within the squares below. The residues showing the most important chemical shift perturbations are shown in the MIF structure in the figure 3.

Supplementary Figure 6

A

sample	sex	age	braak	amyloid	pmd [h]	ph	weight	apoe
AD 1	f	72	6	C	5,92	6,54	960	32
AD 2	f	68	6	C	3,83	6,50	985	32
AD 3	f	84	6	C	4,83	6,06	960	33
AD 4	f	86	6	C	5,25	6,20	1090	33
AD 5	m	87	5	C	6,17	6,14	1047	33
AD 6	f	90	6	C	4,75	6,39	1045	33
AD 7	m	71	6	C	6,58	6,20	1092	33
AD 8	m	70	6	C	6,33	6,40	1261	43
AD 9	f	89	6	C	4	6,40	977	43
AD 10	m	81	6	C	7,83	6,15	1120	43
N 1	f	60	0	O	8,17	6,58	1310	32
N 2	f	75	1	A	9,17	6,57	1305	32
N 3	f	72	1	A	6,83	7,22	1165	33
N 4	f	78	1	A	7,17	6,32	1120	33
N 5	m	90	2	O	5,75	6,35	1215	33
N 6	m	73	2	A	8	5,37	1553	33
N 7	f	88	3	A	5,42	6,33	1010	33
N 8	f	91	3	A	4,17	6,58	1052	43
N 9	m	83	1	B	5,75	6,35	1590	43
N 10	m	102	3	A	5	6,64	1124	43

B



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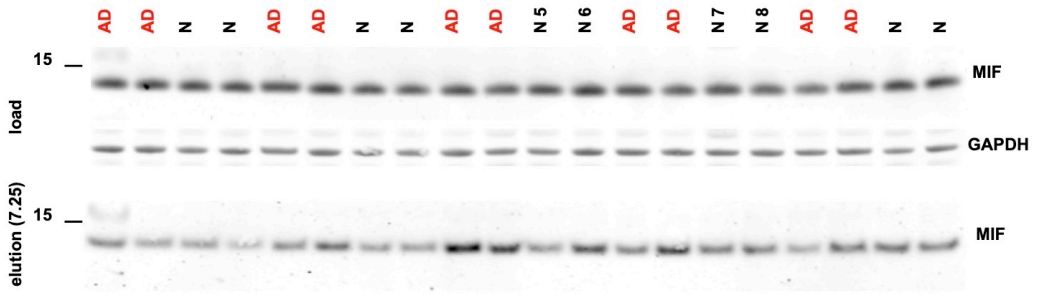
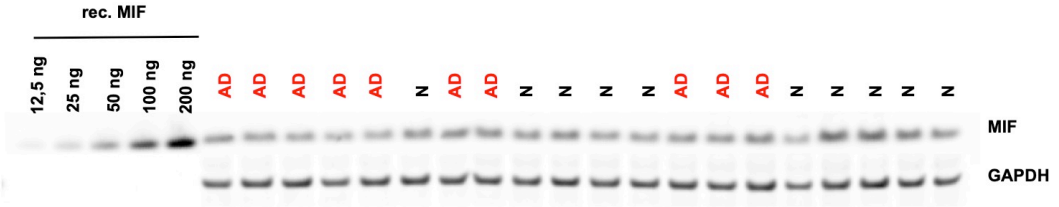


D



Supplementary Figure 6 (related to Figure 4)

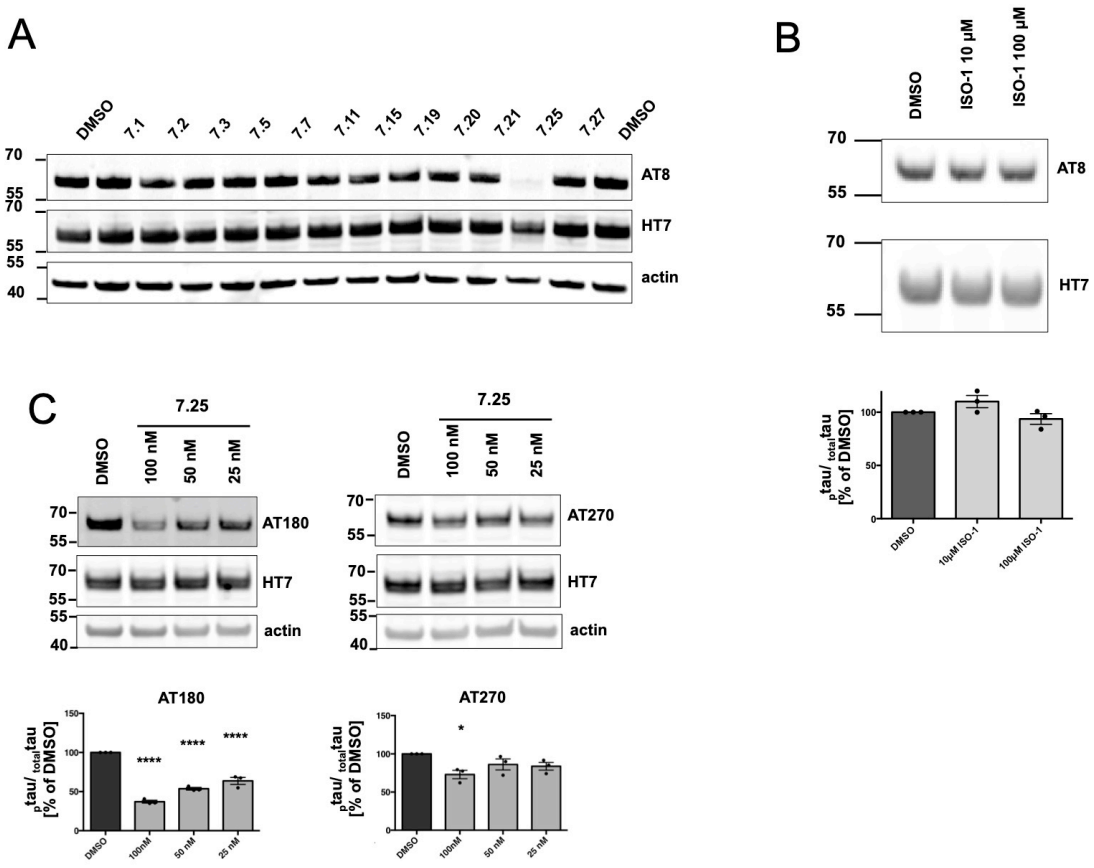
A Table describing the brain samples retrieved from the Amsterdam brain collection regarding, sex, age, tau pathology (braak), amyloid pathology (according to Thal stages), post mortem time (pmd), pH of sample, brain weight and ApoE gene status.

B All AD samples displayed severe tau (i) as well as amyloid pathology (ii) and show an equal distribution of age (iii) and no differences in pmd (iv) or pH (v). The weight of the brains from the AD patients was significantly reduced compared to the controls (vi).

C Representative complete Western Blot of DRAC analysis of brain samples shown in Fig. 4A.). The upper panel displays the loading controls (MIF and GAPDH) of brain homogenates and the lower panel the precipitated and 7.25-eluted MIF.

D Representative quantitative Western Blot of brain samples used for sandwich ELISA shown in Fig. 4C. Increasing concentrations (12,5 ng to 200 ng) of recombinant expressed human MIF (Suppl. Fig. 5A) were used to generate a standard curve allowing the quantification of MIF within the brain samples.

Supplementary Figure 7



Supplementary Figure 7 (related to Figure 5)

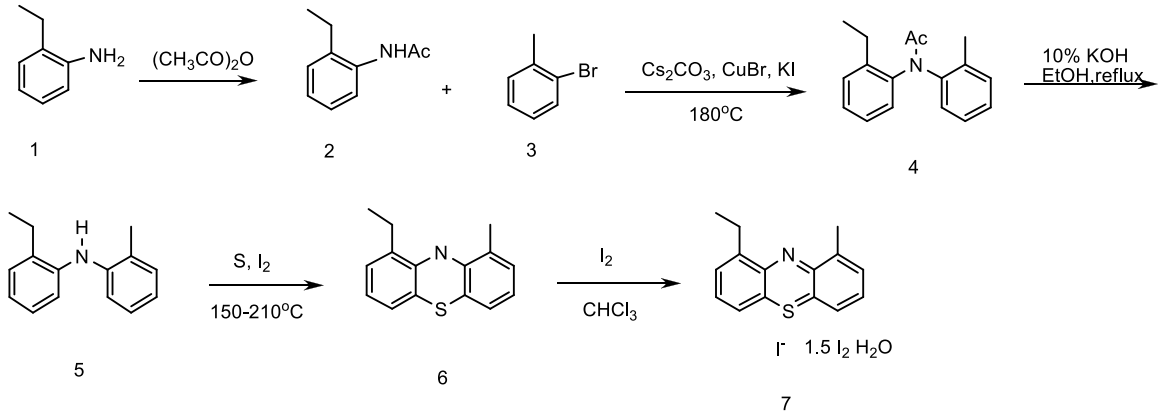
A Representative Western Blot of results shown in Fig. 5C.

B Up to 100 µM of the MIF inhibitor ISO-1 did not modulate tau phosphorylation (AT8) in SH-SY5Y-tau P301S cells (upper diagram), whereas 1.32 showed a slight but significant reduction (lower diagram). The compounds were analyzed in different set-ups. Values from three independent experiments (n=3) each are presented as mean +/- SEM. Two-tailed t-test *p<0.05.

C 7.25 reduced tau phosphorylation at specific sites including those recognized by AT180 and AT270. No reduction was detected at position 396 and 404. The diagrams show the signals of the phosphor-tau specific antibodies normalized to total tau (HT7) from three (n=3) independent experiments. Data are presented as mean +/- SEM. One-way ANOVA (Dunnet´s post-hoc) ****p<0.0001; *p<0.05.

Supplementary methods: Chemical syntheses

1-Ethyl-9-methylphenothiazin-5-ium tetraiodide hydrate



a. N-Acetyl-2-ethylaniline (2): commercial 2-ethylaniline (1) (50mL, 0.40 mol) was dissolved in acetic anhydride (160mL, 1.70 mol) and stirred at room temperature for 2h. Then the reaction mixture was poured into H_2O, the whole was extracted with ethyl acetate (2x200mL). The combined organic extracts were washed with 5% aqueous $NaHCO_3$, brine, dried (K_2CO_3), filtered and concentrated to provide the title compound as a white solid (60.0 g, 92%).

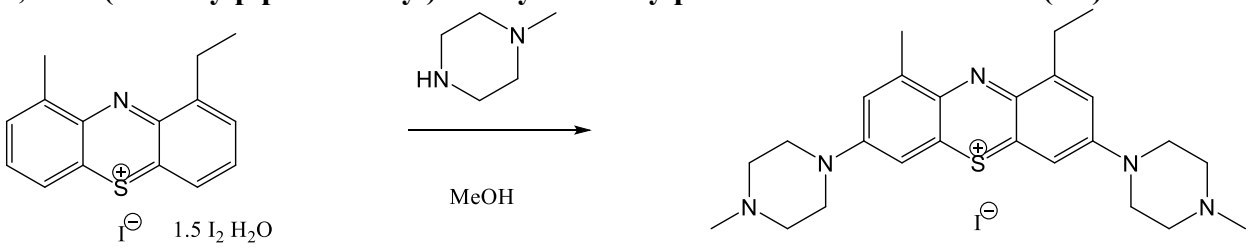
b. N-Acetyl-2-ethyl-2'-methyldiphenylamine (4): a mixture of the N-acetyl-2-ethylaniline (35.0 g, 215 mmol), anhydrous Cs_2CO_3 (70.0 g, 215 mmol), CuBr (2.86 g, 20 mmol), KI (3.33 g, 20 mmol) and 2-bromotoluene (3) (78mL, 640 mmol) was stirred and heated at 175-180^{0}C under an argon atmosphere for 48h. After cooling the reaction mixture was poured into ice-H_2O and extracted with ethyl acetate (2x200mL), the combined organic extracts were washed with brine, dried over anhydrous K_2CO_3, filtered and concentrated to dryness. The obtained crude material was purified by flash chromatography (using ethyl acetate - hexane as an eluent) to afford the N-acetyl-2-ethyl-2'-methyldiphenylamine (35.4 g, 65%).

c. 2-Ethyl-2'-methyldiphenylamine (5): a solution of the N-acetyl-2-ethyl-2'-methyldiphenyl-amine (32.5 g, 128 mmol) in 10% KOH (72 g, 1.28 mol)/EtOH (120mL) was stirred and refluxed for 6 h, then poured into H_2O. The mixture was extracted with ethyl acetate (2x100mL). The combined organic layers were washed with brine, dried (Na_2SO_4), filtered and concentrated to dryness, gave dark red oil (21.1g, 78%).

d. 1-Ethyl-9-methyl-10H-phenothiazine (6): to a 2-ethyl-2'-methyldiphenylamine (3.0 g, 14.2 mmol), sulfur (909 mg, 28.4 mmol) and iodine (601 mg, 4.7 mmol) were added. Vial was charged with balloon for discharge. The heating block was preheated (150^{0}C). The vial was heated on the heating block and after 15 min. temperature was increased to 210^{0}C, reaction mixture was stirred and heated for an additional 1 h. The mixture was allowed to cool to 90^{0}C. The dark solid material was dissolved in mixture methanol/chloroform and purified by flash chromatography (ethyl acetate - hexane as an eluent) to afford the desired product (790 mg, 23%).

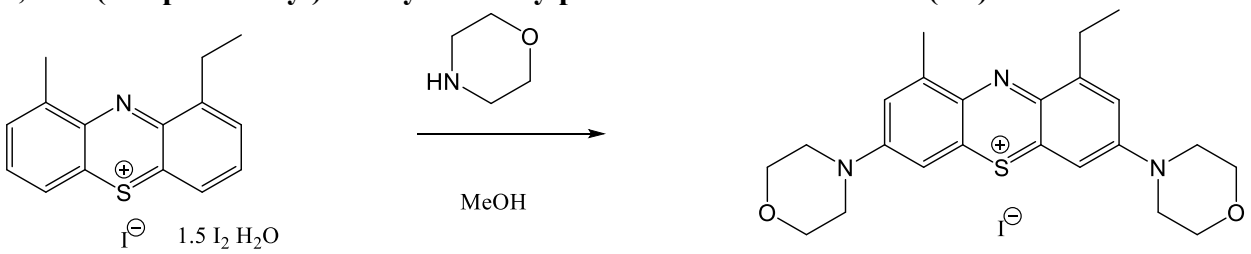
e. 1-Ethyl-9-methylphenothiazin-5-ium tetraiodide hydrate (7) : a solution of 1-ethyl-9-methyl-10H-phenothiazine (4.83g, 20 mmol) in anhydrous chloroform (50mL) was stirred at 5^{0}C and the solution of iodine (15.25 g, 60 mmol) in $CHCl_3$ (300mL) was added drop wise over 3h. The resulting dark solution was stirred for an additional 3h at 5^{0}C, monitored by TLC. After the disappearance of the starting material, the resulting precipitate was filtered, washed with a copious amount of chloroform, dried overnight in vacuum to afford a dark solid (9.18 g, 60%).

3,7-Di- (4-methylpiperazin-1-yl)-1-ethyl-9-methylphenothiazin-5-ium iodide (7.1)



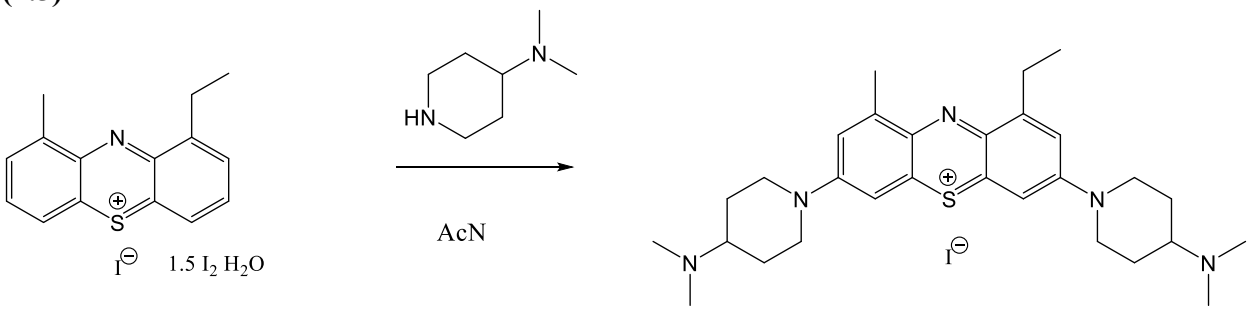
A solution of 1-ethyl-9-methylphenothiazin-5-ium tetraiodide hydrate (50 mg, 0.07 mmol) in methanol (10mL) and 1-methylpiperazine (30 mg, 0.3 mmol) was stirred for 2 h at room temperature. The resulting mixture was concentrated to dryness and purified by flash chromatography using the methanol-chloroform gradient to provide the title compound.

3,7-Di-(morpholin-1-yl)-1-ethyl-9-methylphenothiazin-5-ium iodide (7.3)



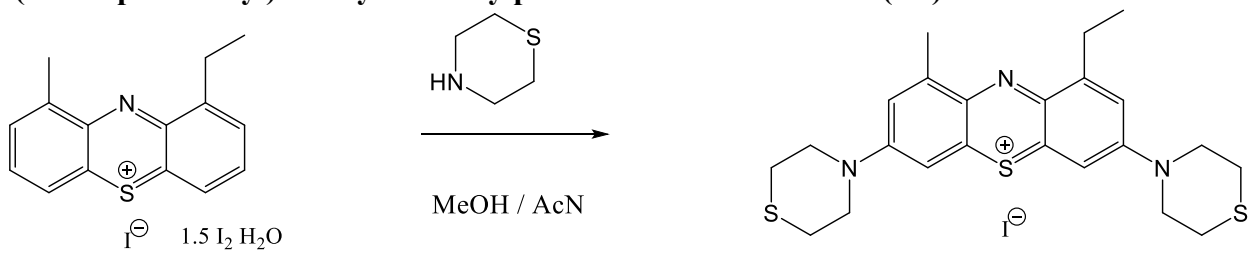
A solution of 1-ethyl-9-methylphenothiazin-5-ium tetraiodide hydrate (50 mg, 0.07 mmol) in methanol (10mL) and morpholine (0.05 mL, 0.5 mmol) was stirred for 1 h at room temperature. The resulting mixture was concentrated to dryness and purified by flash chromatography using the methanol-chloroform gradient to provide the title compound.

3,7-Di-(4-(dimethylamino)piperidin-1-yl)-1-ethyl-9-methylphenothiazin-5-ium iodide (7.5)



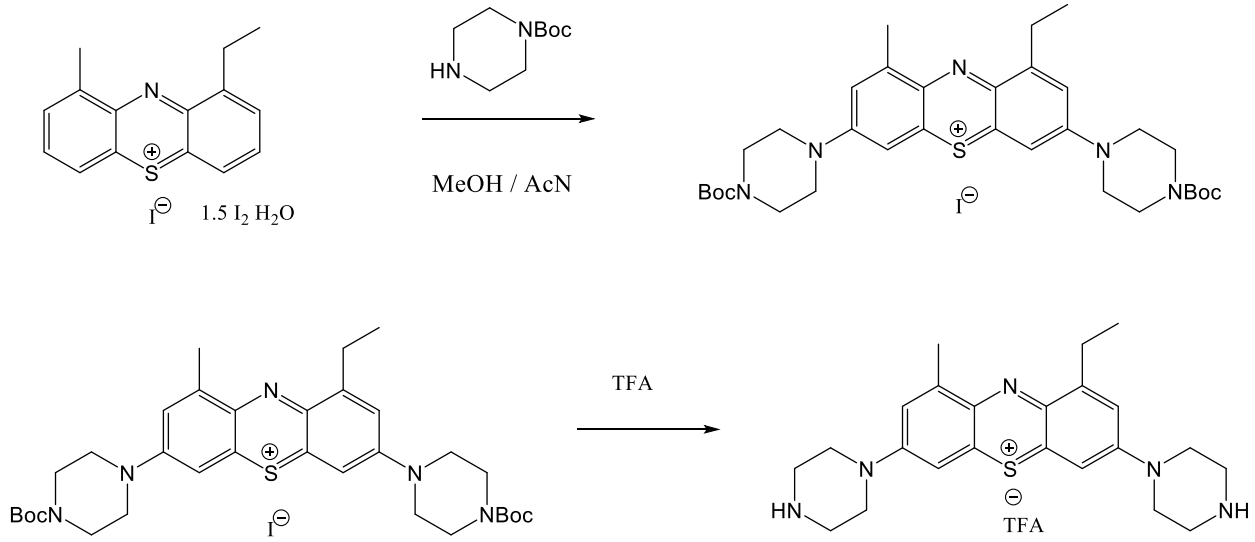
A solution of 1-ethyl-9-methylphenothiazin-5-ium tetraiodide hydrate (382 mg, 0.5 mmol) in acetonitrile (10mL) and 4-(dimethylamino)piperidine (192 mg, 1.5 mmol) was stirred for 1 h at room temperature. The resulting mixture was concentrated to dryness and purified by flash chromatography using the methanol-chloroform gradient to provide the title compound.

3,7-Di-(tiomorpholin-1-yl)-1-ethyl-9-methylphenothiazin-5-ium iodide (7.7)



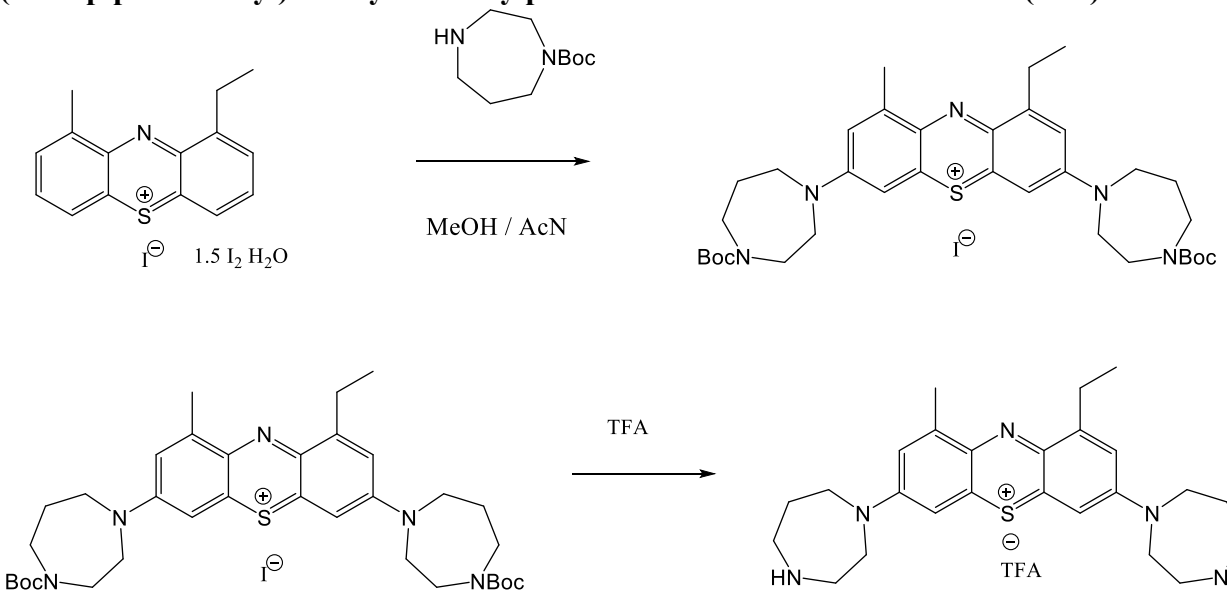
A solution of 1-ethyl-9-methylphenothiazin-5-ium tetraiodide hydrate (191 mg, 0.25 mmol) in mixture methanol and acetonitrile (1:1) (10mL) and tiomorpholine (0.1 mL, 1.0 mmol) was stirred for 1 h at room temperature. The resulting mixture was concentrated to dryness and purified by flash chromatography using the methanol-chloroform gradient to provide the title compound.

3,7-Di(piperazin-1-yl)-1-ethyl-9-methylphenothiazin-5-ium trifluoroacetate (7.19)



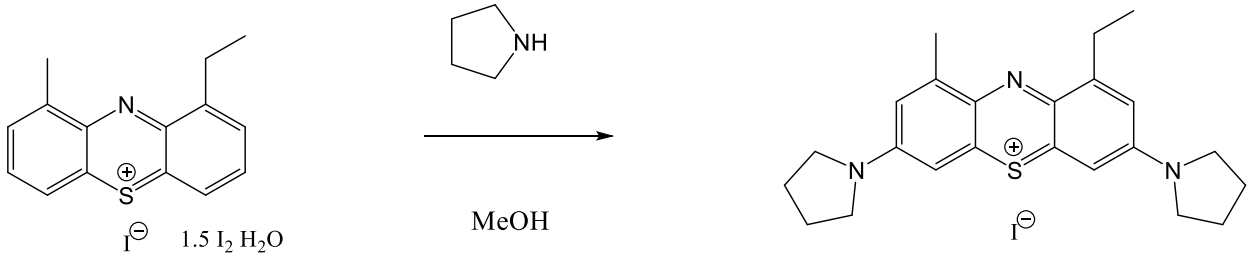
A solution of 1-ethyl-9-methylphenothiazin-5-ium tetraiodide hydrate (153 mg, 0.2 mmol) in mixture methanol and acetonitrile (1:1) (10mL) and N-Bocpiperazine (186 mg, 1.0 mmol) was stirred for 1 h at room temperature. The resulting mixture was concentrated to dryness and dissolved in DCM. TFA (1 mL) was added with stirring. After 30 min. mixture was concentrated and purified by prep-HPLC to provide the title compound.

3,7-Di(homopiperazin-1-yl)-1-ethyl-9-methylphenothiazin-5-ium trifluoroacetate (7.15)



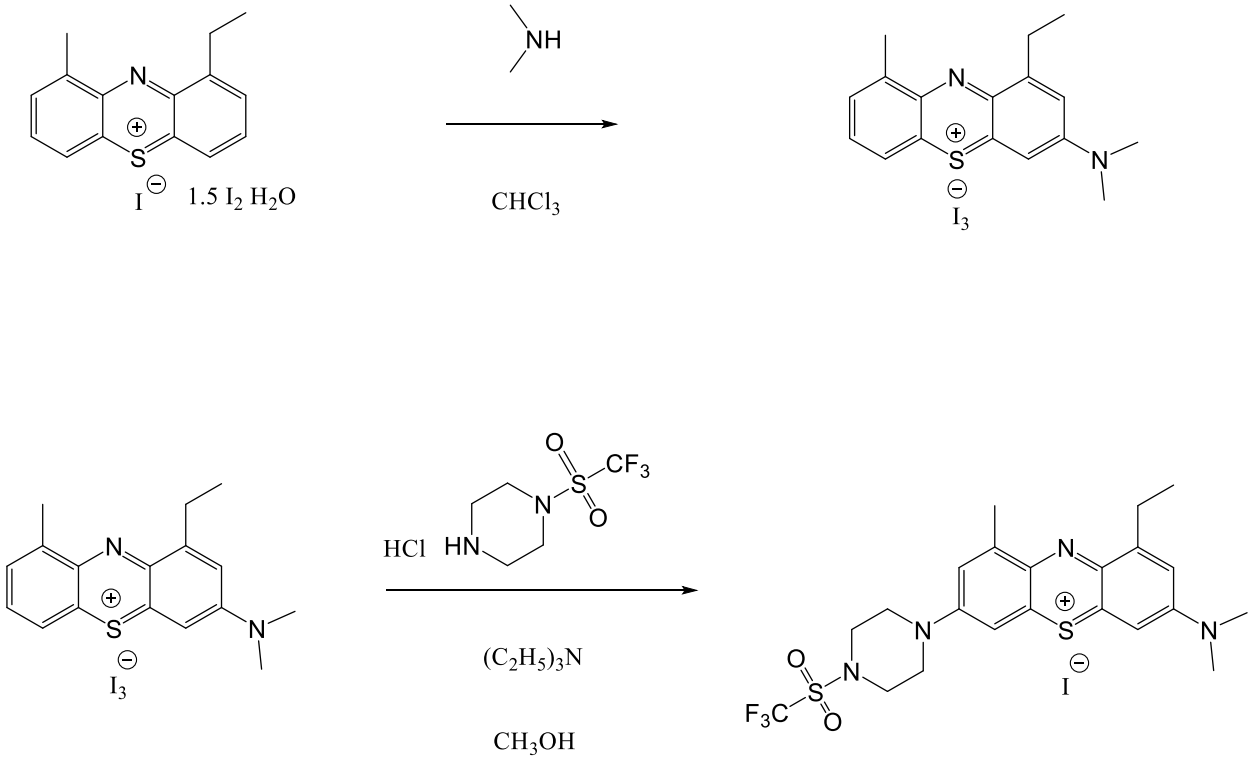
A solution of 1-ethyl-9-methylphenothiazin-5-ium tetraiodide hydrate (153 mg, 0.2 mmol) in mixture methanol and acetonitrile (1:1) (10mL) and N-Bochomopiperazine (200 mg, 1.0 mmol) was stirred for 1 h at room temperature. The resulting mixture was concentrated to dryness and dissolved in DCM. TFA (1 mL) was added with stirring. After 30 min. mixture was concentrated and purified by prep-HPLC to provide the title compound.

3,7-Di(pyrrolidine-1-yl) 1-ethyl-9-methyl-phenothiazin-5-ium iodide (7.25)



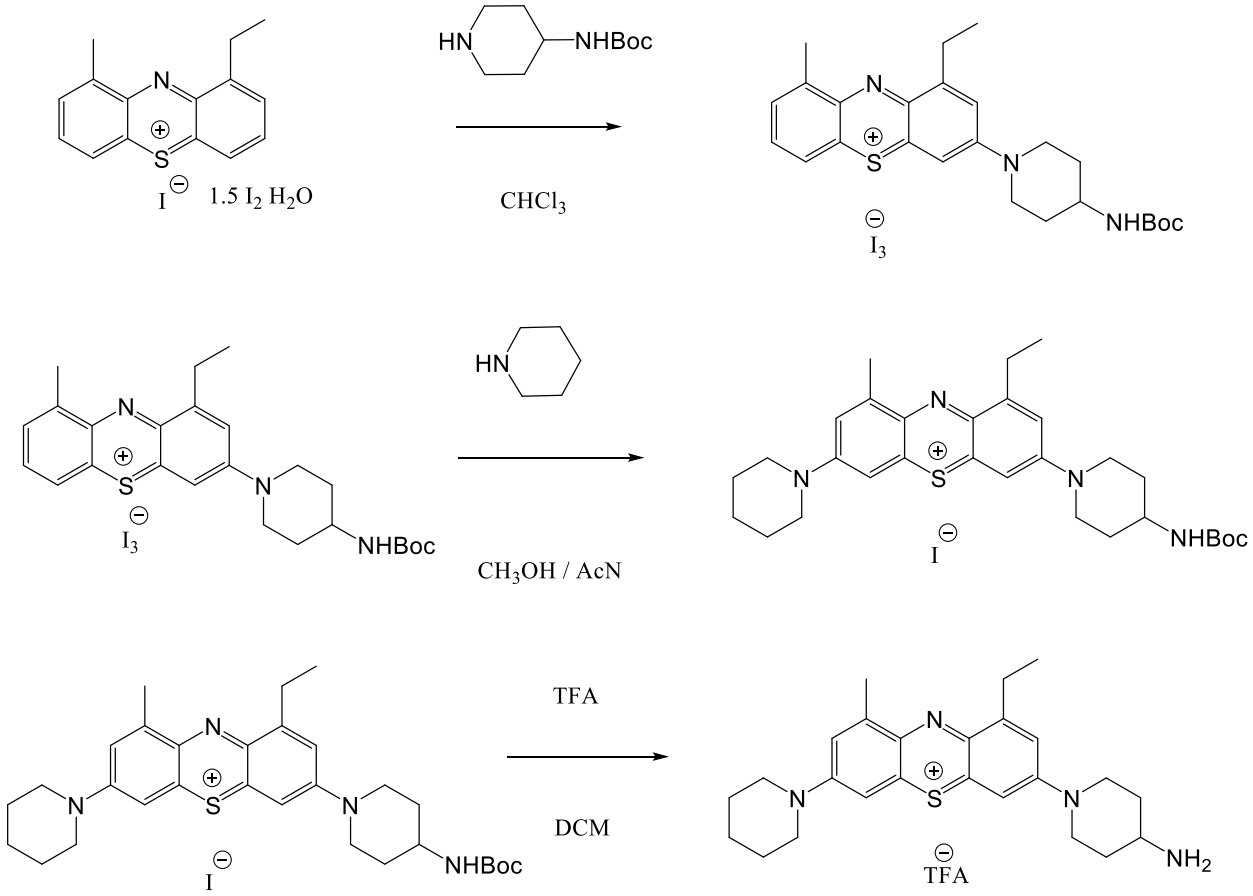
To the stirred mixture of 1-ethyl-9-methylphenothiazin-5-ium tetraiodide hydrate (383 mg, 0.5 mmol) in methanol (20mL) pyrrolidine (142 mg, 2.0 mmol) was added drop wise. The resulting mixture was stirred at room temperature 1 h, concentrated to dryness. Compound was purified with prep-HPLC.

3-(Dimethylamino)-1-ethyl-9-methyl-7-(4-(trifluoromethylsulfonyl)piperazin-1-yl)phenothiazin-5-ium iodide (7.2)



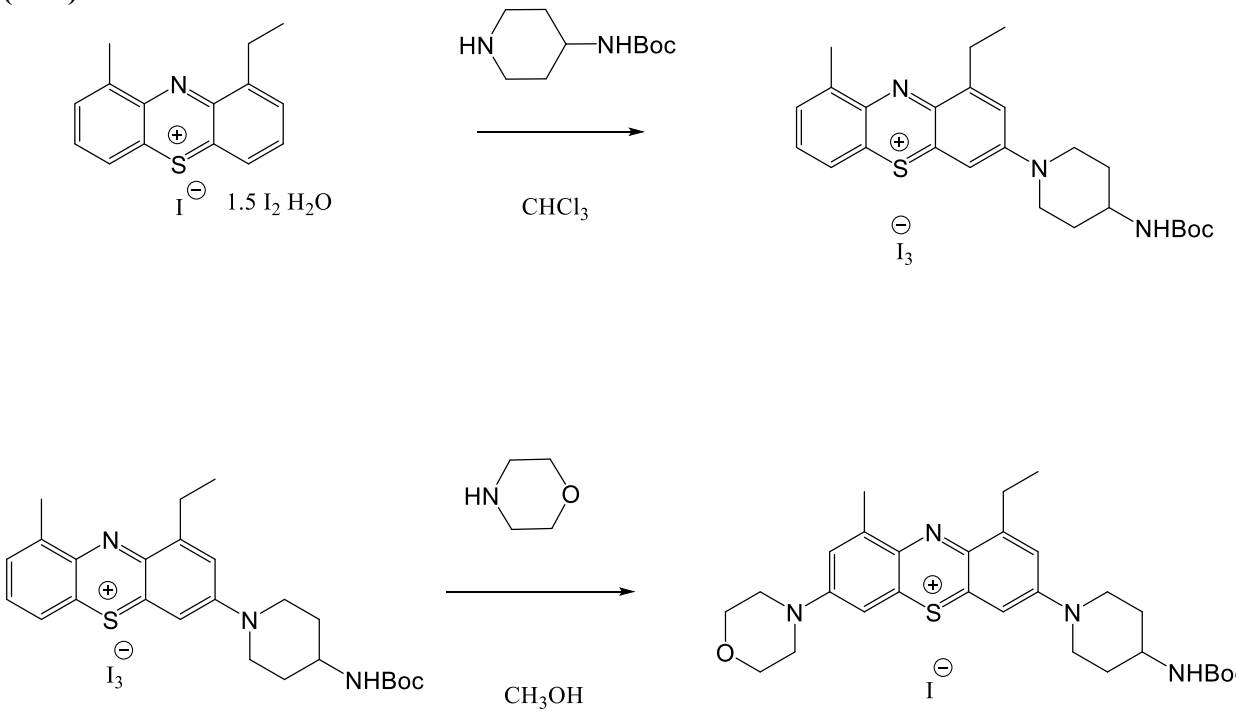
To the stirred mixture of 1-ethyl-9-methylphenothiazin-5-ium tetraiodide hydrate (383 mg, 0.5 mmol) (52) in anhydrous $CHCl_3$ (20mL) dimethylamine (0.5mL, 1.0 mmol, 2M solution in THF) was added drop wise over 0.5 h. The resulting mixture was stirred at room temperature 1 h and concentrated to dryness.
A solution of 3-(dimethylamino)-1-ethyl-9-methylphenothiazin-5-ium triiodide (100 mg, 0.15 mmol) in methanol (10mL), (piperazin-1-yl)trifluoromethyl sulfone hydrochloride (115 mg, 0.45 mmol) and triethylamine (0.5mL) was stirred for 2 h at room temperature. The resulting mixture was concentrated to dryness and purified by flash chromatography using the methanol-chloroform gradient to provide the title compound.

1-Ethyl-9-methyl-7-(piperidin-1-yl)-3-(4-(Bocamino)piperidin-1-yl)phenothiazin-5-ium trifluoroacetate (7.11)



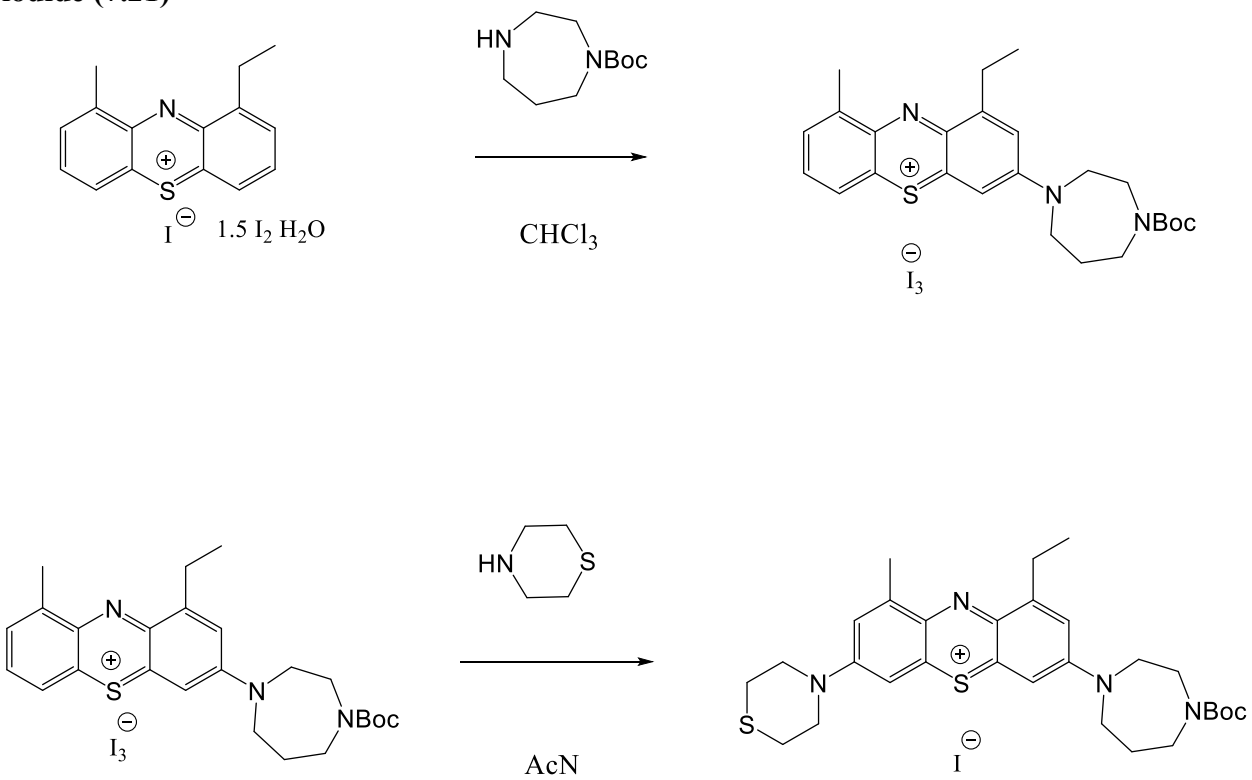
To the stirred mixture of 1-ethyl-9-methylphenothiazin-5-ium tetraiodide hydrate (153 mg, 0.2 mmol) in anhydrous CHCl3 (10mL) 4-Bocaminopiperidine (60 mg, 0.3 mmol) was added with stirring. The resulting mixture was stirred at room temperature overnight, concentrated to dryness. A solution of 1-ethyl-9-methyl-3-(4-Bocamino)piperidin-1yl)phenothiazin-5-ium triiodide (100 mg, 0.12 mmol) in mixture acetonitrile-methanol (1:1) (10mL) and piperidine (0.03 mL, 0.3 mmol) was stirred for 1 h at room temperature. The resulting mixture was concentrated to dryness and purified by flash chromatography using the methanol-chloroform gradient. Product was concentrated to dryness and dissolved in DCM. TFA (1 mL) was added with stirring. After 30 min. mixture was concentrated and purified by prep-HPLC to provide the title compound.

1-Ethyl-9-methyl-7-morpholino-3-(4-Bocaminopiperidin-1-yl)phenothiazin-5-ium iodide (7.20)



To the stirred mixture of 1-ethyl-9-methylphenothiazin-5-ium tetraiodide hydrate (153 mg, 0.2 mmol) in anhydrous $CHCl_3$ (10mL) 4-Bocaminopiperidine (60 mg, 0.3 mmol) was added with stirring. The resulting mixture was stirred at room temperature overnight, concentrated to dryness. A solution of 1-ethyl-9-methyl-3-(4-Bocaminopiperidin-1yl)phenothiazin-5-ium triiodide (165 mg, 0.2 mmol) in methanol (10mL) and morpholine (17.4 mg, 0.2 mmol) was stirred for 4 h at room temperature. The resulting mixture was concentrated to dryness and purified by flash chromatography using the methanol-chloroform gradient.

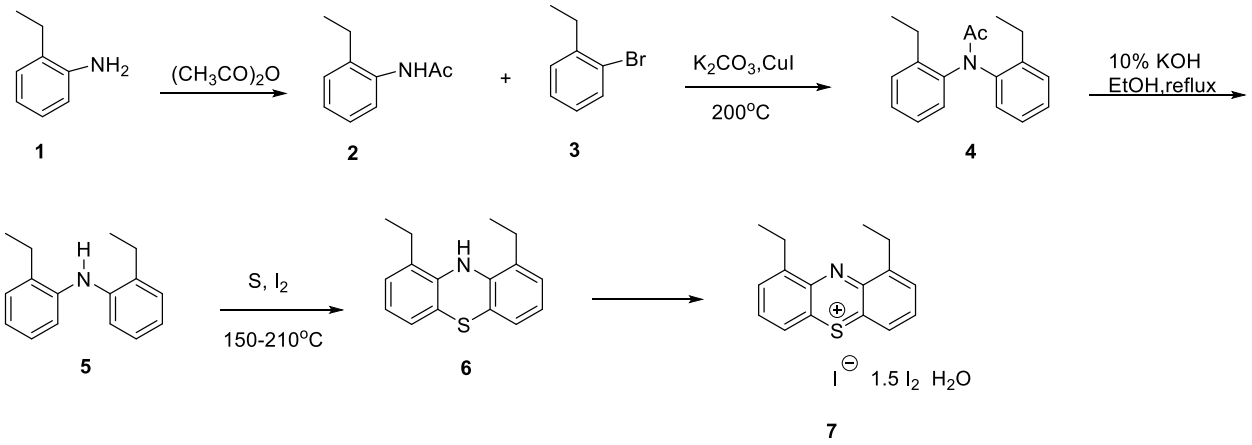
1-Ethyl-9-methyl-7-tiomorpholino-3-(4-Boc-1,4-diazepane-1-yl)phenothiazin-5-ium iodide (7.21)



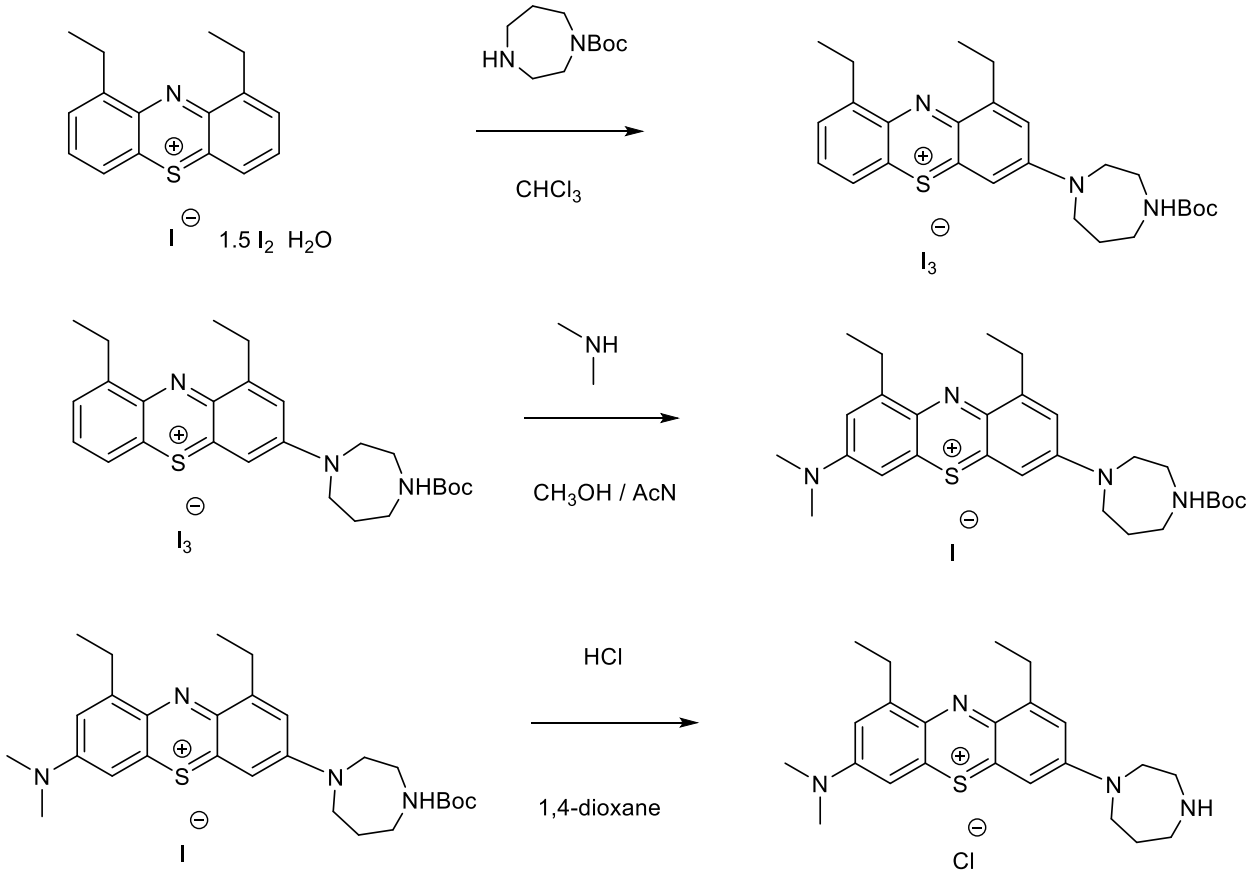
To the stirred mixture of 1-ethyl-9-methylphenothiazin-5-ium tetraiodide hydrate (780 mg, 1.0 mmol) in anhydrous $CHCl_3$ (15 mL) 1-Boc-1,4-diazepane (400 mg, 2.0 mmol) was added at room temperature. The resulting mixture was stirred at this temperature for 4 h. Solvent was removed under vacuum. A solution of 1-ethyl-9-methyl-3-(4-Boc-1,4-diazepane-1-yl)phenothiazin-5-ium triiodide (165 mg, 0.2 mmol) in acetonitrile (10mL) and tiomorpholine (72 mg, 0.8 mmol) was stirred for 4 h at room temperature. The resulting mixture was concentrated to dryness and purified by flash chromatography using the methanol-chloroform gradient.

1,9-Diethylphenothiazin-5-ium tetraiodide hydrate

The same scheme and procedures like for 1-ethyl-9-methylphenothiazinium salt. Compound 3 is 2-ethylbromobenzene.

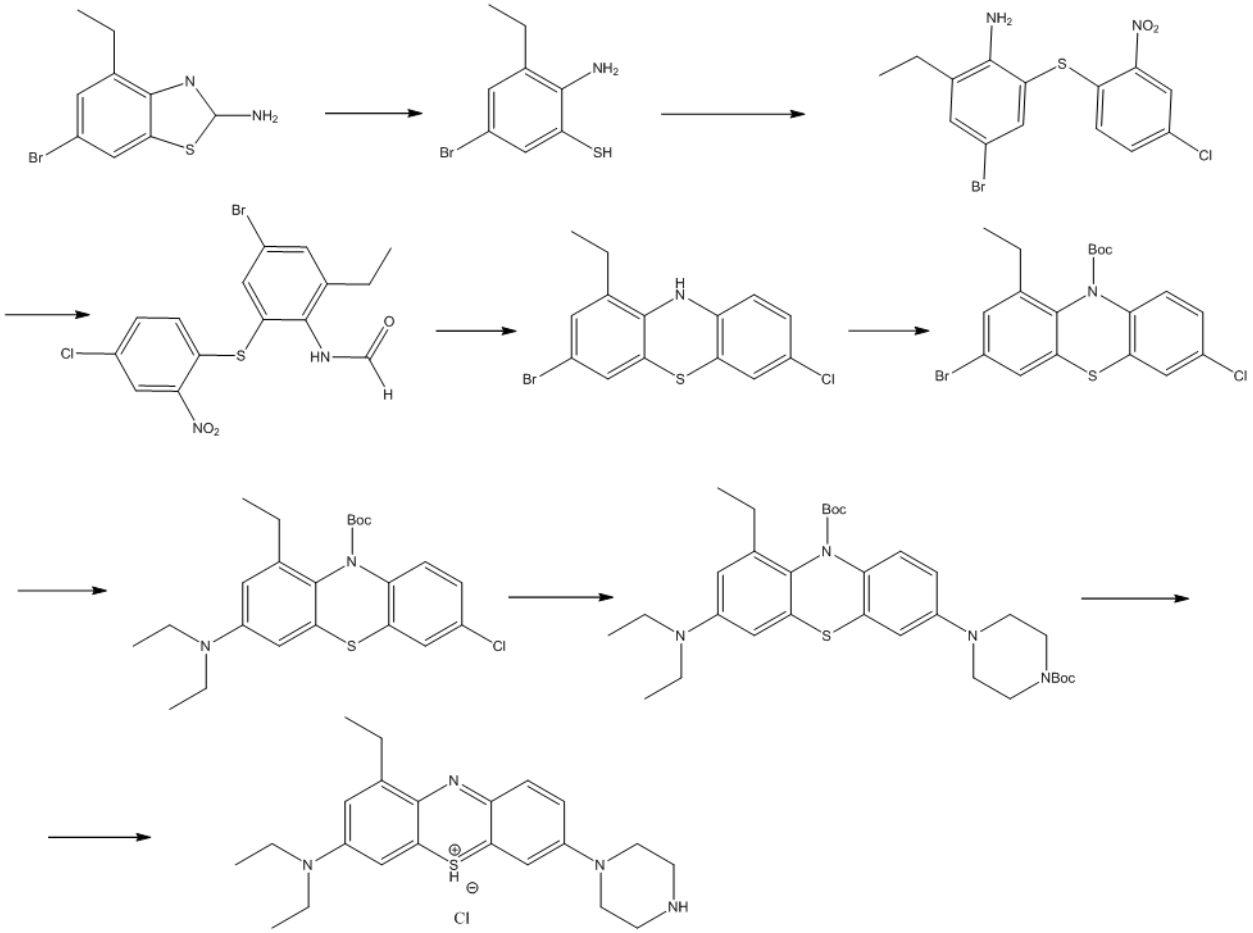


1,9-Diethyl-3-(1,4-diazepane-1-yl)-7-dimethylaminophenothiazin-5-ium iodide (7.27)



To the stirred mixture of 1,9-diethylphenothiazin-5-ium tetraiodide hydrate (3.9 g, 5.0 mmol) in anhydrous CHCl3 (100 mL) 1-Boc-1,4-diazepane (1.2 g, 6.0 mmol) was added at room temperature. The resulting mixture was stirred at this temperature for 2 h. Solvent was removed under vacuum. A solution of 1,9-diethyl-3-(4-Boc-1,4-diazepane-1-yl)phenothiazin-5-ium triiodide in mixture methanol-acetonitrile (1:1) (150mL) and dimethylamine (10 mL 2 M sol. in THF) was stirred for 1 h at room temperature. The resulting mixture was concentrated to dryness and purified by flash chromatography using the methanol-chloroform gradient. Product (300 mg) was concentrated to dryness. HCl (5 mL 4 M solution in 1,4-dioxane) was added with stirring. After 30 min. mixture was concentrated and purified by prep-HPLC to provide the title compound.

1-Ethyl-7-(piperazin-1-yl)-3-diethylaminophenothiazin-5-ium chloride (1.32)



2-Amino-5-bromo-3-ethyl-benzenethiol: 6-Bromo-4-ethyl-1,3-benzothiazol-2-amine (2.0 g, 7.7 mmol) was added to a solution of KOH (13.5 g, 240 mmol) and H2O (25 mL) and the reaction mixture was heated to 150oC overnight. The reaction was monitored by LCMS for consumption of starting material. At the completion of the reaction, the reaction was allowed to cool to RT and ice bath was added as the mixture was slowly neutralized with conc. HCl to pH= 6. The solid was filtered off and dried under vacuum overnight. Both the solid and the filtrate were washed with Et2O and the organic layers were combined. The resulting organic layer was washed with brine, dried over MgSO4, filtered and evaporated to give the product, which was used without further purification. LCMS, M+H=232.0.
4-Bromo-2-(4-chloro-2-nitro-phenyl)sulfanyl-6-ethyl-aniline: 2-Amino-5-bromo-3-ethylbenzenethiol (2.3 g, 10 mmol) was combined with 1,4-dichloro-2-nitrobenzene (2.03 g, 10.6 mmol), Cs2CO3 (10.29 g, 31.6 mmol) and acetonitrile (50mL) and stirred at room temperature overnight. The starting material was monitored by TLC. At the completion of the reaction, the reaction was filtered, and the solvent evaporated to give a residue. The residue was purified by flash silica gel chromatography to give the desired product. LCMS: M+H=388
N-[4-Bromo-2-(4-chloro-2-nitro-phenyl)sulfanyl-6-ethyl-phenyl]formamide: 4-Bromo-2-ethyl-6-
(4-chloro-2-nitro-phenyl)sulfanyl-aniline (14.6 g, 39.22 mmol) was dissolved in formic acid (50mL) and heated at 100oC overnight. The reaction was allowed to cool to RT and ice water was added, keeping the temperature near 0oC. The resulting solid was filtered off, washed with cold water and dried overnight under vacuum. LCMS, M+H=416

3-Bromo-7-chloro-1-ethyl-10H-phenothiazine: N-[4-Bromo-2-ethyl-6-(4-chloro-2-nitrophenyl)
sulfanyl-phenyl]formamide was combined with acetone (20 mL) and heated to reflux and an alcoholic solution of KOH (0.7 g,12.4 mmol) in EtOH (20ml) was added. The resulting solution was refluxed for 30 minutes to 1 hour. Another portion of alcoholic KOH (0.7 g,12.4 mmol) was added and the resulting reaction was refluxed for 4 hours. The mixture was allowed to cool to room temperature. The solvent was evaporated, and the residue was extracted with CHCl3 and brine. The combined organic layers were dried over MgSO4, filtered, and evaporated to give a residue. The crude material was purified by flash silica gel chromatography to give the desired compound. LCMS, M+H=341
tert-Butyl 3-bromo-7-chloro-1-ethyl-phenothiazine-10-carboxylate: 3-Bromo-1-ethyl-7-chloro-
10H-phenothiazine (1.5 g, 4.40 mmol), was combined with Boc2O (1.92 g, 8.81 mmol), DMAP
(0.54 g, 4.40 mmol), and acetonitrile (30 mL). The resulting reaction mixture was stirred and, heated to reflux overnight. After cooling the reaction mixture was concentrated to dryness and purified on the ISCO using EtOAc / Hexanes gradient to afford the desired compound as a waxy solid. M+H =441
tert-Butyl 7-chloro-3-diethylamino-1-ethylphenothiazine-10-carboxylate: To a mixture of Na t-OBu (23 mg, 0.237mmol), Pd(dba)2 (2.3 mg, 0.004mmol), BINAP (2.5 mg, 0.004mmol), diethylamine (17 mg, 0.237mmol) and the tert-butyl 3-bromo-7-chloro-phenothiazine-10-carboxylate (85 mg, 0.206mmol) was added dioxane (2.5mL) (all in a flame dried screw top vial). The mixture was stirred, under argon, at 100oC for 4h (take an aliquot after 2h and check for completeness). Upon completion the reaction was cooled diluted with dioxane (5mL) and filtered through a small celite plug. The filtrate was rotary evaporated to dryness and the residue purified by the flash-chromatography. Yield 75mg (91%) LCMS, M+H=433.
tert-butyl 7-(N-Bocpiperazin-1-yl)-3-diethylamino-1-ethylphenothiazine-10-carboxylate: To a mixture of Na t-OBu (23 mg, 0.237mmol), PdRuPhos (3 mg, 0.004 mmol), RuPhos (2 mg, 0.004 mmol), N-Bocpiperazine (47 mg, 0.237mmol) and tert-Butyl 7-chloro-3-diethylamino-1-ethylphenothiazine-10-carboxylate (83mg, 0.2mmol) was added THF (2.5mL) (all in a flame dried screw top vial). The mixture was stirred under argon at 85oC for 4h (take an aliquot after 2h and check for completeness). Upon completion the reaction was cooled diluted with 10ml of THF and filtered through a small celite plug. The filtrate was rotary evaporated to dryness and the residue purified on the ISCO. Yield 72mg (80%) LCMS, M+H=583.
3-Diethylamino-7-(piperazin-1-yl)-1-ethylphenothiazin-5-ium: A 10mg sample of tert-butyl 3-diethylamino-7-(piperazin-1-yl)-1-ethylphenothiazine-10-carboxylate was treated with 200ul of 4N HCl in dioxane for 1h with stirring. The mixture was then rotary evaporated to dryness. Yield=7.5 mg as HCl salt. LCMS, M+H=418