Supplementary Information

Selective blockade of the lyso-PS lipase ABHD12 stimulates immune responses *in vivo*

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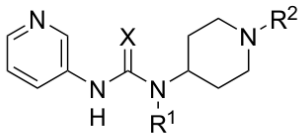
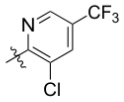
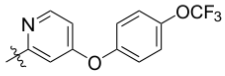
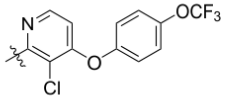
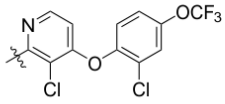
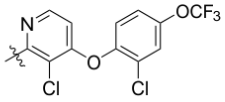
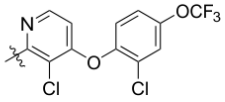
Supplementary Table 1. Information on high-throughput screen for discovery of ABHD12 inhibitors.

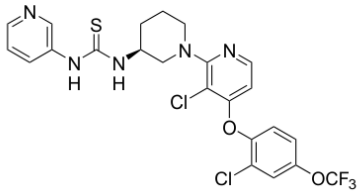
Category	Parameter	Description
Assay	Type of assay	Enzyme-coupled fluorescence assay
	Target	ABHD12
	Primary measurement	ABHD12-mediated hydrolysis of lysophosphatidic acid (lyso-PA) by oxidation of the glycerol phosphate product to generate H_2O_2, which then undergoes a horseradish peroxidase-catalyzed reaction with Ampliflu Red to furnish the fluorescent product resorufin
	Key reagents	ABHD12-transfected HEK293F lysate, lysophosphatidic acid, glycerol-3-phosphate oxidase, horse radish peroxidase, Ampliflu Red
	Assay protocol	10 µL of 0.3 mg/mL mABHD12 or mock lysates prepared as described above was plated in 384-well plates (Greiner Bio-One, 788076). Tetrahydrolipstatin (THL) was used as a control inhibitor in the screening. 50 nL of 2 mM compounds in DMSO was dispensed, and the plates were incubated for 1 h at 37 °C on a shaker at 150 rpm. After the incubation, 1 µL of 11x substrate cocktail solution (330 µM 17:0 LPA, 4.4 U/mL glycerol 3-phosphate oxidase, 3.3 U/mL horseradish peroxidase, 110 µM Ampliflu Red, 0.12 % Triton X-100) was dispensed into each well, and the plates were incubated for 45 min at room temperature. Fluorescence was measured at 571 nm excitation and 585 nm emission.
	Additional comments	The assay is based on the ability of ABHD12 to hydrolyze lyso-PA.
Library	Library size	16,000 compounds arrayed on 384-wellplates as single compounds at 2 mM in DMSO
	Library composition	The compounds represent the drug-like diversity of the Maybridge Screening Collection, and all compounds meet Lipinski guidelines for “drug-likeness”
	Source	Maybridge HitFinder™ Collection
	Additional comments	All compounds have purity greater than 90%
Screen	Format	384-well plate (Greiner Bio-One, 788076)
	Concentration(s) tested	10 µM
	Plate controls	Use of mock-transfected lysate to measure background signals and THL (10 µM) as a control ABHD12 inhibitor
	Reagent/ compound dispensing system	Biomek FXP (Bechman Coulter) for dispensing 50 nL of compounds. BioRAPTR FRD™ Workstation (Bechman Coulter) for dispensing substrate cocktail
	Detection instrument and software	EnVision™ 2014 Multilabel Reader (PerkinElmer) and its accompanying software (Wallac EnVision Manager 1.12) were used for detecting fluorescence
	Assay validation/QC	Average Z’ and S/B values for the screen were 0.78 and 5.5, respectively
	Correction factors	Correction was not performed

	Normalization	% inhibition = 100 x (Average ABHD12 signal – sample result) / (Average ABHD12 signal – Average of THL-treated ABHD12 signal)
	Additional comments	
Post-HTS analysis	Hit criteria	50% inhibition
	Hit rate	1.2%
	Additional assay(s)	Two orthogonal assays (LC/MS-based lyso-PS hydrolysis assay and a competitive ABPP assay using FP-Rh) were used for hit validation (**Supplementary Fig. 3**).
	Confirmation of hit purity and structure	The hit compound was repurchased, resynthesized and retested.
	Additional comments	The structure of the hit compound had been mis-assigned as a semithiocarbazide instead of a thiourea (**Supplementary Fig. 4**)

Supplementary Table 2. Optimization of DO130 and discovery of inactive control compound (S)-DO271. IC_{50} values were determined by gel-based competitive ABPP using the JJH350 probe (see **Supplementary Fig. 7** for representative gels). n = 4 independent experiments for DO130, DO230 and DO253, and n = 3 independent experiments for DO264, DO276, DO277 and (S)-DO271.



compound	X	R^1	R^2	IC_{50} (μM)	95% CI (μM)
DO130 (**1**)	S	H		1.1	0.95 – 1.3
DO230 (**4**)	S	H		0.41	0.34 – 0.48
DO253 (**5**)	S	H		0.025	0.022 – 0.030
DO264 (**6**)	S	H		0.011	0.0096 – 0.013
DO276 (**7**)	S	Me		1.1	0.93 – 1.2
DO277 (**8**)	O	H		0.17	0.14 – 0.19

compound	structure	IC_{50} (μM)	95% CI (μM)
(S)-DO271 (**9**)		>100	N.D.

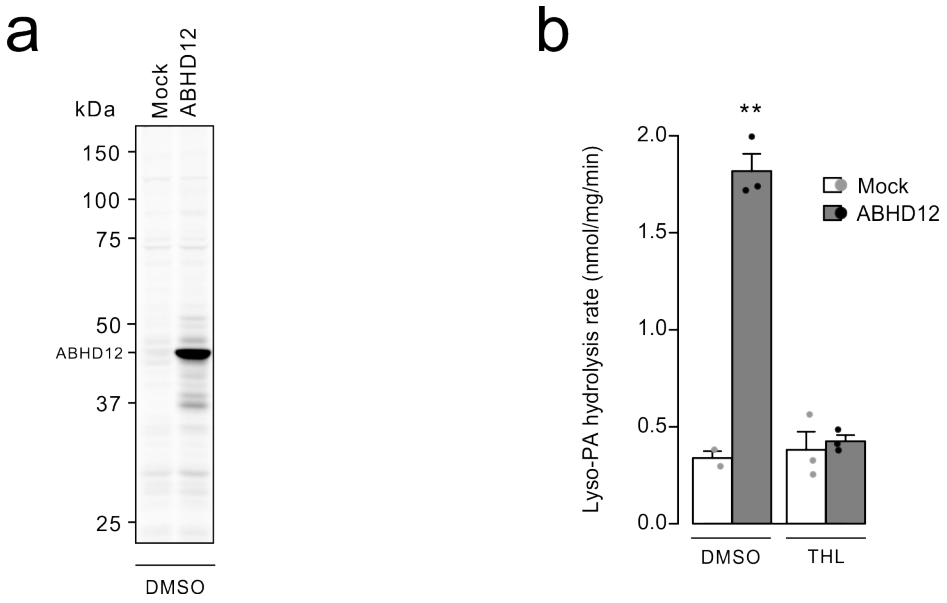
Supplementary Table 3. Complete mass spectrometry-based ABPP data.

See accompanying Excel file.

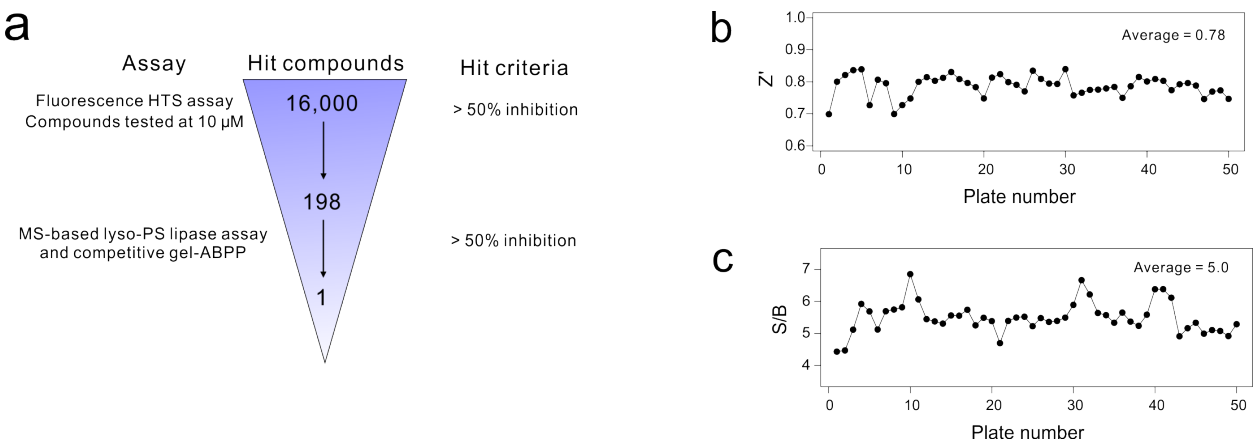
Supplementary Table 4. Complete targeted lipidomics data.

See accompanying Excel file.

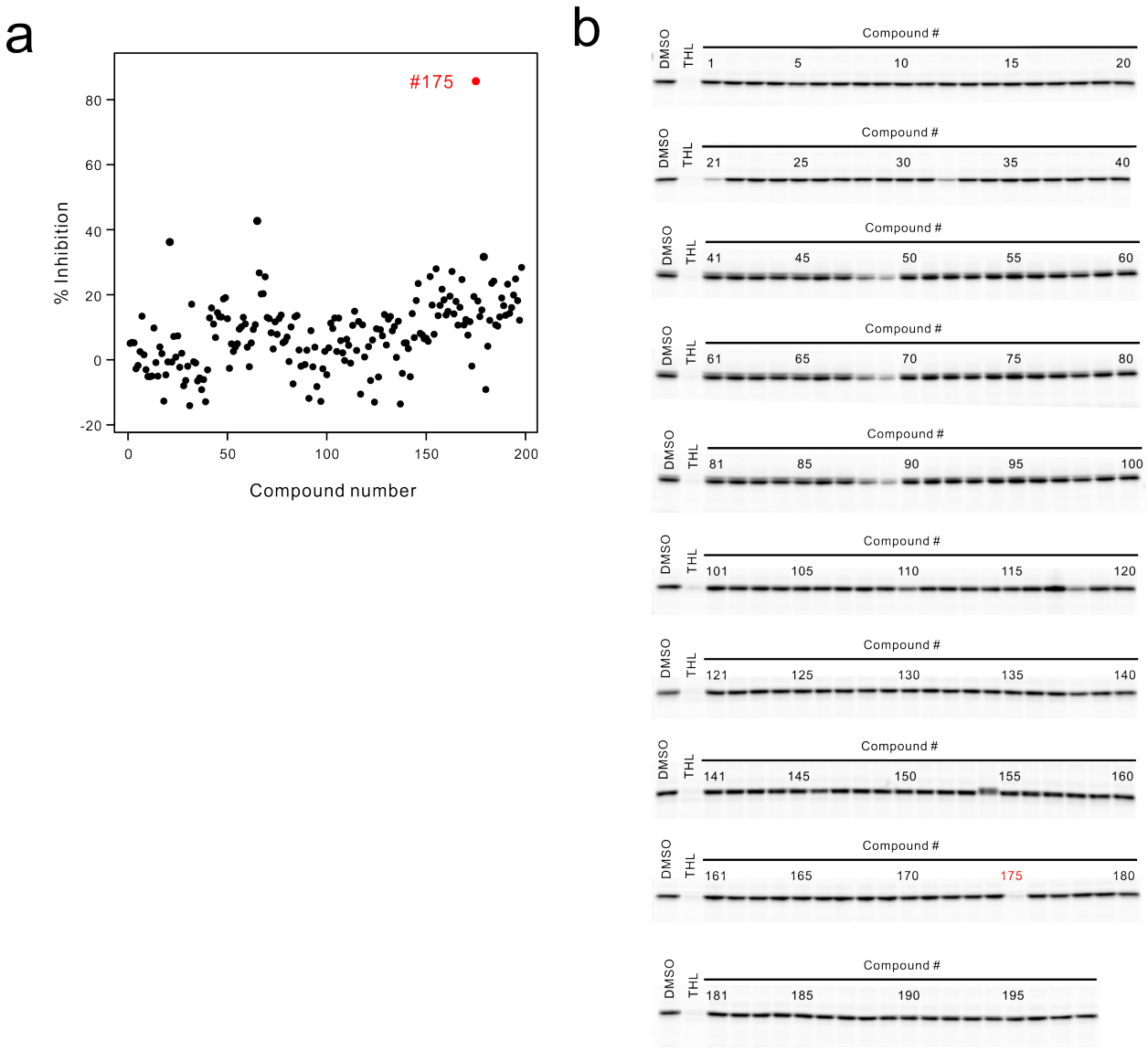
Supplementary Figures



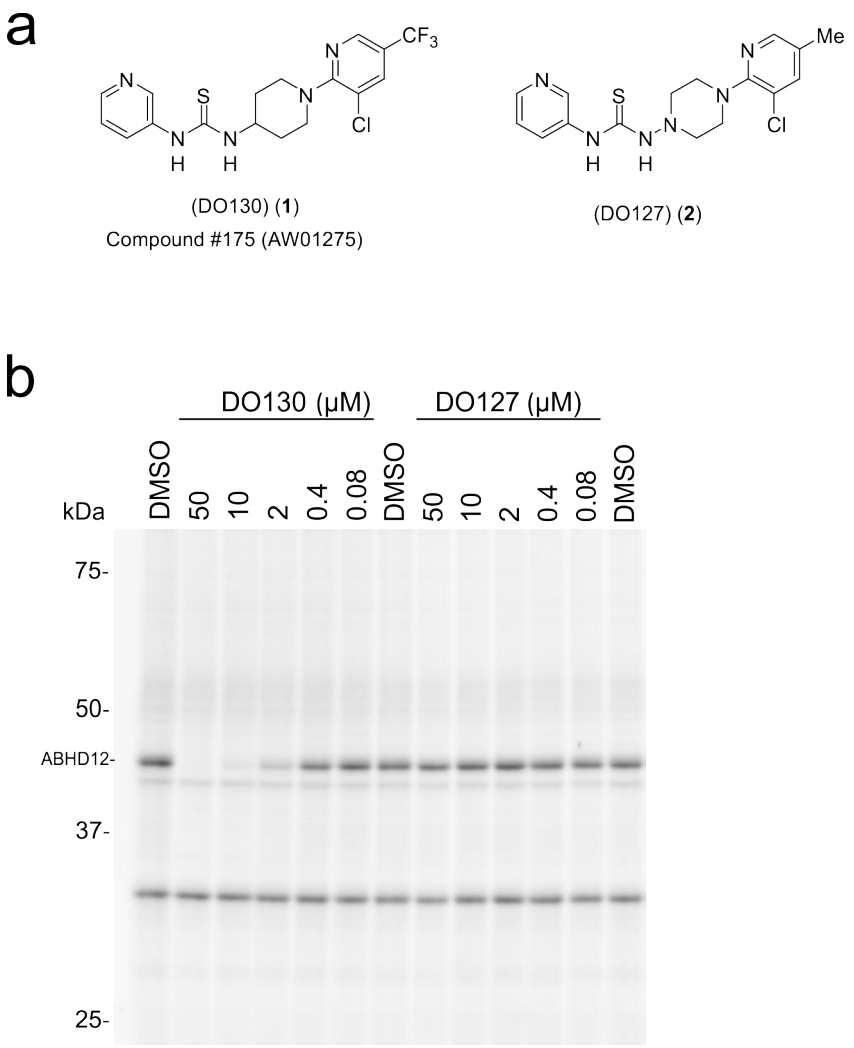
Supplementary Figure 1. Lyso-PA hydrolysis activity of recombinant ABHD12. (**a**) Gel-based ABPP using the FP-Rh probe (1 µM, 30 min, 37 ºC) of membrane proteomes (1 mg/mL) from mock- or ABHD12-transfected HEK293T cells. The result is a representative of three independent experiments. (**b**) Lyso-PA hydrolysis activity of membrane proteomes (0.2 mg/mL) from mock- and ABHD12-transfected HEK293T cells pre-treated with DMSO or THL (10 µM, 30 min). Lyso-PA hydrolysis was measured by LC-MS using 17:0 lyso-PA (100 µM, 30 min, 37 °C) as a substrate and monitoring the production of 17:0 FFA. Data represent average values ± SEM (n = 3 independent experiments). ***p* = 0.0011 (Two-sided Student's *t*-test performed relative to mock control).



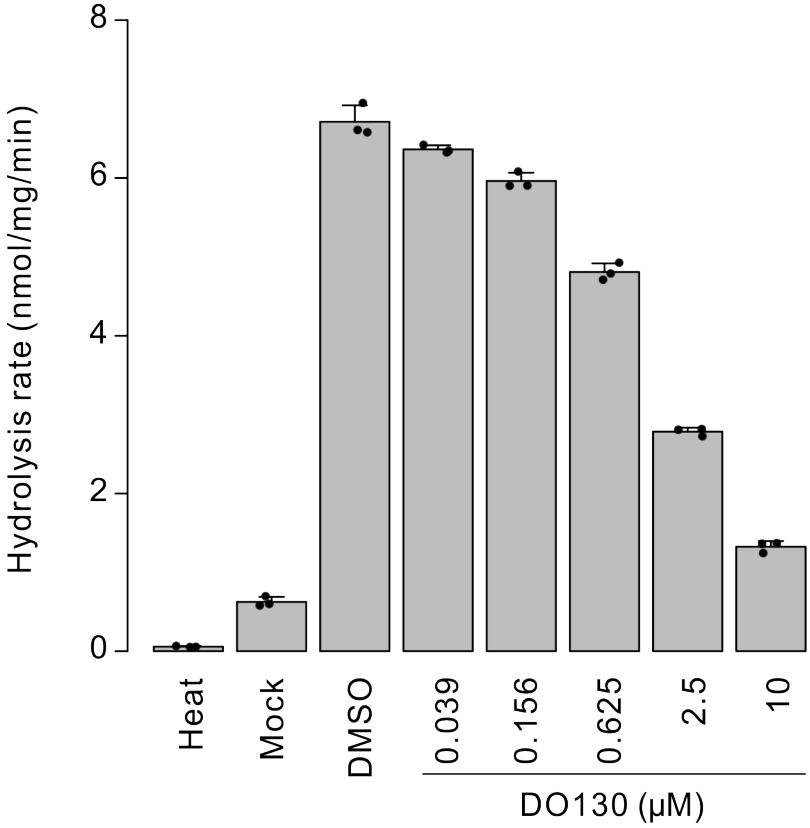
Supplementary Figure 2. Workflow for screen to discover ABHD12 inhibitors and summary statistics for the HTS assay. (**a**) Screening workflow showing hit criteria for primary and secondary assays and number of compounds that satisfied these criteria. (**b, c**) Z' (**b**) and S/B (**c**) values for each plate in the screen. The experiment was performed once.



Supplementary Figure 3. Secondary assay characterization of 198 hit compounds from the enzyme-coupled HTS analysis. (**a**) Secondary assay measuring lyso-PS lipase activity of lysates from ABHD12-transfected cells following pre-treatment with hit compounds (10 μM, 30 min, 37 °C). 17:1 Lyso-PS (100 μM, 20 min, 37 ºC) was used as a substrate. (**b**) Secondary assay measuring ABHD12 activity by gel-based competitive ABPP, where lysates from ABHD12-transfected cells were pre-treated with hit compounds (10 μM, 30 min, 37 °C) and then incubated with the FP-Rh probe (1 μM, 30 min, 37 °C). THL (10 μM) was included as a control. The single hit compound that showed activity in both secondary assays – compound 175 – is marked in red. The experiment was performed once.

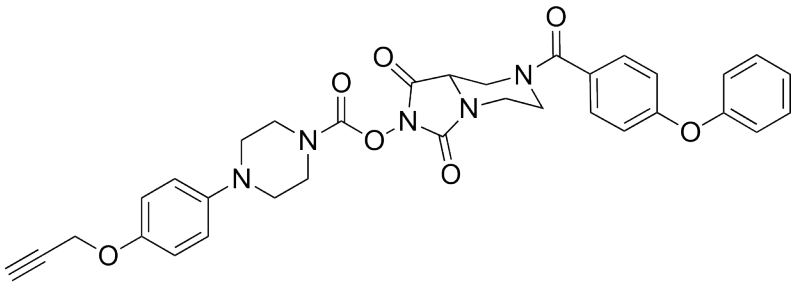


Supplementary Figure 4. Chemical structures and ABHD12 inhibitory activities of correct and mis-assigned structures of the hit compound 175. (**a**) The structure of compound 175 was originally assigned as a semithiocarbazide (DO127), but confirmed by resynthesis and analytical characterization as a thiourea (DO130). (**b**) DO130, but not DO127 blocked ABHD12 activity, as measured by gel-based competitive ABPP of mouse brain membrane proteome (1 mg/mL protein) using the ABHD12-directed probe JJH350 (2 μM, 45 min, 37 ^{o}C). See **Supplementary Fig. 6** for structure of JJH350. The result is a representative of two independent experiments.



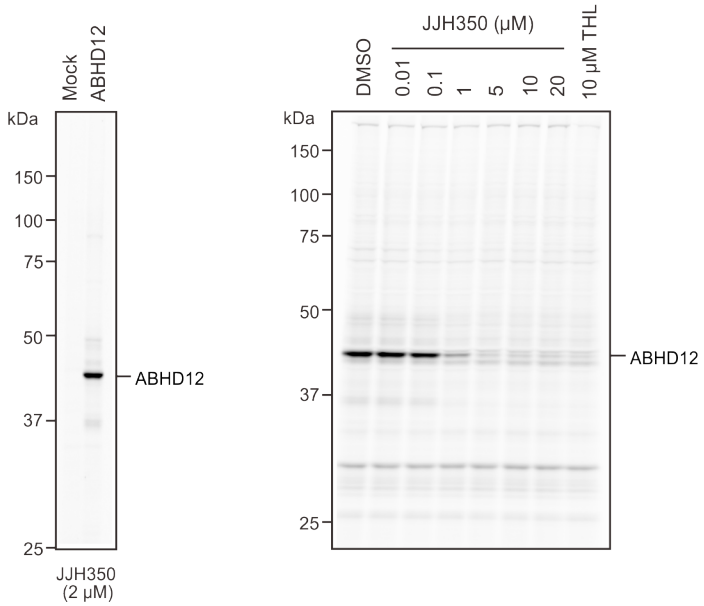
Supplementary Figure 5. Bar graph representation of lyso-PS hydrolysis results shown in **Figure 1e** for membrane lysates from ABHD12-transfected HEK293T cells treated with the indicated concentrations of DO130. Data represent average values ± SD (n = 3 independent experiments). Heat: heat-inactivated proteome; Mock: mock-transfected HEK293T membrane proteome.

a

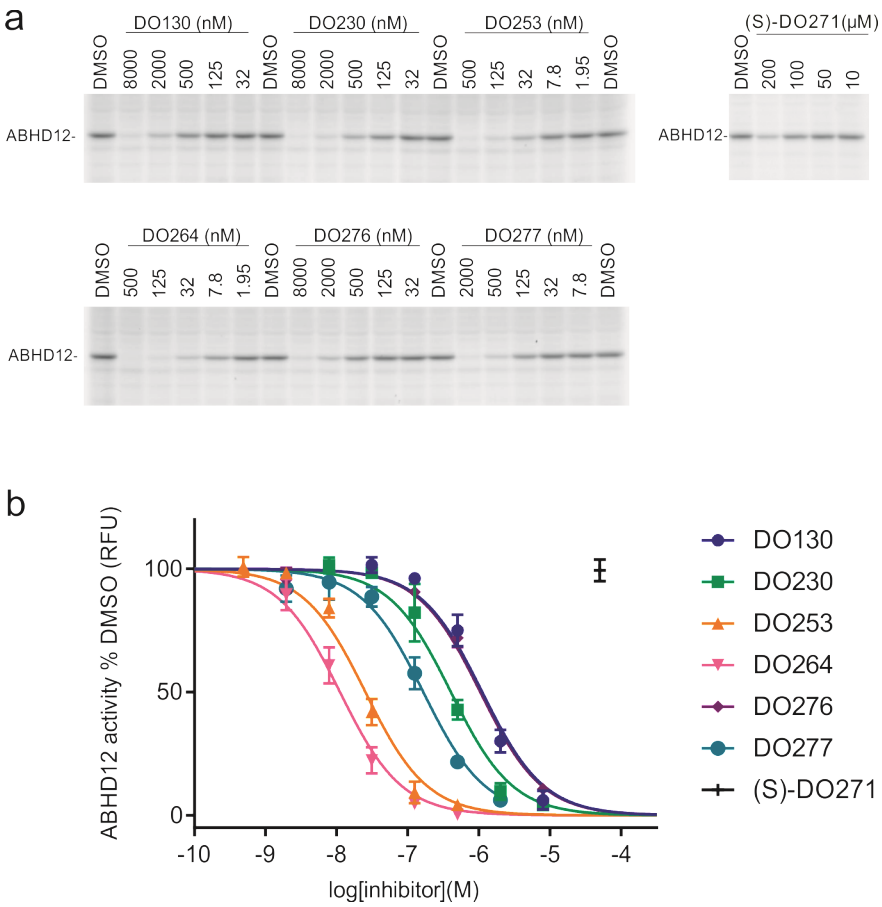


JJH350 (**3**)

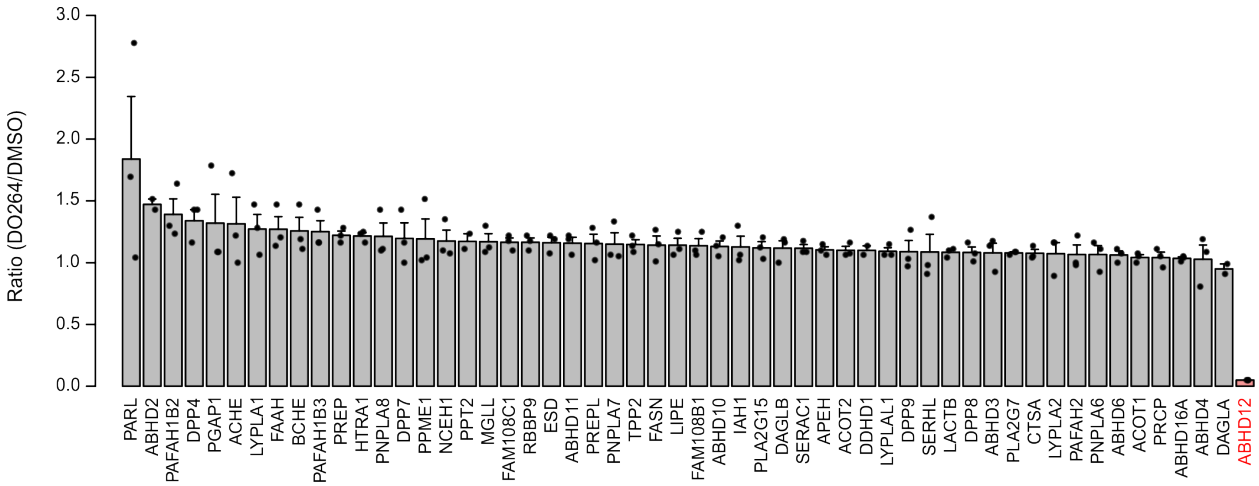
b



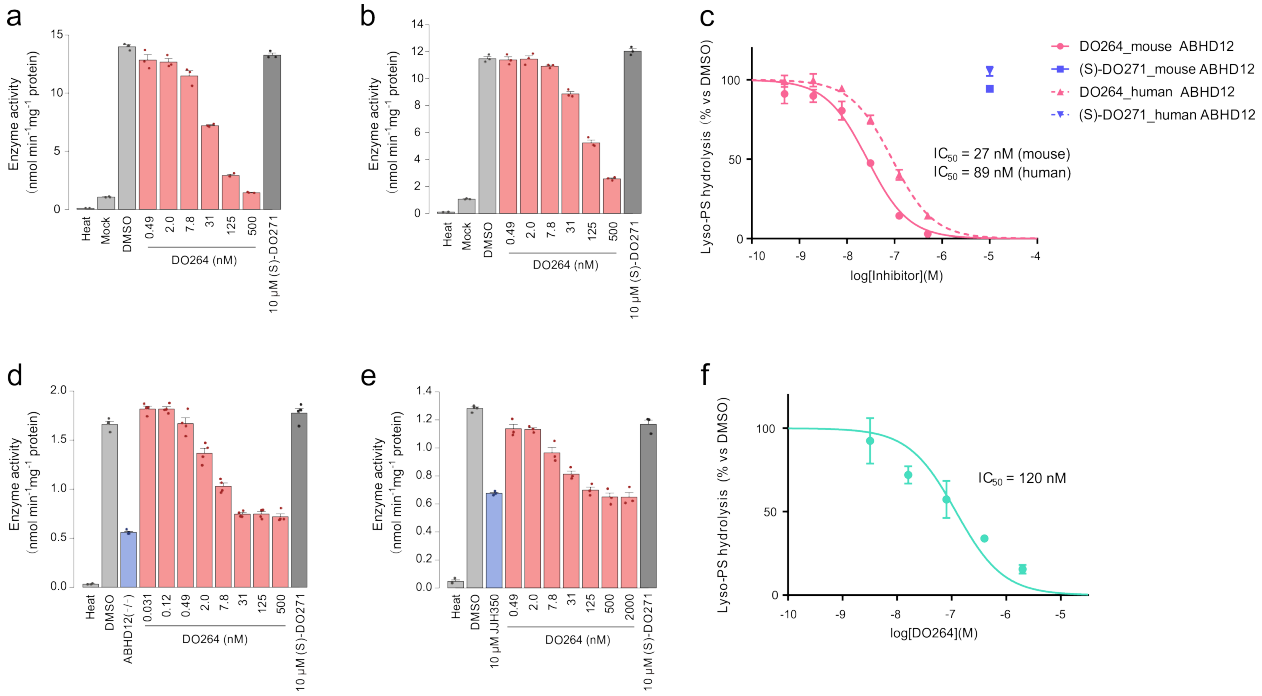
Supplementary Figure 6. Structure and characterization of JJH350, a tailored activity-based probe for ABHD12. (**a**) Chemical structure of JJH350. (**b**) *Left*, Protein labeling profile for JJH350 (2 μM, 45 min, 37 °C) in membrane lysates of mock- or ABHD12-transfected HEK293T cells, where JJH350-labeled proteins were visualized by copper-catalyzed azide-alkyne cycloaddition (CuAAC) with an N_3-rhodamine reporter tag followed by SDS-PAGE and in-gel fluorescence scanning. *Right*, Gel-based competitive ABPP showing concentration-dependent blockade of FP-Rh labeling (1 μM, 30 min, 37 °C) of ABHD12 by JJH350 in membrane lysates from ABHD12-transfected HEK293T cells. The result is a representative of three independent experiments.



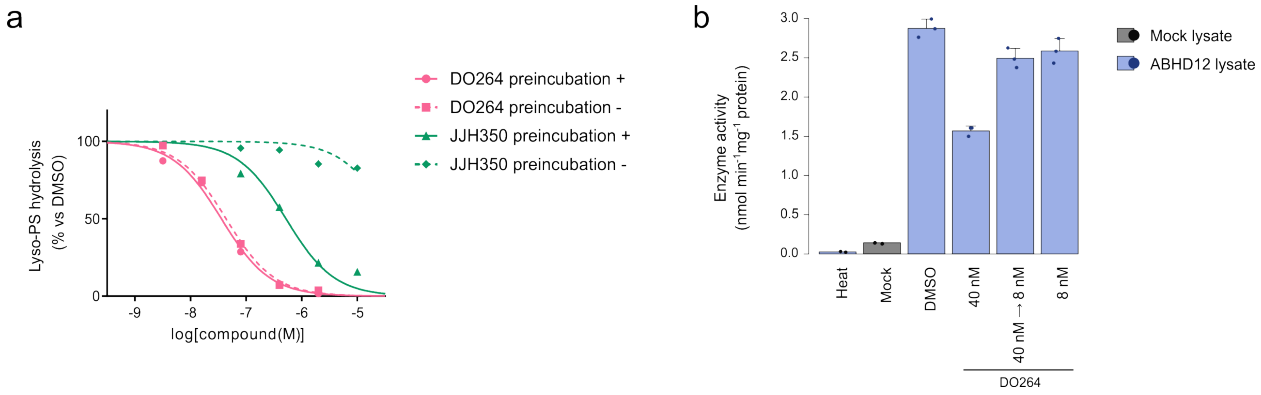
Supplementary Figure 7. Representative gel-based competitive ABPP and IC_{50} curves for results shown in **Supplementary Table 2**. (**a**) Representative competitive ABPP data for indicated compounds (20 min pre-treatment, room temperature) performed with mouse brain membrane proteome (1 mg/mL protein) and the JJH350 probe (2 μM, 45 min, 37 °C). The result is a representative of three independent experiments. (**b**) IC_{50} curves for ABHD12 inhibitors calculated from band intensities in gel-based ABPP experiments. Data represent average values ± SD. n =4 independent experiments for DO130, DO230 and DO253, and n = 3 independent experiments for DO264, DO276, DO277 and (S)-DO271. RFU: relative fluorescence unit.



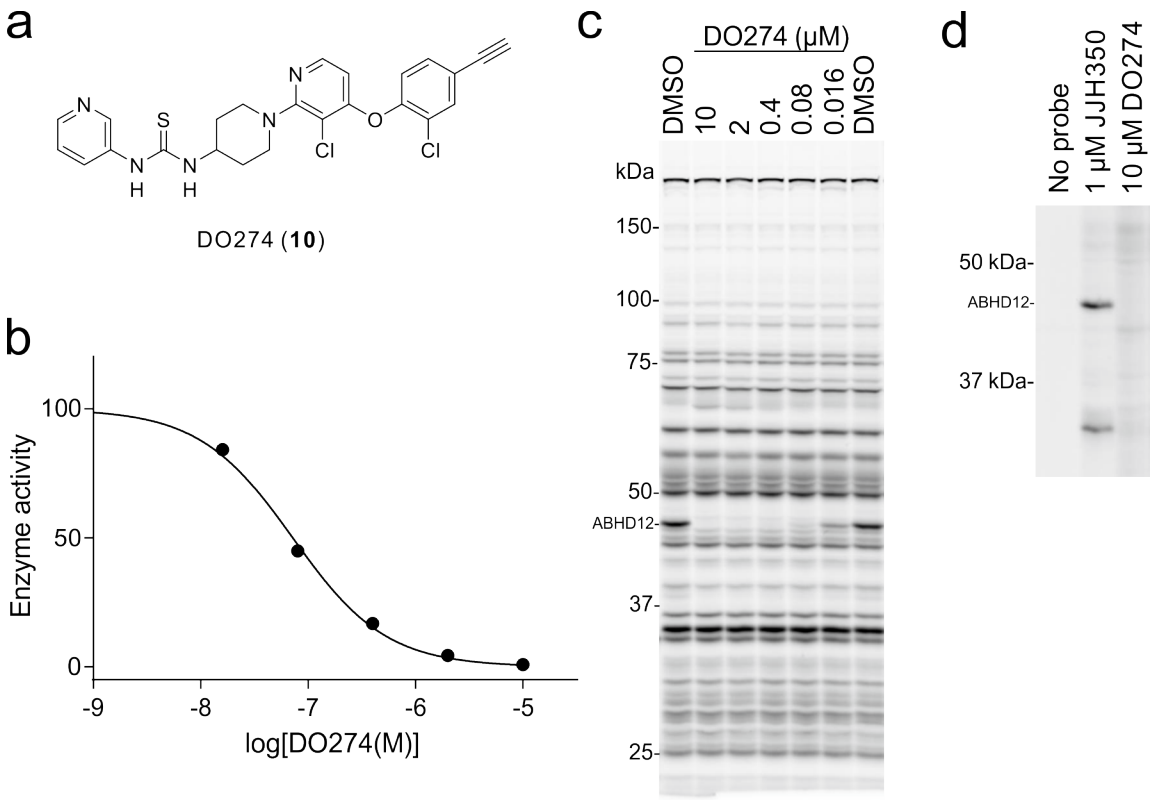
Supplementary Figure 8. Quantitative MS-ABPP analysis of serine hydrolase activities in mouse brain membrane proteome treated with 1 μM DO264 (20 min, room temperature). Data represent the mean of median ratios ± SEM for peptides quantified for each protein (n = 3 independent experiments).



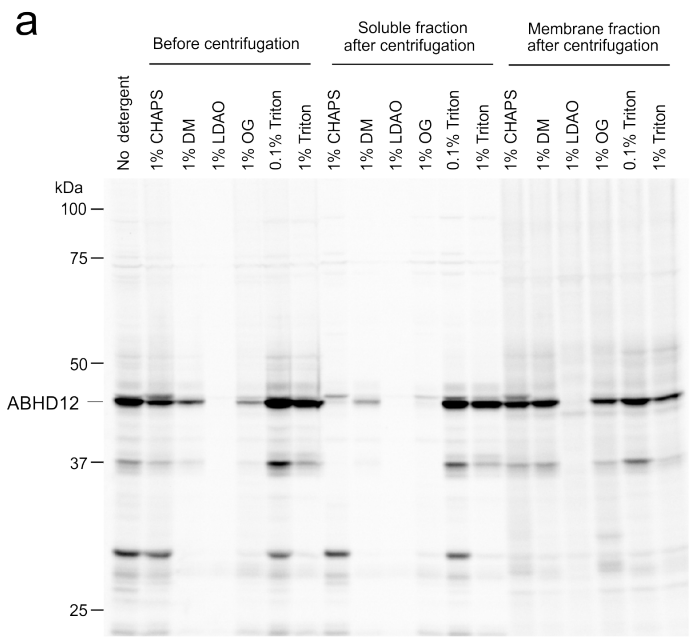
Supplementary Figure 9. Inhibition of recombinant and endogenous mouse and human ABHD12 by DO264. (**a-c**) Bar graph representation (**a** and **b**) and IC_{50} curves (**c**) for concentration-dependent inhibition of lyso-PS hydrolysis activities of membrane proteomic lysates (0.2 mg/mL) from mouse (**a**) and human (**b**) ABHD12-transfected HEK293T cells as determined by using 17:1 lyso-PS as a substrate. (**c**) IC_{50} values of 27 nM (23 nM–31 nM 95% CI) and 89 nM (82 nM–97 nM 95% CI) were calculated for DO264 inhibition of mouse and human ABHD12, respectively. (**d, e**) Bar graph representations of results in **Figure 2c** and **d** showing concentration-dependent inhibition of lyso-PS hydrolysis activity of mouse brain (**d**) or THP-1 (**e**) membrane proteome (0.4 mg/mL) by DO264 as determined using 17:1 lyso-PS as a substrate. (**f**) DO264 shows a similar IC_{50} value (compare to (**c**)) when tested with lower amounts of recombinant human ABHD12 (0.04 mg/mL membrane proteomic lysate from hABHD12-transfected HEK293T cells), as determined by using 17:1 lyso-PS as a substrate. Heat: heat-inactivated proteome; Mock: mock-transfected HEK293T membrane proteome. n = 3 independent experiments except for **d** (n = 4 independent experiments). Data represent mean ± SD.



Supplementary Figure 10. DO264 acts as a reversible inhibitor of ABHD12. (**a**) JJ350, but not DO264 shows time-dependent increases in potency of inhibition of ABHD12. Membrane proteomic lysate (0.2 mg/mL) of ABHD12-transfected HEK293T cells was treated with the indicated concentrations of DO264 and JJH350 for 30-45 min or without preincubation, after which lyso-PS hydrolysis activity was measured using 17:1 lyso-PS as a substrate. (**b**) The lyso-PS hydrolysis activity of ABHD12 is regained after dilution of DO264-treated samples. Membrane proteomic lysate (0.2 mg/mL) of ABHD12-transfected HEK293T cells was preincubated with DO264 for 20 min and, either assayed directly for, or diluted five-fold in buffer prior to measuring, lyso-PS hydrolysis activity. For **a**, data represent mean values of n = 2 independent experiments. For **b**, data represent mean ± SD of n = 3 independent experiments. . Heat: heat-inactivated ABHD12 proteome; Mock: mock-transfected HEK293T membrane proteome.

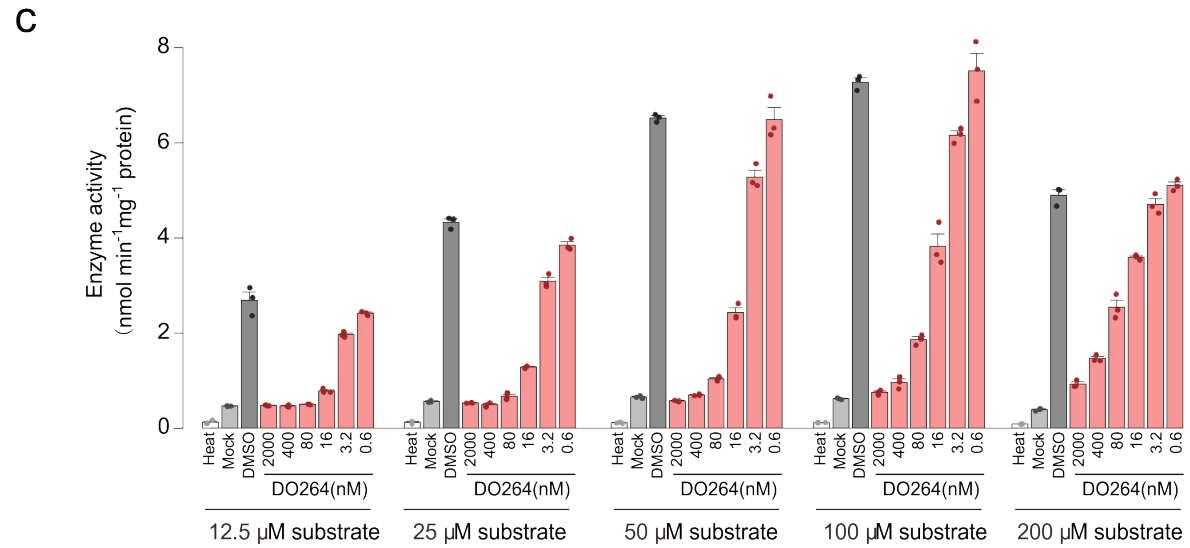


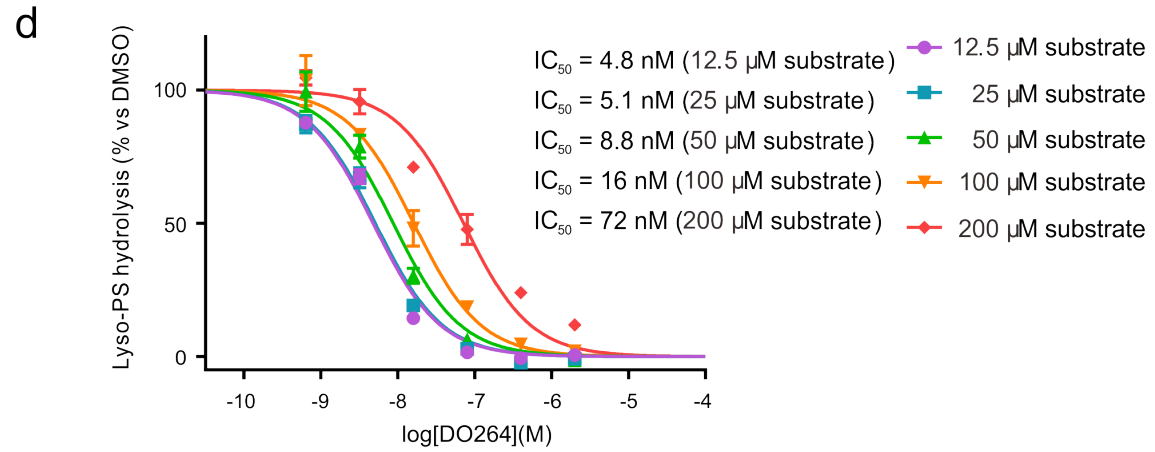
Supplementary Figure 11. Structure and characterization of DO274 (**10**), an alkynylated analogue of DO264. (**a**) Chemical structure of DO274. (**b**) Concentration-dependent inhibition of lyso-PS hydrolysis activity of membrane proteomic lysates (0.2 mg/mL) of ABHD12-transfected HEK293T cells as determined using 17:1 lyso-PS as a substrate. Data represent mean values of n = 2 independent experiments. An IC_{50} value of 72 nM (66 nM–79 nM 95% CI) was calculated for DO274 inhibition of ABHD12. (**c**) Concentration-dependent inhibition of mouse brain ABHD12 activity by DO274 as determined by gel-based competitive ABPP using the FP-Rh probe (1 μM, 45 min, 37 °C). (**d**) JJH350, but not DO274 (indicated concentrations, 45 min, 37 °C) covalently modify mouse brain ABHD12 as visualized by CuAAC with Rh-N_3. For **c** and **d**, the experiment was performed once.



b

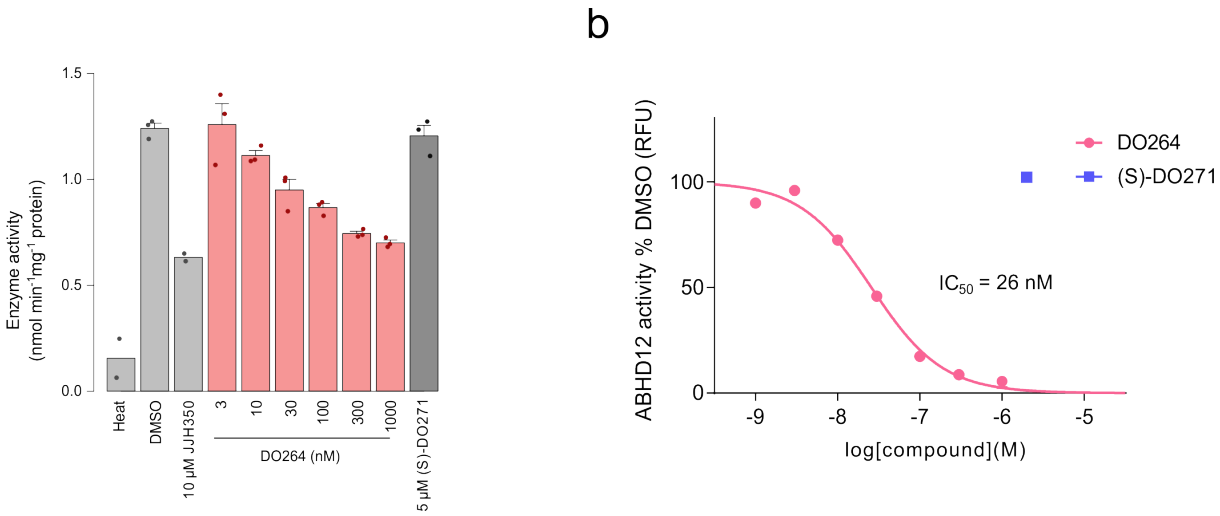
Lysate	Hydrolysis rate ± SEM (nmol/mg/min)
Mock + DMSO	0.76 ± 0.03
mABHD12 + DMSO	5.27 ± 0.10
mABHD12 + 1 μM DO264	0.75 ± 0.01
mABHD12 + 1 μM (S)-DO271	5.14 ± 0.02
Triton-solubilized mABHD12	0.56 ± 0.02



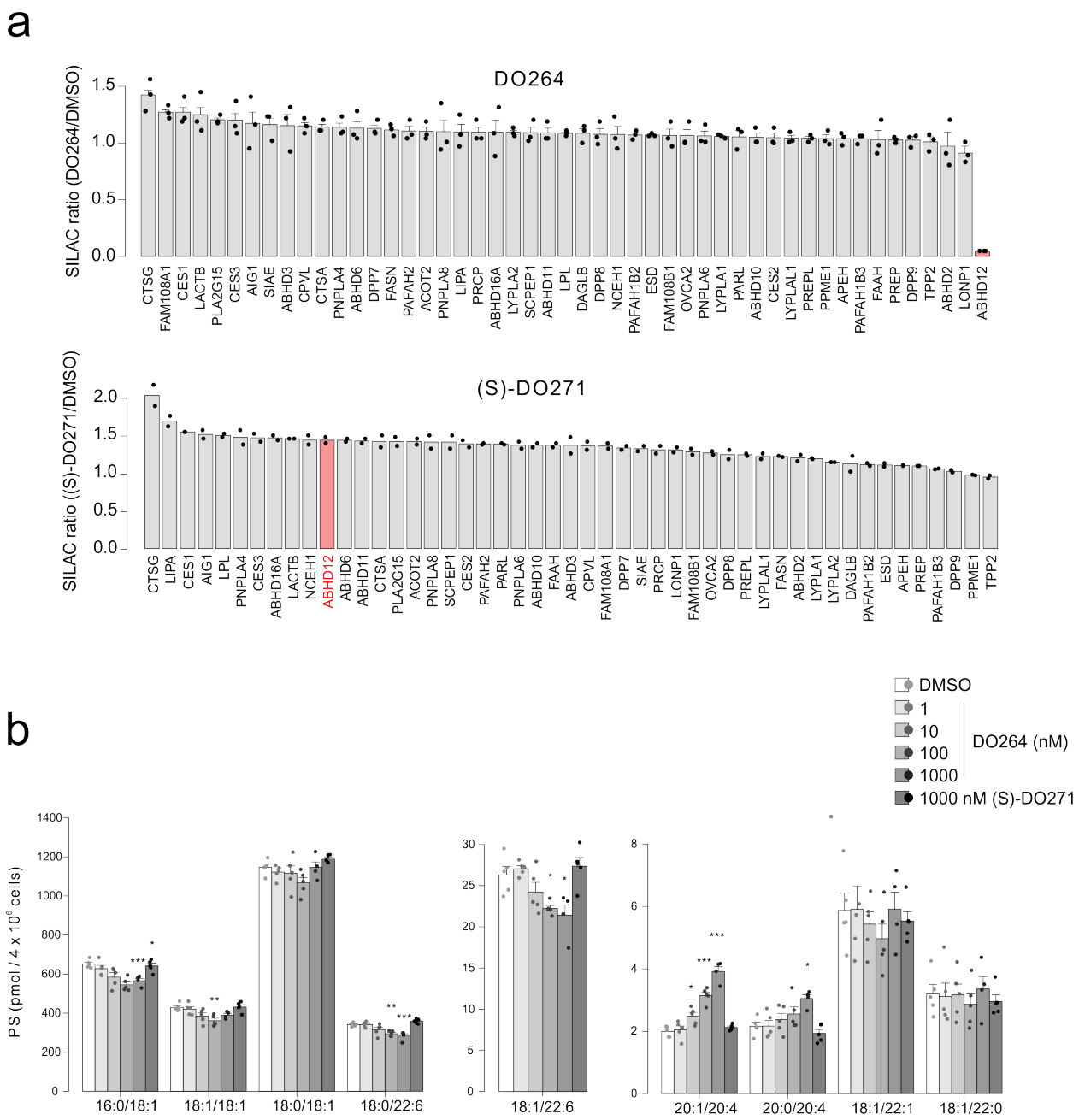


Supplementary Figure 12. Attempts to solubilize recombinant ABHD12 and evidence that DO264 acts as a competitive inhibitor of ABHD12. (**a**) Detergent solubilization of ABHD12 in membrane lysates form ABHD12-transfected HEK293F cells. Lysate was incubated with the indicated detergents (1 h, 4 °C), fractionated by

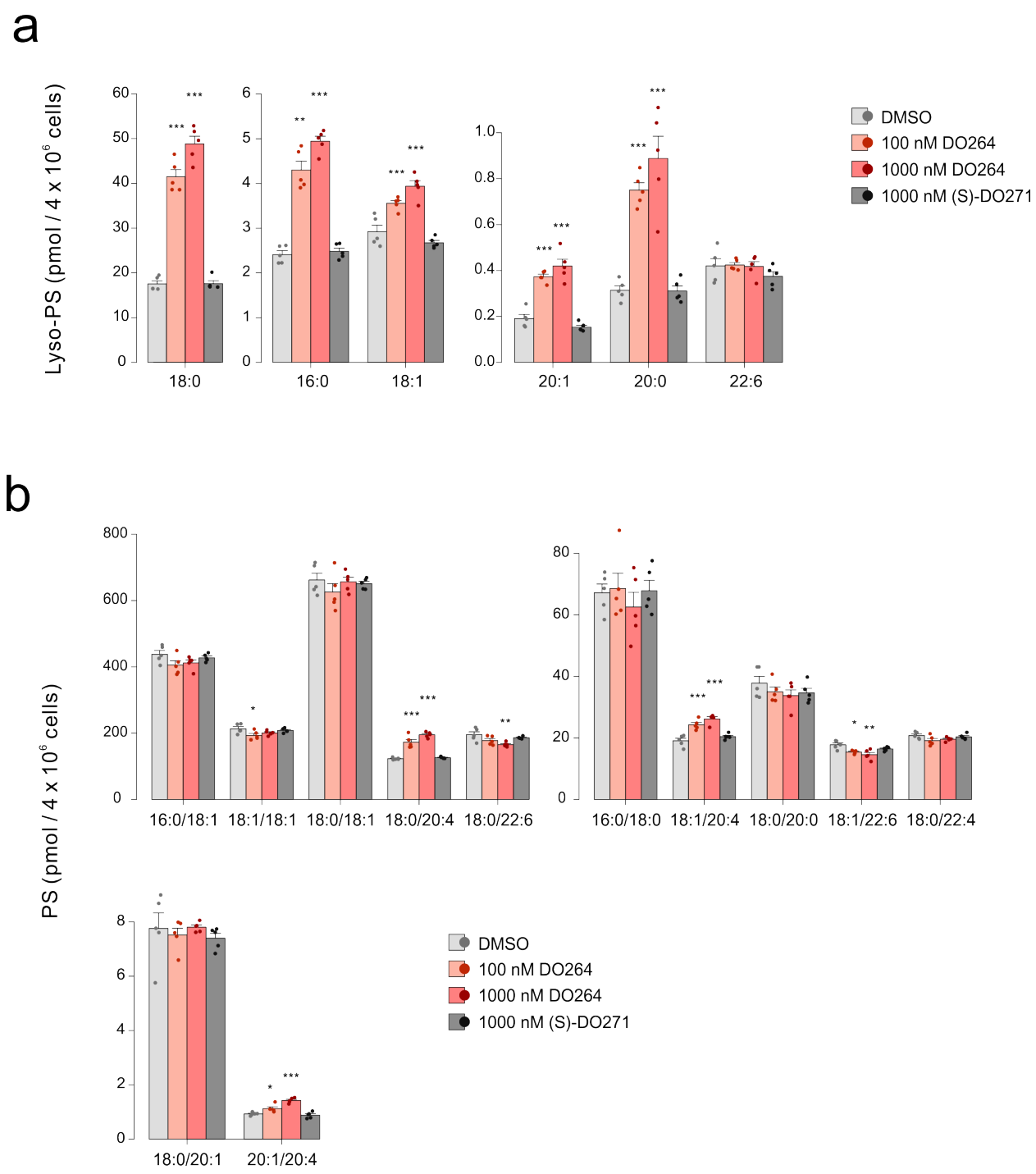
centrifugation, and ABHD12 solubilization evaluated in the soluble and particulate fractions by ABPP (1 μM FP-Rh, 30 min, 37 °C). The result is a representative of two independent experiments. (**b**) Triton-solubilized ABHD12 (0.1% Triton) showed dramatically reduced 17:1 lyso-PS hydrolysis activity (>10X lower activity compared to unsolubilized ABHD12). Data represent mean ± SEM for n = 3 independent experiments. (**c**, **d**) Shift in IC_{50} value of DO264 in assays with increasing concentrations of lyso-PS substrate. (**c**) Bar graph representation for concentration-dependent inhibition of lyso-PS hydrolysis of membrane proteomic lysates (0.2 mg/mL) from ABHD12-transfected HEK293T cells as determined by using various concentrations of 17:1 lyso-PS (12.5 μM – 200 μM) as a substrate. Heat: heat-inactivated proteome; Mock: mock-transfected HEK293 membrane proteome. (**d**) IC_{50} values of 4.8 nM (4.1 nM–5.6 nM 95% CI), 5.1 nM (4.5 nM–5.8 nM 95% CI), 8.8 nM (7.4 nM–10 nM 95% CI), 16 nM (14 nM–19 nM 95% CI) and 72 nM (54 nM–94 nM 95% CI) were calculated for DO264 inhibition of ABHD12 with 12.5 μM, 25 μM, 50 μM, 100 μM and 200 μM lyso-PS substrate, respectively. Data represent average values ± SD for n = 3 independent experiments. DM: n-dodecyl β-D-maltoside; LDAO: lauryldimethylamine oxide; OG: n-octyl-β-D-glucoside



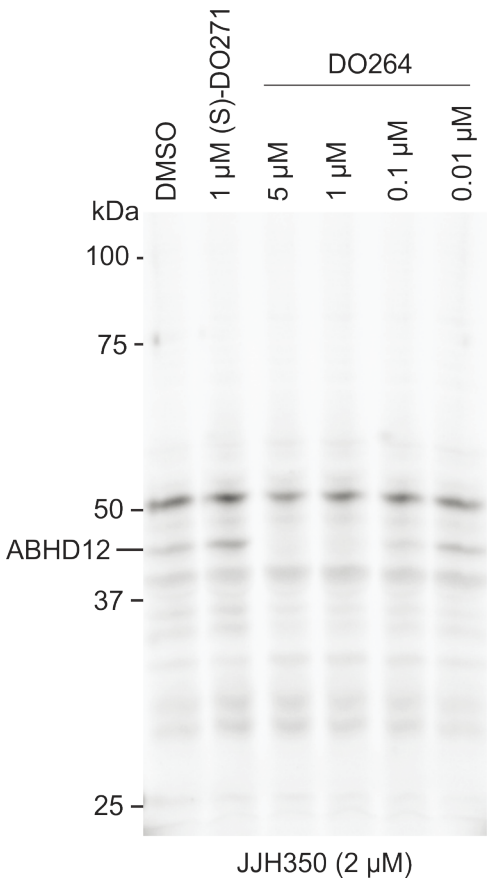
Supplementary Figure 13. *In situ* ABHD12 inhibitory activity of DO264. (**a)** Bar graph representations of results in **Figure 3b** showing concentration-dependent inhibition of ABHD12 in THP-1 cells treated *in situ* with DO264. Data represent mean ± SD for three independent experiments. Heat: heat-inactivated proteome. (**b**) *In situ* inhibition of ABHD12 by DO264 as determined by gel-based ABPP. The IC_{50} value was calculated based on ABHD12 band intensity in membrane proteome (1 mg/mL) of THP-1 cells treated with DO264 *in situ* as measured by gel-based competitive ABPP using JJH350 (2 µM, 45 min, 37 °C). Data represent mean for n = 2 independent experiments. The IC_{50} value was calculated as 26 nM (20 nM–33 nM 95% CI). RFU: relative fluorescence unit.



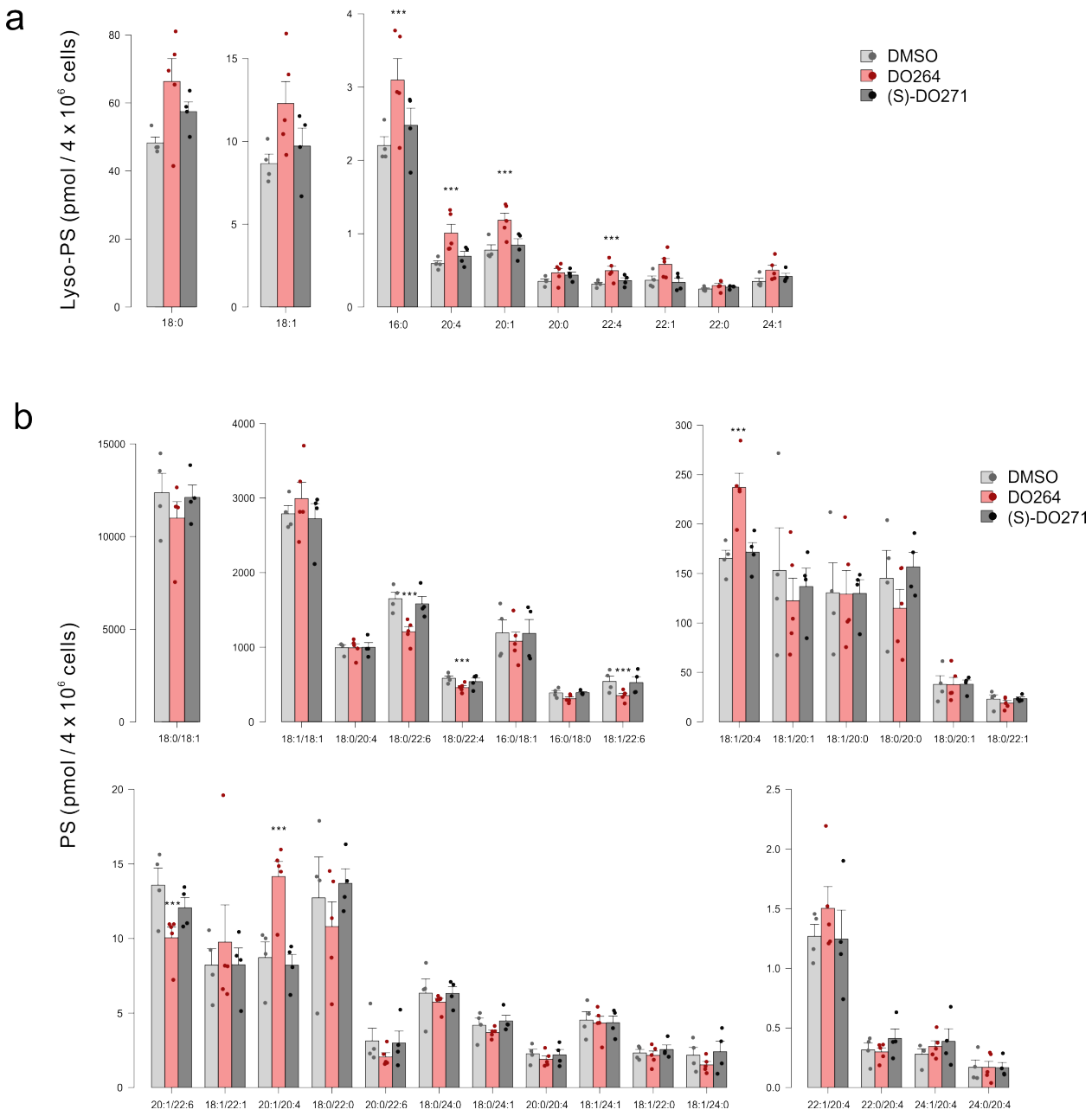
Supplementary Figure 14. (**a**) ABPP-SILAC analysis of serine hydrolase activities in whole proteomic lysates from THP-1 cells treated *in situ* with DO264 (upper panel) or (S)-DO271 (lower panel) (1 μM of each compound, 4 h) versus DMSO control. Data represent the mean of median SILAC ratios ± SEM for peptides quantified for each protein from 3 (DO264) and 2 ((S)-DO271) independent samples. (**b**) Quantification by targeted LC-MS analysis of the PS content of THP-1 cells treated with the DMSO or the indicated concentrations of DO264 or (S)-DO271 (4 h treatment at 37 °C). Data are related to results shown in **Figure 3c** and represent average values ± SEM (n = 5 independent samples per group). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Two-sided Student's *t*-test performed relative to DMSO control). The *p*-values and are provided in **Supplementary Table 4**.



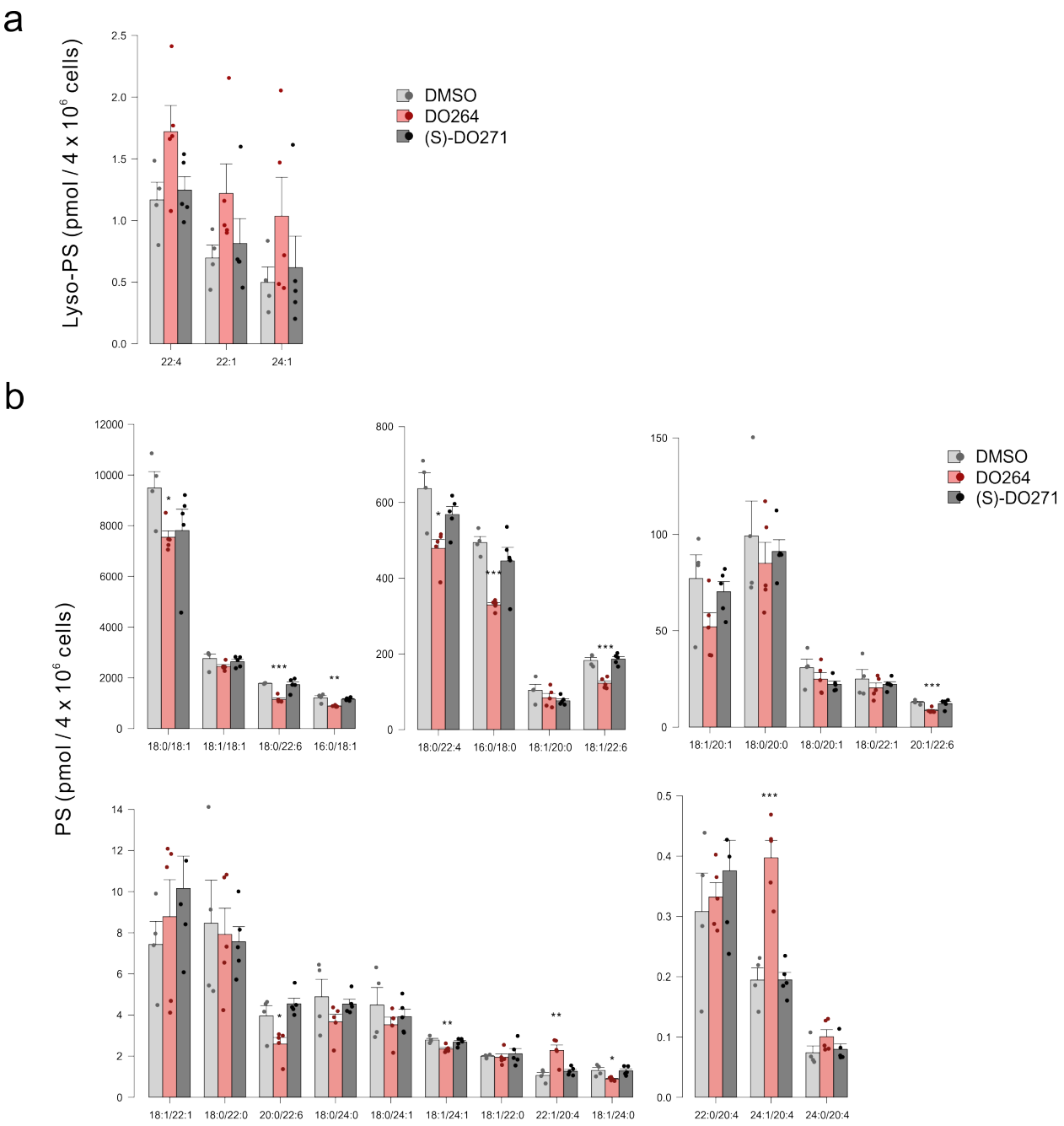
Supplementary Figure 15. Quantification by targeted LC-MS analysis of the lyso-PS (**a**) and PS (**b**) content of PMA-differentiated THP-1 cells treated with the DMSO or the indicated concentrations of DO264 or (S)-DO271 (4 h treatment at 37 °C). Data represent average values ± SEM (n = 5 independent samples per group). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Two-sided Student's *t*-test performed relative to DMSO control). The *p*-values and are provided in **Supplementary Table 4**.



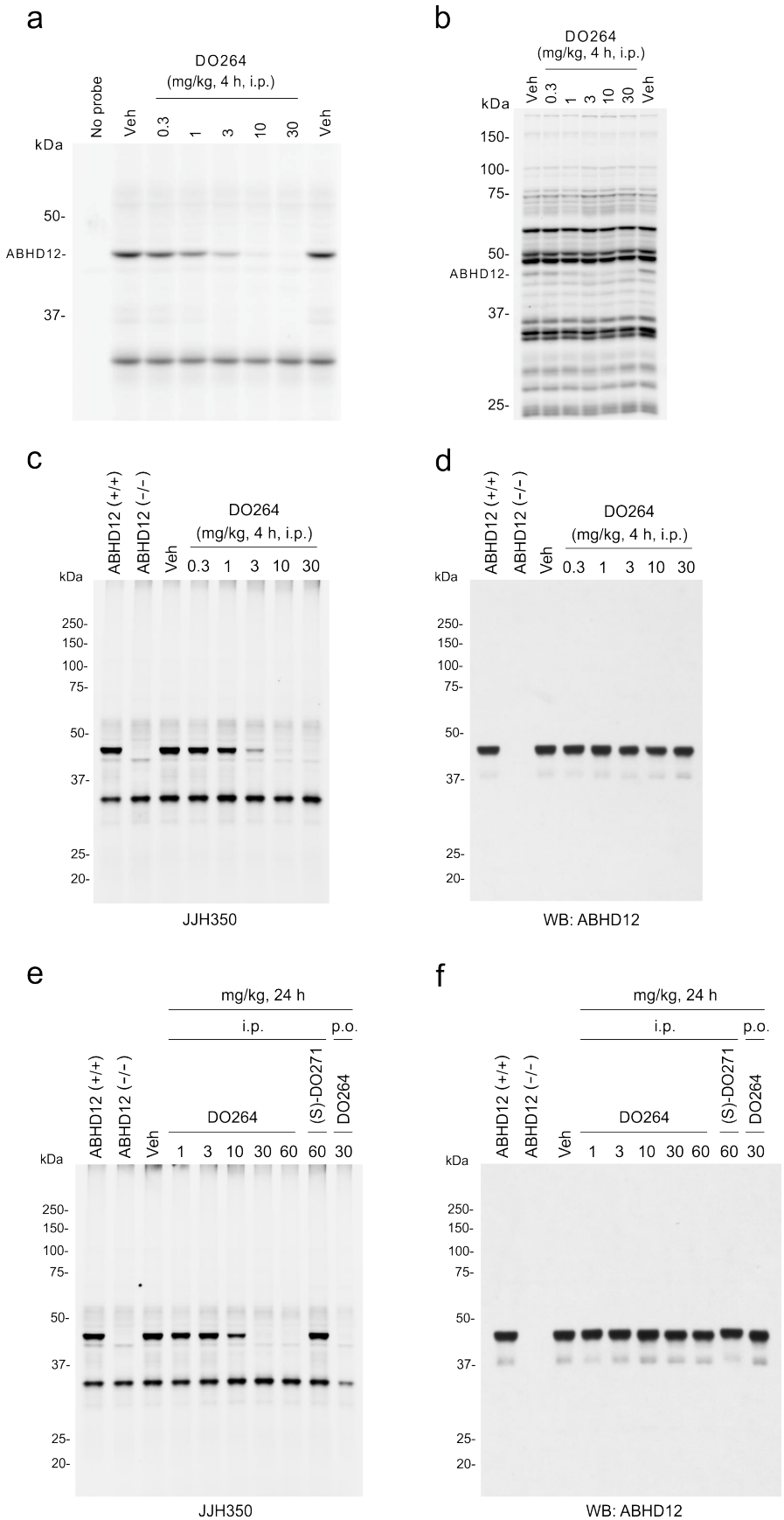
Supplementary Figure 16. Concentration-dependent inhibition of ABHD12 in primary human macrophages by DO264 (24 h treatment). ABHD12 activity in whole cell lysates was determined by gel-based competitive ABPP using the JJH350 probe (2 µM, 45 min, 37 °C). The result is a representative of two independent experiments.



Supplementary Figure 17. Quantification by targeted LC-MS analysis of the lyso-PS (**a**) and PS (**b**) content of primary human macrophages treated with the DMSO, DO264, or (S)-DO271 (1 µM compound, 4 h treatment at 37 °C). Data represent average values ± SEM (n = 4 independent samples for DMSO and (S)-DO271 and n = 5 independent samples for DO264). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Two-sided Student's *t*-test performed relative to DMSO control). The *p*-values and are provided in **Supplementary Table 4**.

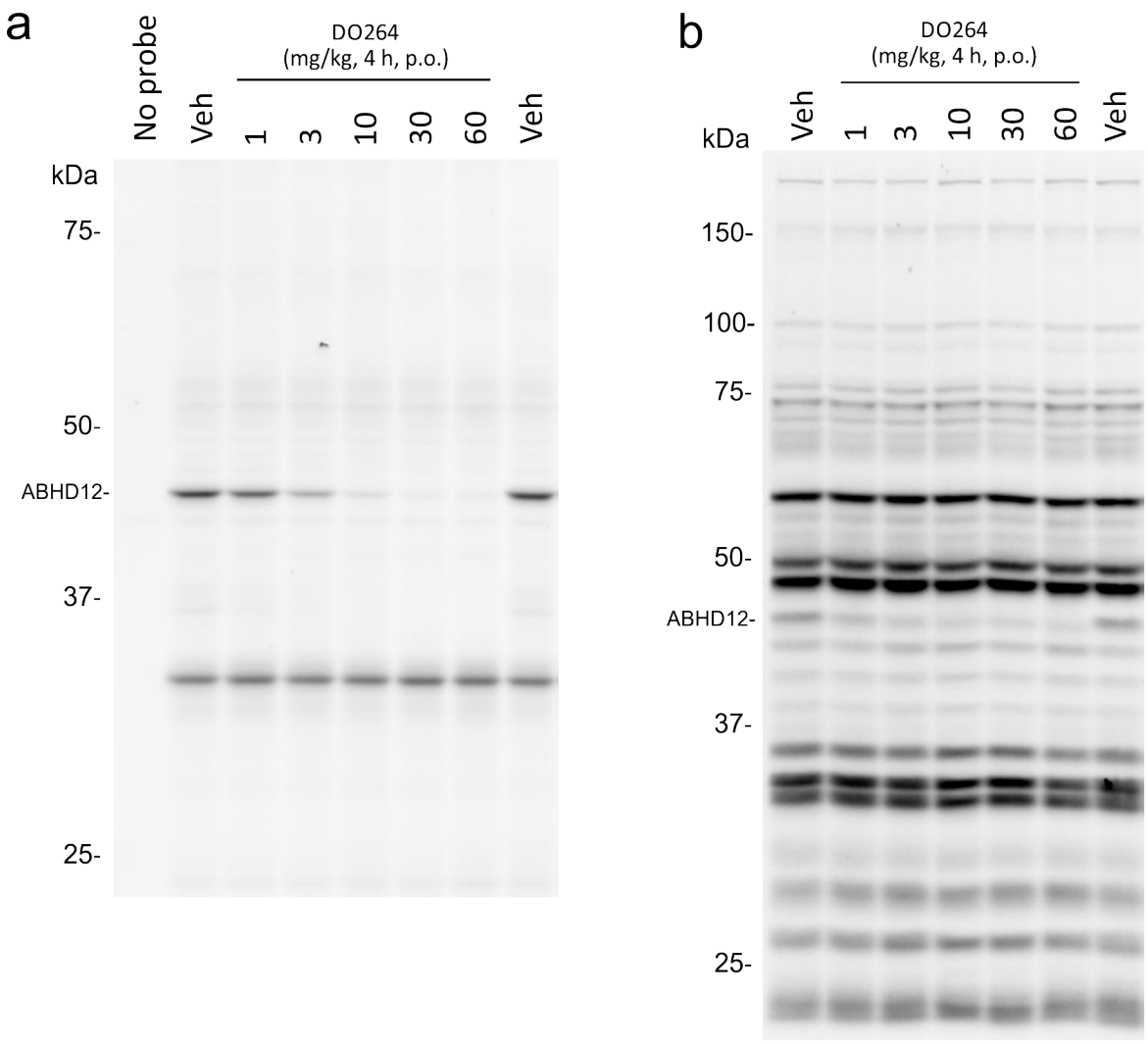


Supplementary Figure 18. Quantification by targeted LC-MS analysis of the lyso-PS (**a**) and PS (**b**) content of primary human macrophages treated with the DMSO, DO264, or (S)-DO271 (1 µM compound, 24 h treatment at 37 °C). Data are related to **Figure 3e** and represent average values ± SEM (n = 4 independent samples for DMSO and (S)-DO271 and n = 5 independent samples for DO264). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Two-sided Student's *t*-test performed relative to DMSO control). The *p*-values and are provided in **Supplementary Table 4**.

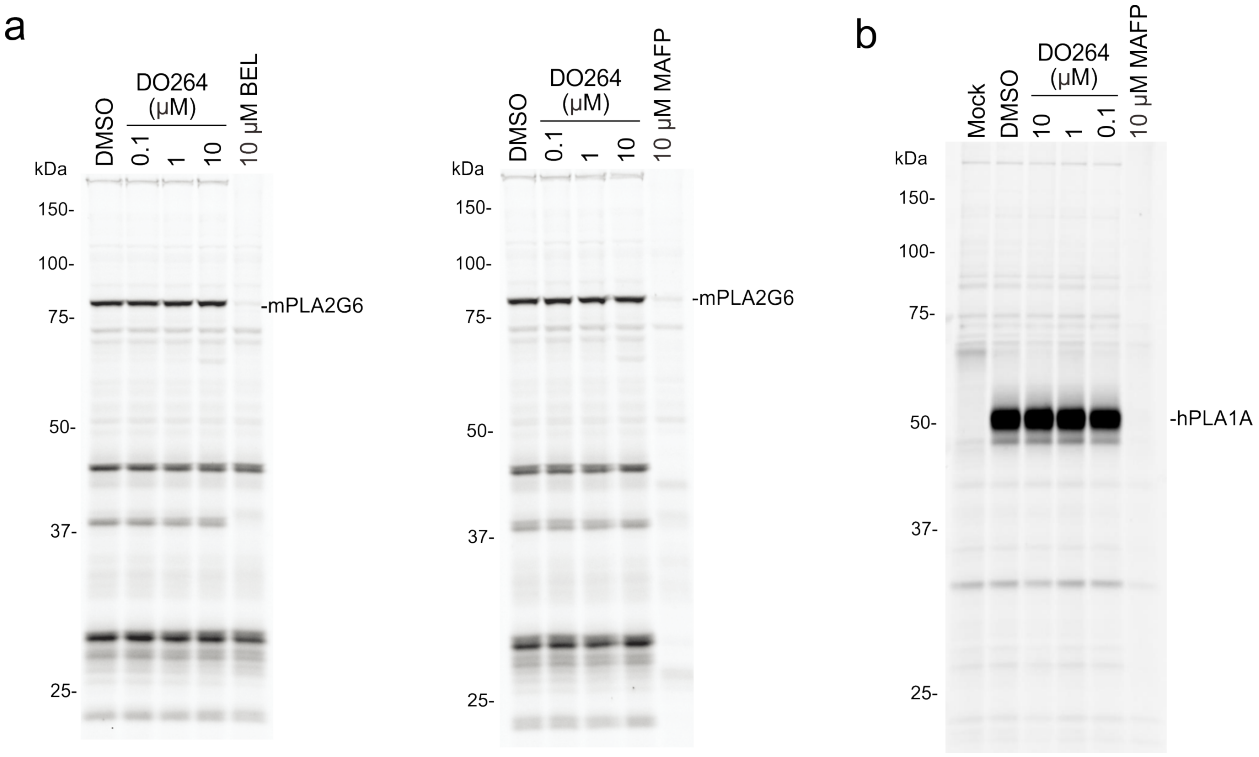


Supplementary Figure 19. (**a** and **b**) Dose-dependent inhibition of ABHD12 in the brain proteome of mice treated with DO264 (4 h, i.p.). Doses are indicated in the figure, and inhibition of ABHD12 was determined by competitive ABPP using (**a**) the JJH350 probe (2 μM, 45 min, 37 ºC) or (**b**) FP-Rh probe (1 μM, 45 min, 37 °C).

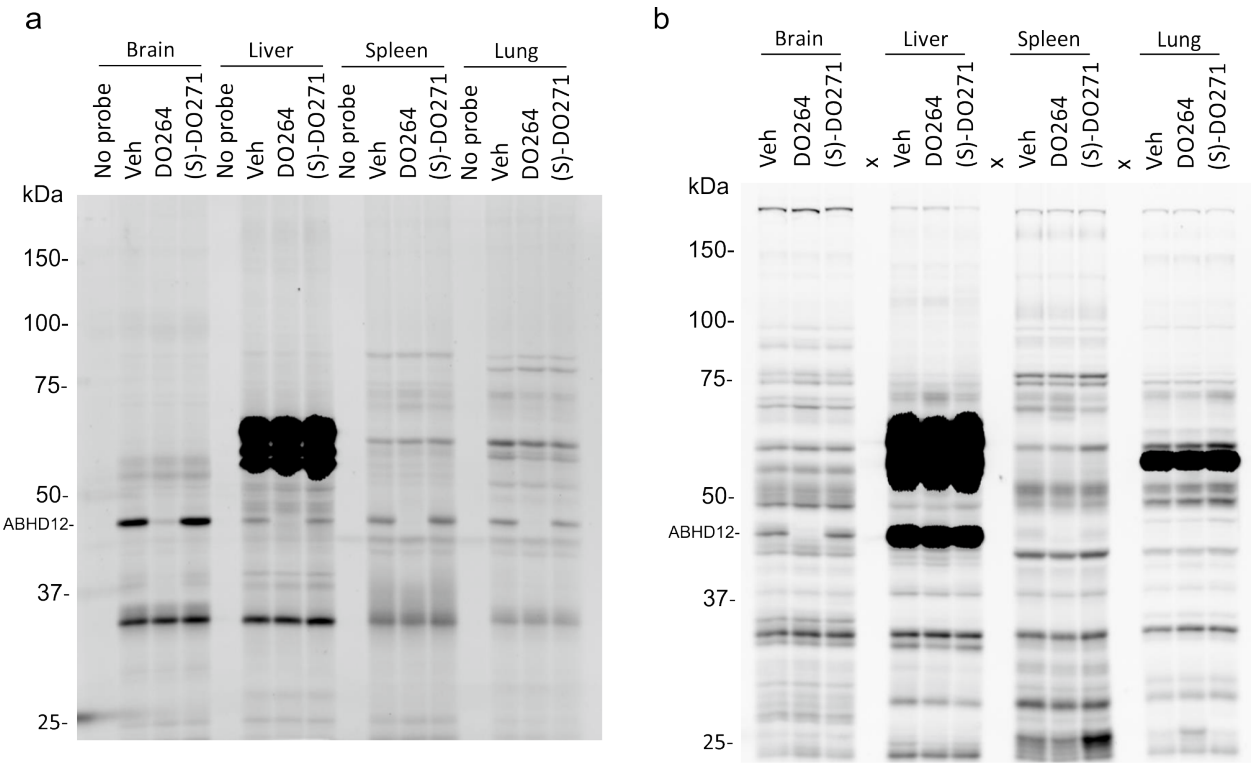
(**c-f**) ABHD12 expression was not affected by DO264 or (S)-DO271 treatment. Brain proteomes from ABHD12 (+/+) and ABHD12(–/–) mice treated with DO264 and (S)-DO271 (indicated doses, i.p.) for 4 h (**c**) and 24 h (**e**) were analyzed by gel-based competitive ABPP using JJH350 probe (2 μM, 45 min, 37 °C) and western blotting (**d** and **f**) using an ABHD12 antibody. The result is a representative of two independent experiments.



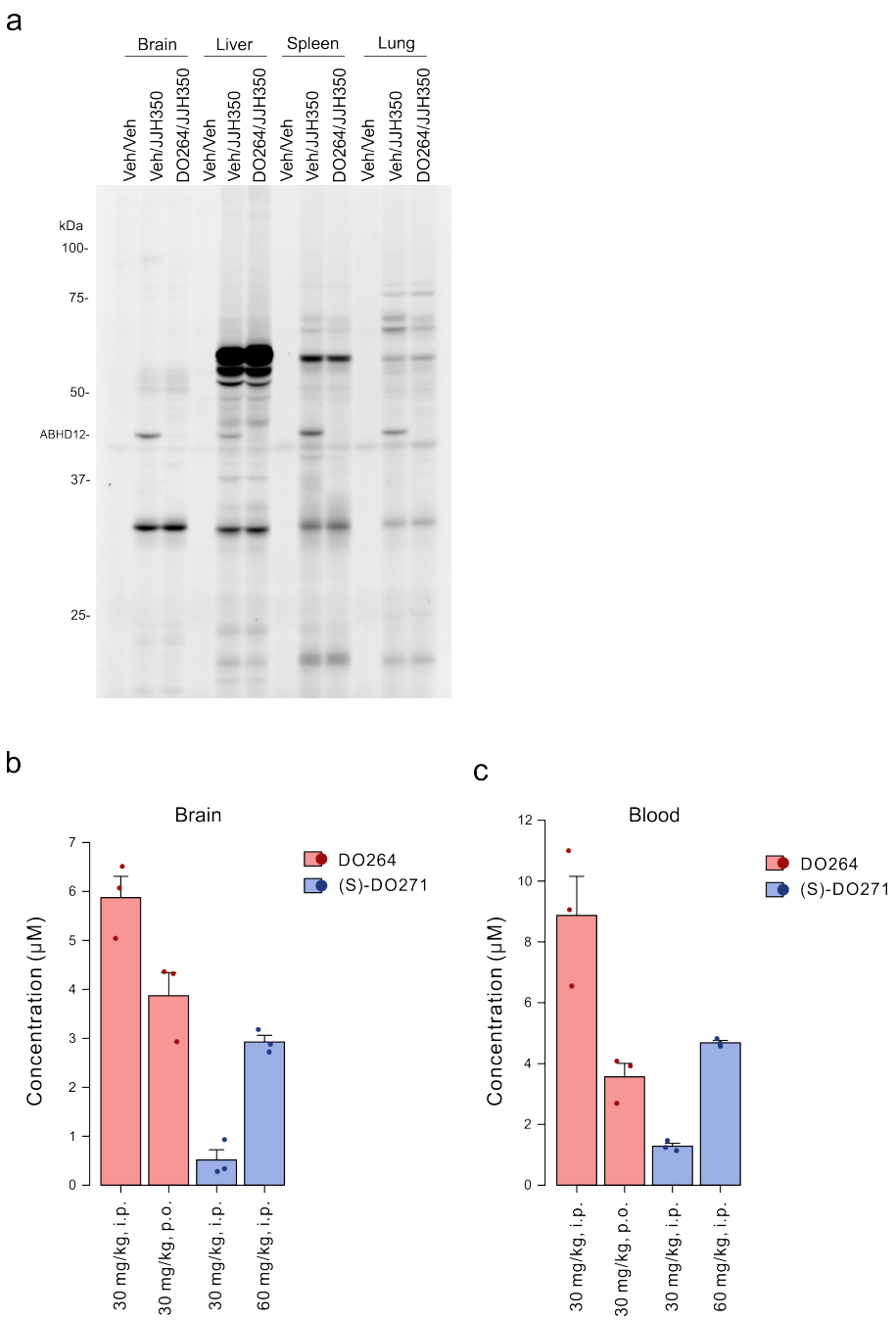
Supplementary Figure 20. Dose-dependent inhibition of ABHD12 in the brain proteome of mice treated orally (p.o.) with DO264 for 4 h. Doses are indicated in the figure, and inhibition of ABHD12 was determined by competitive ABPP using (**a**) the JJH350 probe (2 μM, 45 min, 37 ºC) or (**b**) FP-Rh probe (1 μM, 45 min, 37 °C). The result is a representative of two independent experiments.



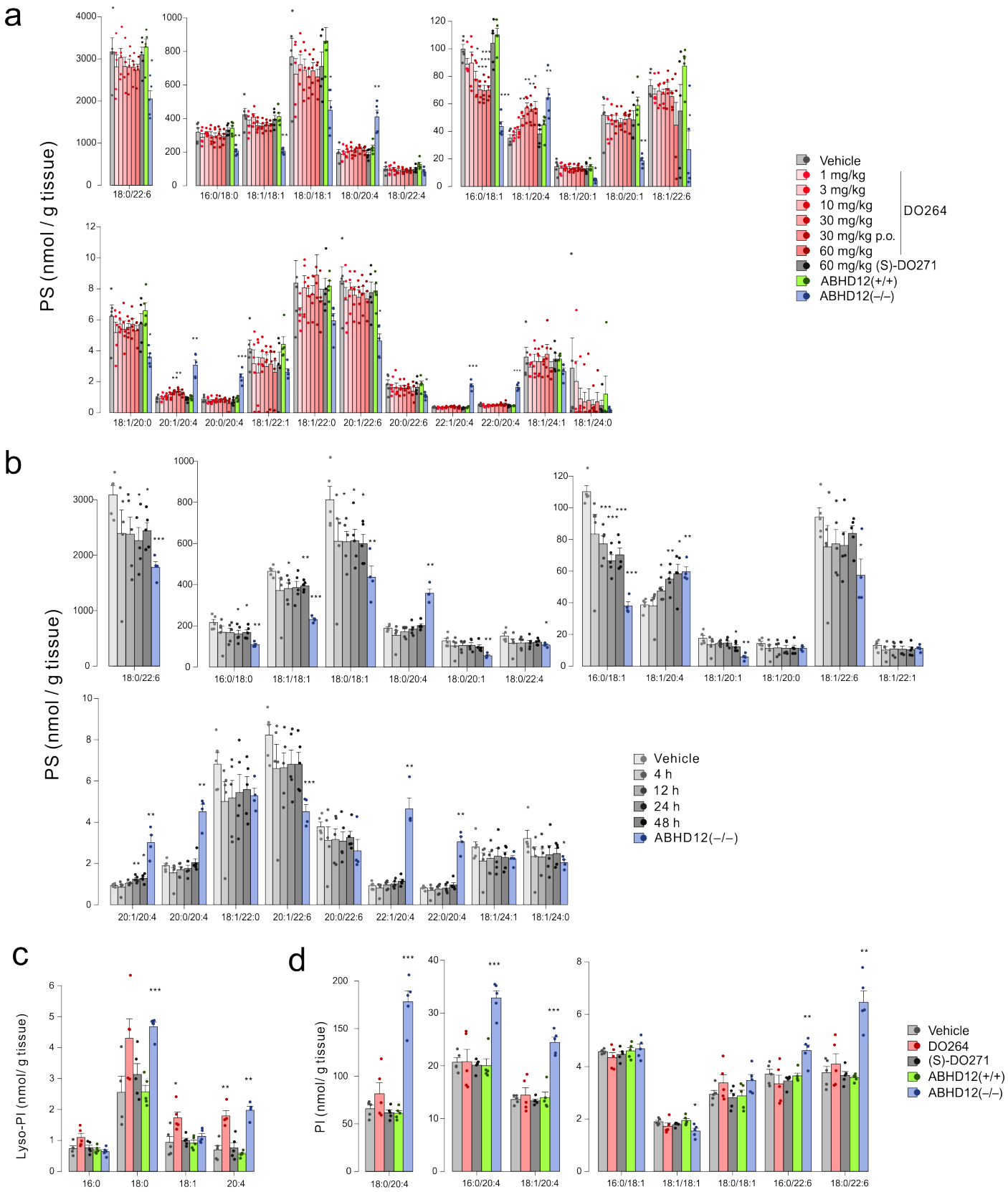
Supplementary Fig. 21. DO264 does not inhibit the activity of mouse PLA2G6 (**a**) or human PLA1A (**b**) as determined by competitive gel ABPP using the FP-Rh probe (1 μ, 37 °C). Recombinantly expressed PLA2G6 and PLA1A were assayed in membrane lysate (1 mg/mL) and conditioned media (0.1 mg/mL), respectively, of transfected HEK293T cells. Note that both PLA2G6 and PLA1A activities were blocked by positive-control inhibitors (e.g., BEL and MAFP). The experiment was performed once. BEL: bromoenol lactone; MAFP: methyl arachidonyl fluorophosphonate.



Supplementary Figure 22. Gel-based competitive ABPP of different tissues from mice treated with DO264 *in vivo*. Mice were treated with compounds (30 mg/kg, i.p., 4 h), sacrificed, and the indicated tissues processed and analyzed by gel-based competitive ABPP using (**a**) the JJH350 probe (2 μM, 45 min, 37 °C) or (**b**) the FP-Rh probe (1 μM, 45 min, 37 °C). Note that ABHD12 signals are better visualized with the tailored ABPP probe JJH350. The result is a representative of two independent experiments.

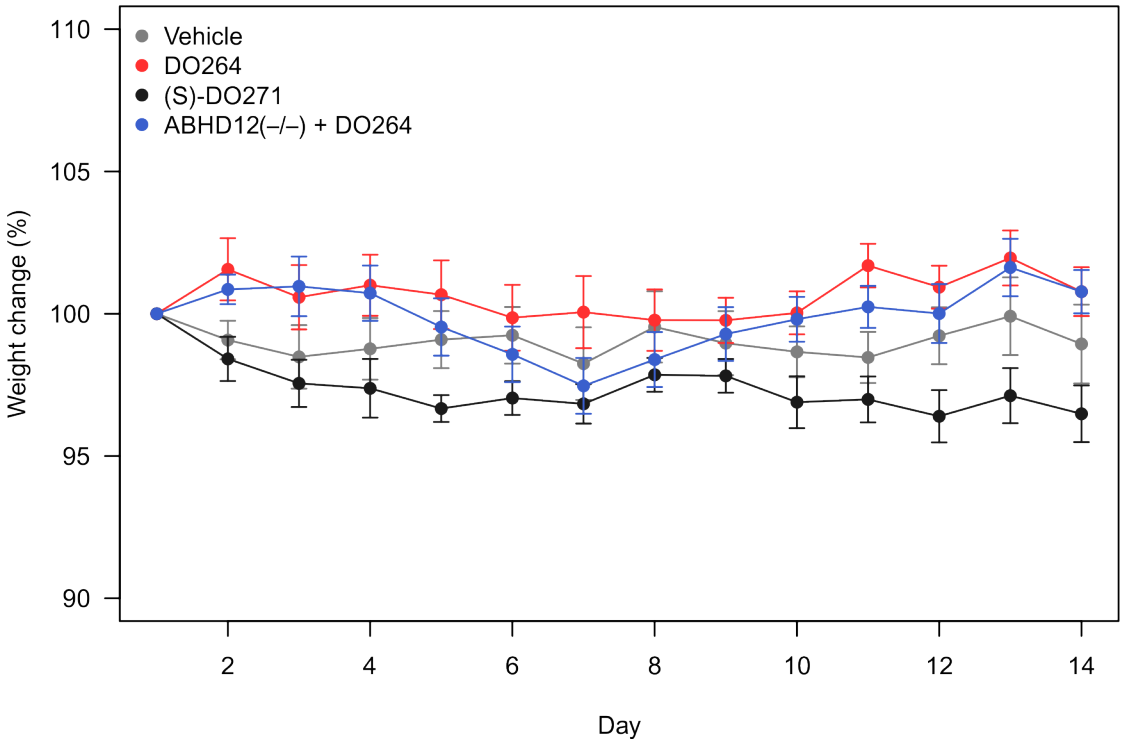


Supplementary Figure 23. Assessment of the target engagement and pharmacokinetics of DO264 *in vivo*. (**a**) Blockade of ABHD12 activity *in vivo* as assessed by gel-based ABPP using the JJH350 probe. Mice were treated with vehicle or DO264 (30 mg/kg, p.o., 4 h) followed by JJH350 probe (30 mg/kg, i.p., 4 h) and then sacrificed and the indicated tissues processed to generate membrane proteomic lysates. The lysates were analyzed by gel-based ABPP as described in **Supplementary Fig. 6**. The experiment was performed once. (**b**, **c**) Concentrations of DO264 and (S)-DO271 in brain (**b**) and blood (**c**) from mice treated with 30mg/kg or 60mg/kg dose of compound for 4h. Data represent mean ± SD for n = 3 independent experiments.

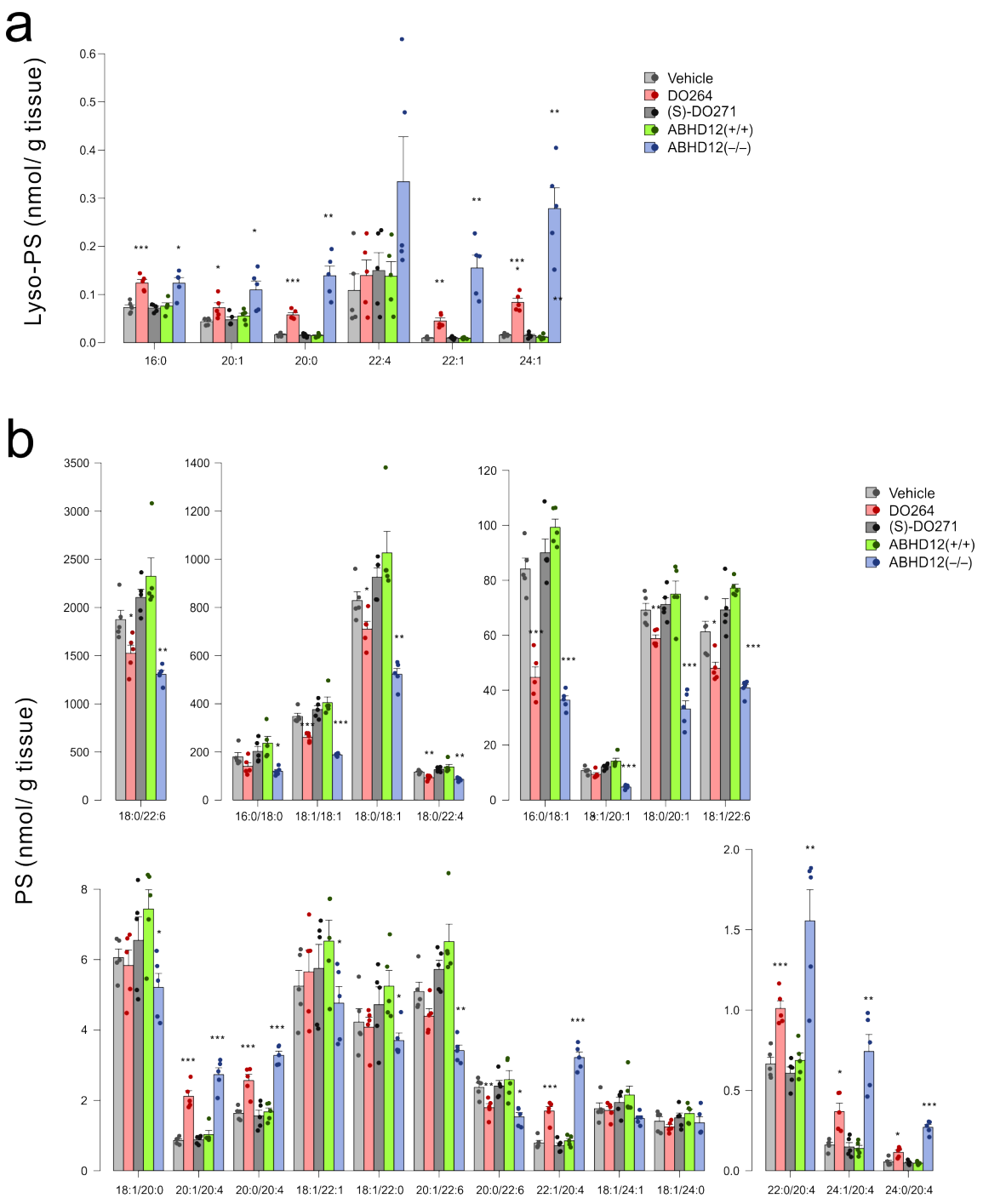


Supplementary Figure 24. Quantification by targeted LC-MS analysis of the PS and (lyso)-PI content of brain tissue from mice treated with DO264. (**a**) Brain PS content of DO264-treated mice (24 h treatment, doses of compound and route of administration are indicated in the figure). Brain samples from ABHD12(+/+) and ABHD12(–/–) mice were included as controls. (**b**) Time-course analysis of brain PS content from DO264-treated mice (30 mg/kg, i.p., indicated time points). (**c**, **d**) Lyso-PI (**c**) and PI (**d**) content of DO264-treated mice

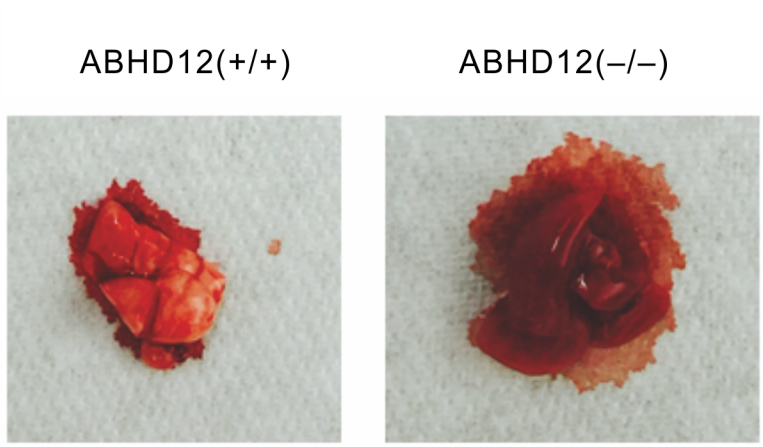
(30 mg/kg compound, p.o., 24 h). For **a-d**, brain samples from ABHD12(–/–) mice were included as a control. Data represent average values ± SEM (n = 5 mice per group). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Two-sided Student's *t*-test performed relative to vehicle-treated mice except for the ABHD12(–/–) mice, which were compared to the ABHD12(+/+) mice. The *p*-values and other lipid species measured by targeted LC-MS are shown in **Supplementary Table 4**.



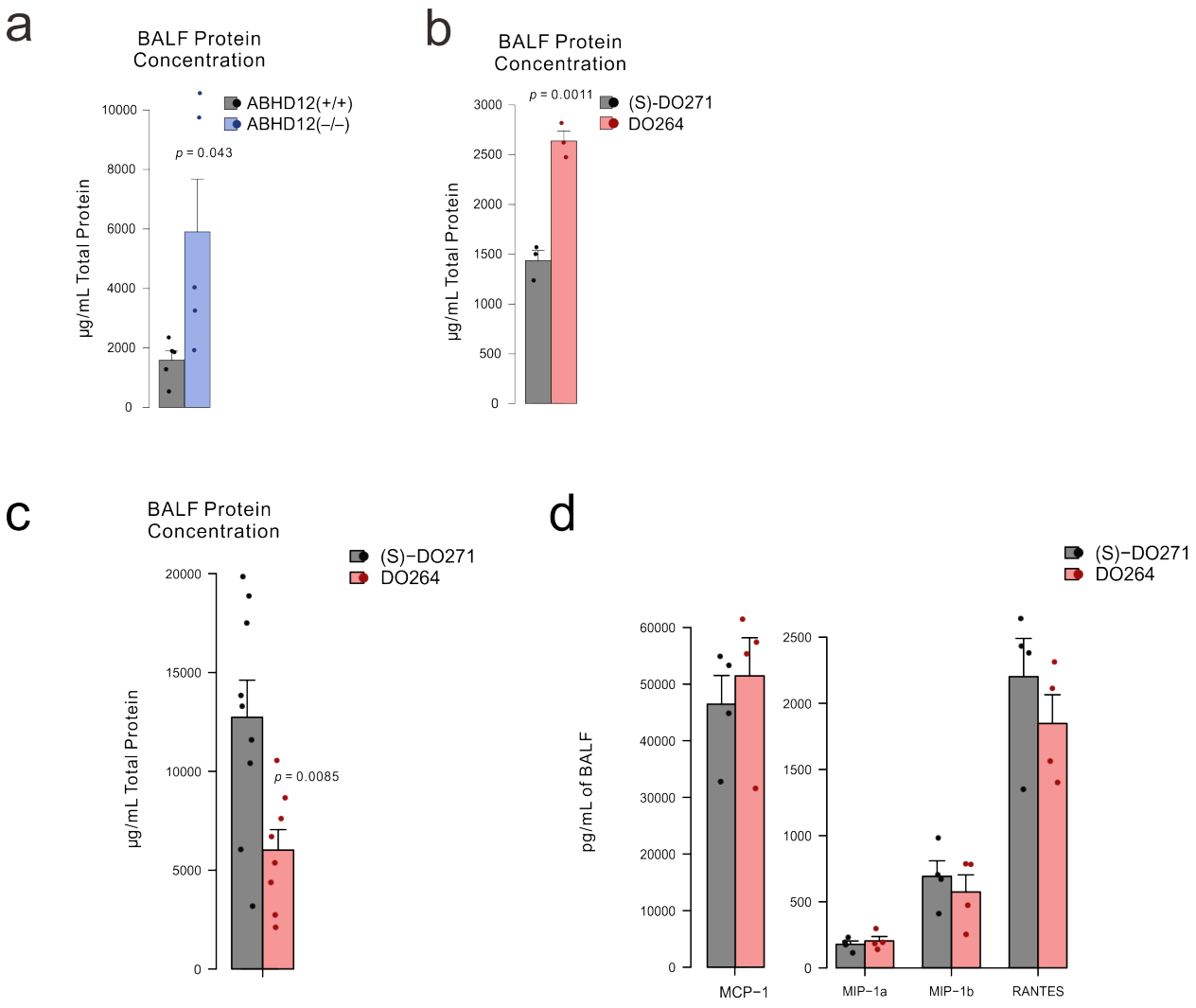
Supplementary Figure 25. Effects of DO264 on body weight of mice. 9-week-old mice were treated with vehicle, DO264, or (S)-DO271 (30 mg/kg compound, p.o., daily for two weeks), and the weight of each mouse measured daily. ABHD12(–/–) mice were also included as a group and treated with DO264. Data represent average values ± SEM (n = 9, 8, 8, and 8 mice for vehicle, DO264, (S)-DO271 and ABHD12(–/–) + DO264 group, respectively).



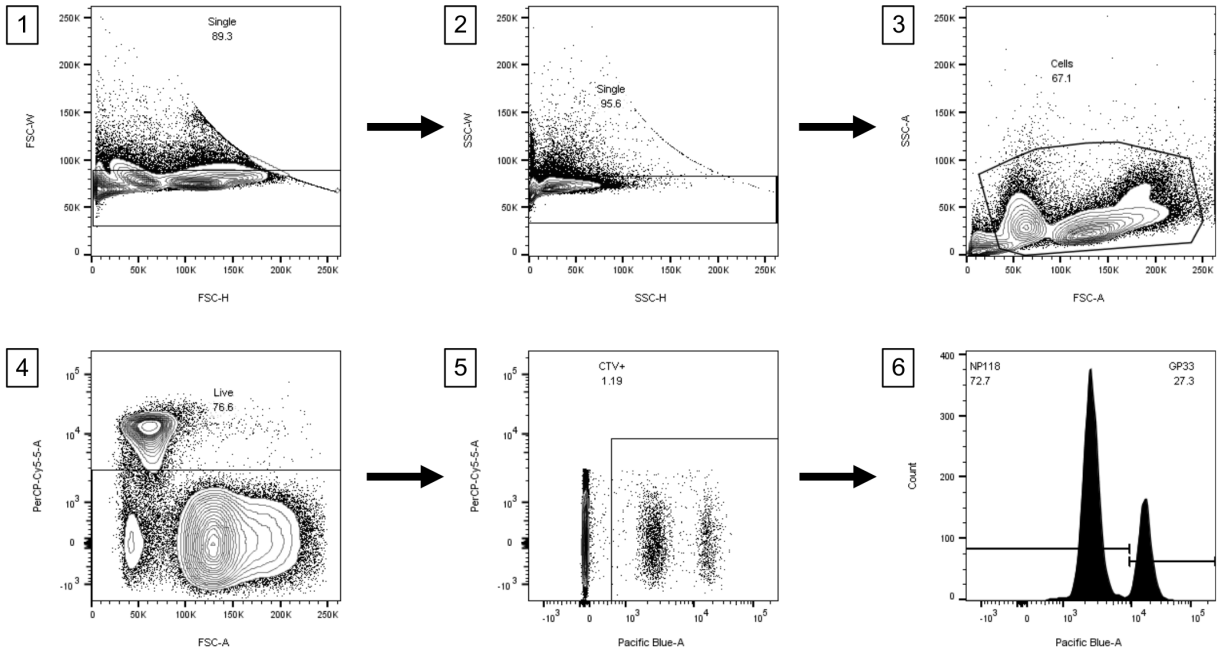
Supplementary Figure 26. Quantification by targeted LC-MS analysis of the brain (**a**) lyso-PS and (**b**) PS content of mice treated for four weeks with DO264. 8-month-old mice were treated with either vehicle, DO264, or (S)-DO271 (30 mg/kg, p.o. daily for 4 weeks). Data represent average values ± SEM (n = 5 mice per group). Brain samples from 9-month-old ABHD12(+/+) and ABHD12(–/–) mice were included as controls. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Two-sided Student's *t*-test performed relative to vehicle-treated mice, except for the ABHD12(–/–) mice, which were compared to ABHD12(+/+) mice. The *p*-values and other lipid species measured by targeted LC-MS are shown in **Supplementary Table 4**



Supplementary Figure 27. ABHD12(–/–) mice infected with LCMV-Clone 13 exhibit increased gross lung pathology. ABHD12(+/+) and ABHD12(–/–) animals were infected with a persistent dose of Clone-13 and day 10 post-infection mice were anesthetized, lungs were perfused, removed and photos taken. The result is a representative of three independent experiments.



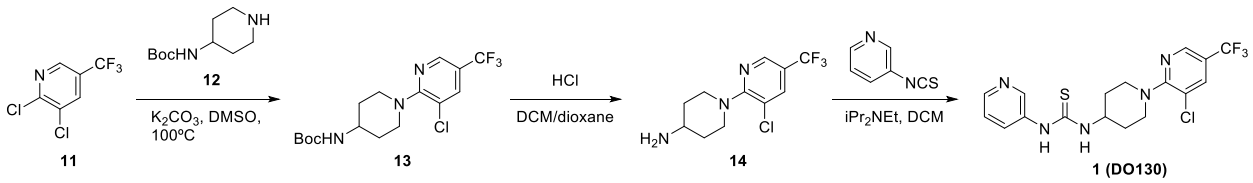
Supplementary Figure 28. Characterization of DO264 in LCMV-infected ABHD12(+/+) and ABHD12(–/–) mice. (**a, b**) Overall protein concentrations in BALF from (**a**) AHBD12(+/+) and ABHD12(–/–) mice or (**b**) DO264 and (S)-DO271-treated mice (30 mg/kg, i.p., daily, dosing started one day prior to infection) evaluated 10 days post-infection with Cl13. Data in **a** are representative of two independent experiments of n=5 mice per group, and **b** are plots representative of mean values of three experiments of n=5 mice per group ± SEM. Two-sided Student's *t*-test performed relative to ABHD12(+/+) or (S)-DO271-treated mice). (**c**, **d**) Characterization of DO264 in LCMV-infected ABHD12(–/–) mice. (**c**) Overall protein and (**d**) chemokine concentrations in BALF from DO264, and (S)-DO271-treated ABHD12 (–/–) mice (30 mg/kg, i.p., daily, dosing started one day prior to infection) evaluated 10 days post-infection with Cl13. For **c**, n = 9 mice for (S)-DO271-treated group and n = 8 mice for DO264-treated group. n = 4 mice per group for **d**. Two-sided Student's *t*-test performed relative to the (S)-DO271-treated group.



Supplementary Figure 29. Gating strategy for identifying CTV^{hi} and CTV^{lo} cell populations for cell lysis calculation. 1) Single cell gate: FSC-H / FSC-W 2) Single cell gate: SSC-H / SSC-W was used to remove doublets 3) Cell gate: FSC-A / SSC-A 4) was used to gate on leukocytes and Live cell gate: FSC-A / 7-AAD (7-AAD- live cells were selected) 5) CTV gate: FSC-A / CTV (CTV+ cells selected (both CTV^{hi} and CTV^{lo} populations) 6) Histogram of CTV^{hi} and CTV^{lo} cells used to calculate percent lysis.

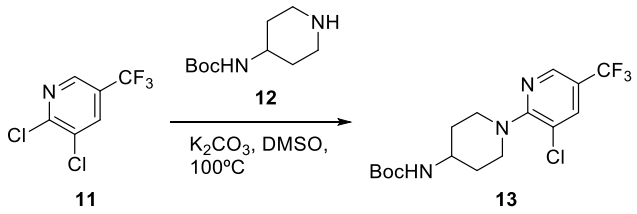
Supplementary Note: Synthetic Chemistry Procedures and Analytical Data

General Information. All chemical reagents were obtained from commercial suppliers and were used without further purification. All the chemical building blocks were purchased from Combi-Blocks. Merck silica gel TLC plates (0.25 mm, 60 F254) were used to monitor reactions. Flash chromatography was performed using SiliaFlash F60 silica gel (40–63 μm, 60 Å). NMR spectra were recorded at room temperature on Bruker DRX-600 spectrometer at 600 (^{1}H) and 150 (^{13}C) MHz using $CDCl_3$ as solvent, unless stated otherwise. Chemical shifts are recorded in ppm relative to tetramethylsilane (TMS) with peaks being reported as follows: chemical shift, multiplicity (s = singlet, brs = broad singlet, d = doublet, t = triplet, q = quartet, m = multiplet), coupling constant (Hz). High-resolution mass spectra (HRMS) were obtained on an Agilent LC/MSD TOF mass spectrometer by electrospray ionization–time-of-flight (ESI-TOF). The single crystal X-ray diffraction studies were carried out on a Bruker Kappa APEX-II CCD diffractometer equipped with Mo Kα radiation (λ = 0.71073 Å).



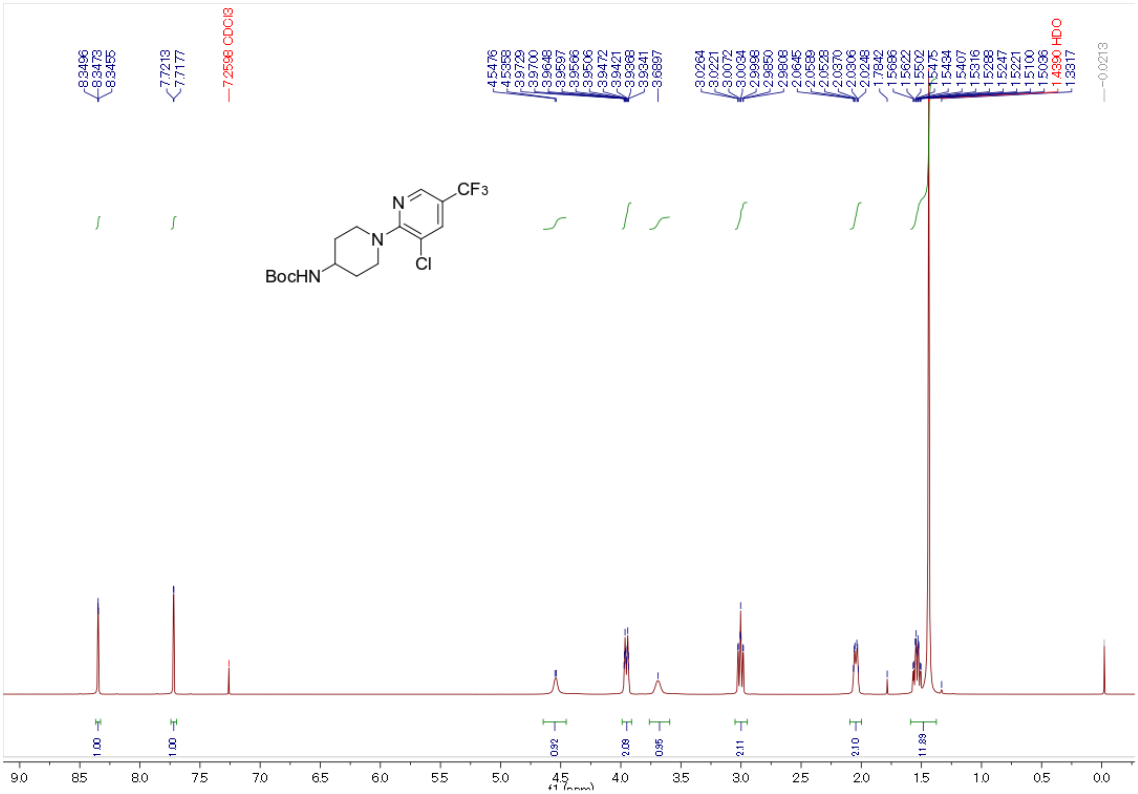
Synthesis of DO130.

tert-butyl [1-{3-chloro-5-(trifluoromethyl)pyridin-2-yl}piperidin-4-yl]carbamate (13)

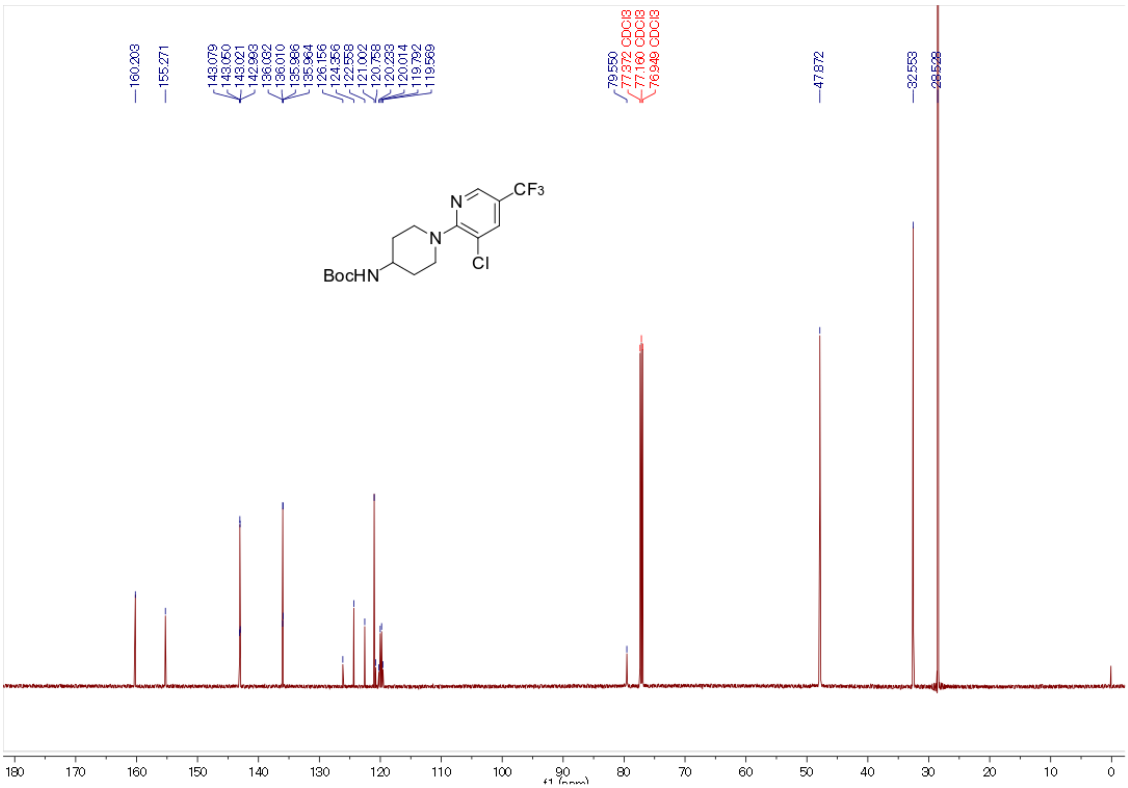


A solution of 2,3-dichloro-5-(trifluoromethyl)pyridin (**11**) (1.0 g, 4.6 mmol), tert-butyl(piperidin-4-yl)carbamate (**12**) (1.4 g, 6.9 mmol) and potassium carbonate (770 mg, 5.6 mmol) in dry DMSO (1.0 mL) was stirred for 2 h at 100 °C. The mixture was diluted with DCM and washed with water and brine. The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by flush column chromatography (ethyl acetate:hexane = 1:8) to afford **13** (1.6 g, 91%) as a white solid. ^{1}H NMR ($CDCl_3$, 600 MHz) δ 8.35 (s, 1H), 7.72 (s, 1H), 4.54 (s, 1H), 3.97 – 3.93 (m, 2H), 3.69 (s, 1H), 3.00 (ddd, 2H, *J* = 12.4, 11.5, 2.5 Hz), 2.06 – 2.02 (m, 2H), 1.54 (dtd, 2H, *J* = 12.7, 11.1, 3.9 Hz), 1.44 (s, 9H). ^{13}C NMR ($CDCl_3$, 150 MHz) 160.20, 155.27, 143.04, 136.00, 123.46 (q, *J* = 269.9 Hz, CF_3), 121.00, 119.89 (q, *J* = 33.2 Hz, $\underline{C}HCF_3$), 79.55, 47.87, 32.55, 28.53. HRMS calculated for $C_{16}H_{21}ClF_3N_3O_2$ $[M+H]^+$ 380.1353, found 380.1360.

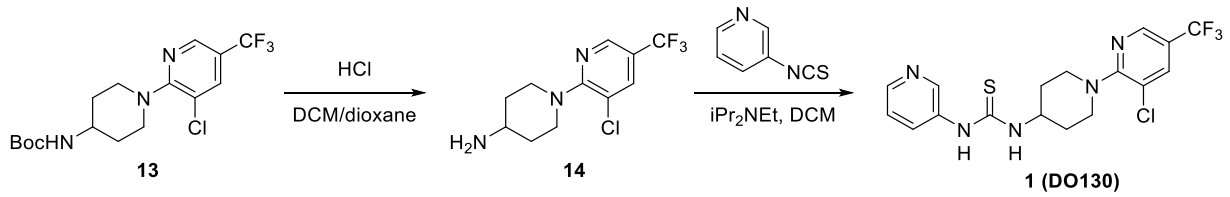
^{1}H NMR of compound 13



^{13}C NMR of compound 13

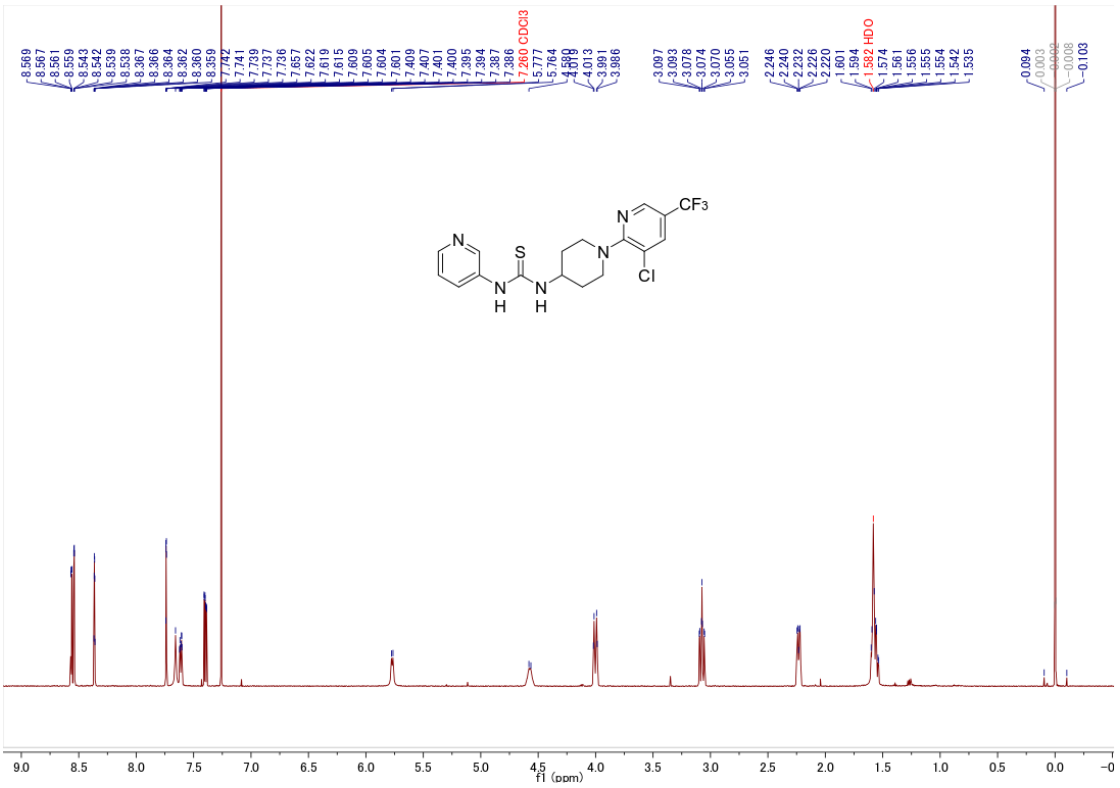


***N*-3-pyridyl-*N'*-[1-{3-chloro-5-(trifluoromethyl)pyridin-2-yl}piperidin-4-yl]thiourea (DO130)**

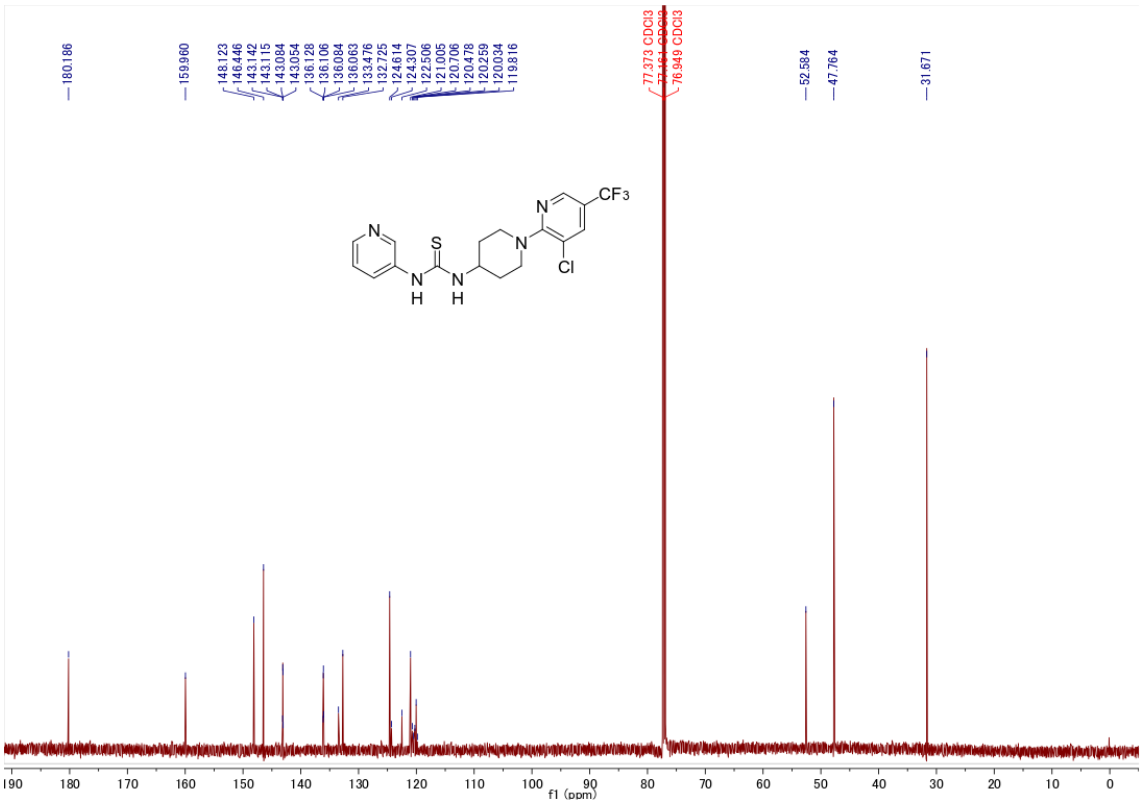


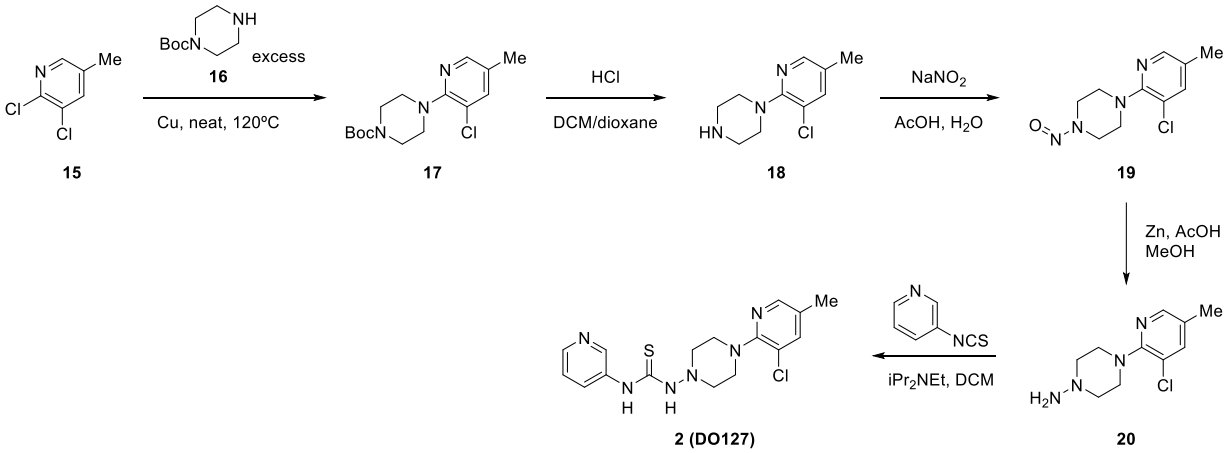
To a solution of **13** (50 mg, 0.13 mmol) in DCM (0.5 mL) was added 4N HCl in dioxane (0.5 mL) in a dropwise fashion and stirred for 2 h at room temperature. The mixture was dried under N_2 stream. The residue was dissolved in DCM (1 mL) with iPr_2NEt (90 µL, 0.51 mmol) and 3-pyridineisothiocyanate (19 mg, 0.14 mmol). The mixture was stirred for 3 h at room temperature. The mixture was diluted with DCM and washed with sat.$NaHCO_3$. The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by preparative TLC (ethylacetate only) to afford **DO130** (50 mg, 94%) as a white solid. ^{1}H NMR ($CDCl_3$, 600 MHz) δ 8.56 (dd, 1H, *J* = 4.8, 1.5 Hz), 8.54 (dd, 1H, *J* = 2.6, 0.8 Hz), 8.37 − 8.36 (m, 1H), 7.74 (d, 1H, *J* = 2.2 Hz), 7.66 (s, 1H), 7.61 (dt, 1H, *J* = 8.3, 2.2 Hz), 7.40 (dd, 1H, *J* = 8.1, 4.7, 0.8 Hz), 5.77 (d, 1H, *J* = 8.1 Hz), 4.60 − 4.54 (1H, m), 4.00 (d, 2H, *J* = 13.7 Hz), 3.07 (ddd, 2H, *J* = 13.7, 11.6, 2.5 Hz), 2.25 − 2.21 (m, 2H), 1.60 − 1.54 (m, 2H). ^{13}C NMR ($CDCl_3$, 150 MHz) 180.19, 148.12, 146.45, 143.09, 136.09, 133.48, 132.73, 124.61, 123.40 (q, *J* = 269.5 Hz, CF_3), 121.01, 120.14 (q, *J* = 33.1 Hz, $\underline{C}HCF_3$), 52.58, 47.76, 31.67. HRMS calculated for $C_{17}H_{17}ClF_3N_5S$ $[M+H]^+$ 416.0924, found 416.0928.

^{1}H NMR of DO130



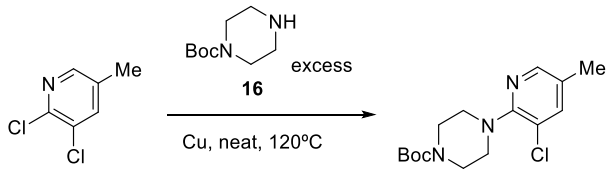
^{13}C NMR of DO130





Synthesis of DO127.

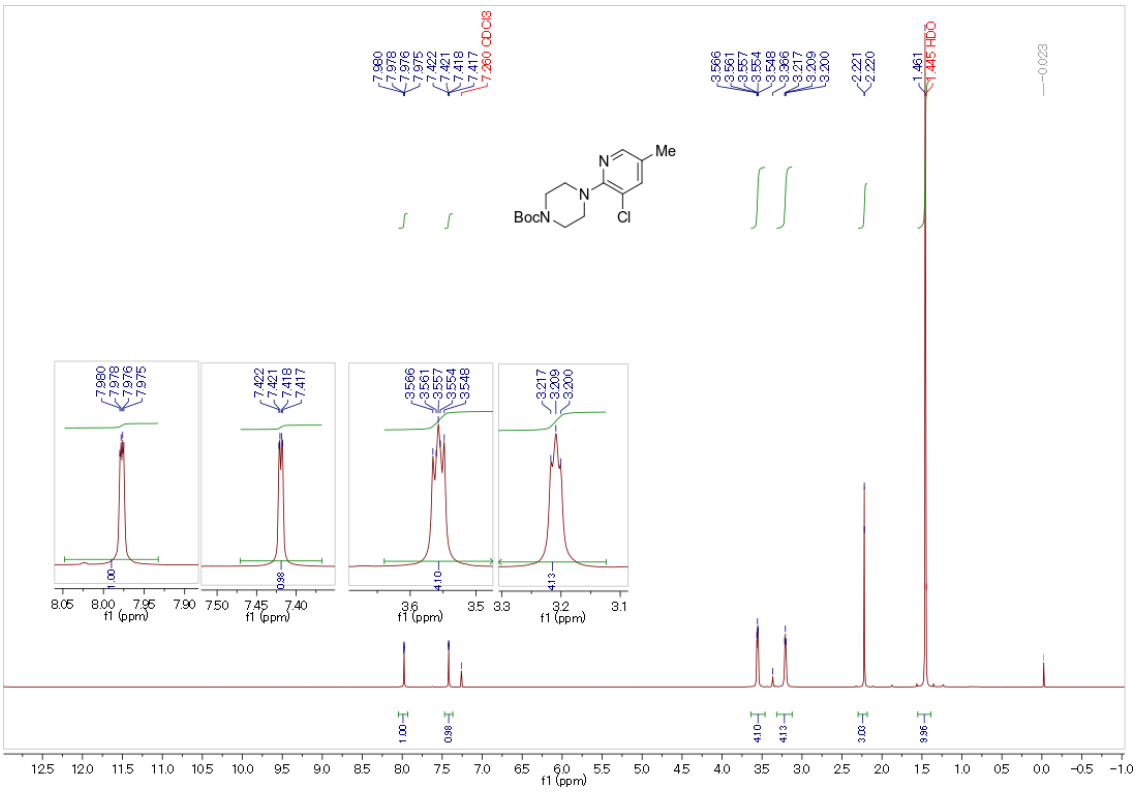
tert-butyl [1-{3-chloro-5-(methyl)pyridin-2-yl}piperazin-4-yl]carbamate (17)



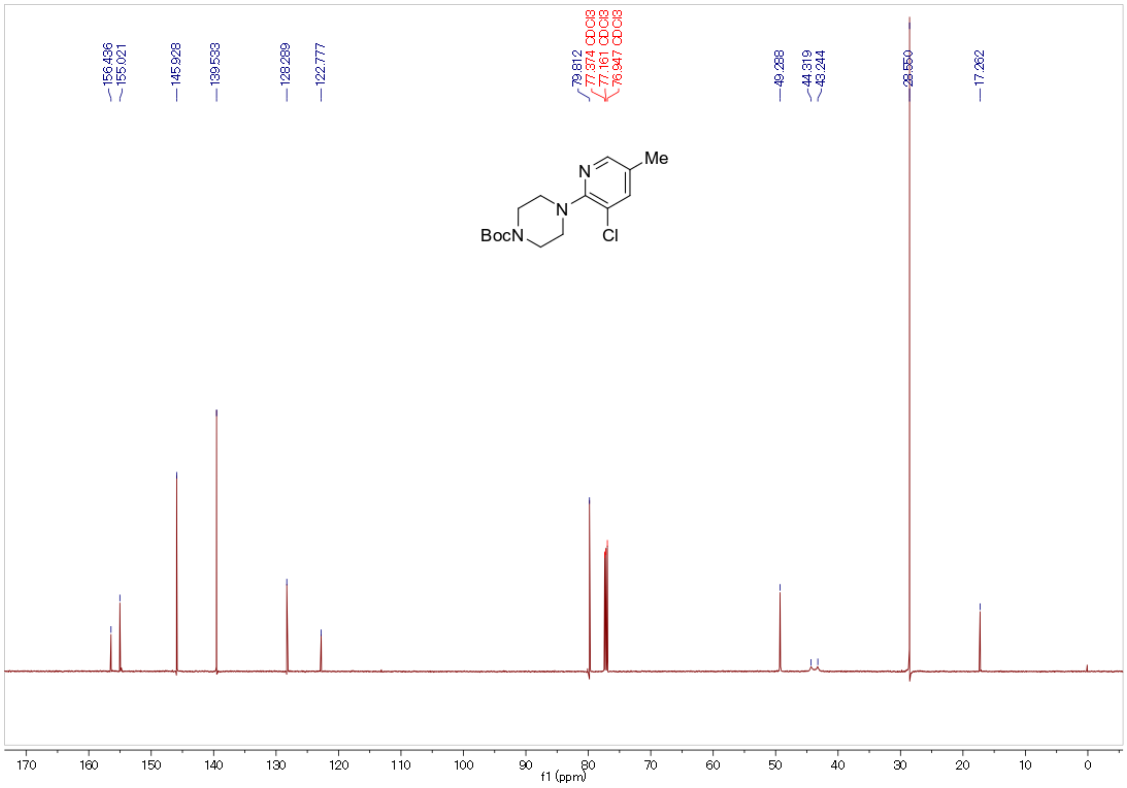
15 17

A solution of 2,3-dichloro-5-(methyl)pyridin (**15**) (500 mg, 3.1 mmol), tert-butyl(piperazin-4-yl)carbamate (**16**) (5.2 g, 28 mmol) and Cu powder (40 mg) was stirred for 18 h at 120 °C. The mixture was diluted with DCM and filtered through a silica pad (ethyl acetate:hexane = 1:1 as a eluent). The eluent was concentrated under reduced pressure. The residue was purified by flash column chromatography (ethyl acetate:hexane = 1:10 to 1:6) to afford **17** (620 mg, 64%) as a white solid. ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.98 (dd, *J* = 2.1, 0.9 Hz, 1H), 7.42 (dd, *J* = 2.1, 0.8 Hz, 1H), 3.57 – 3.55 (m, 4H), 3.21 (t, *J* = 5.1 Hz, 4H), 2.22 (s, 3H), 1.46 (s, 9H). ^{13}C NMR ($CDCl_3$, 150 MHz) 156.44, 155.02, 145.93, 139.53, 128.29, 122.78, 79.81, 49.29, 44.32, 43.24, 28.55, 17.26. HRMS calculated for $C_{15}H_{23}ClN_3O_2$ $[M+H]^+$ 312.1479, found 312.1484.

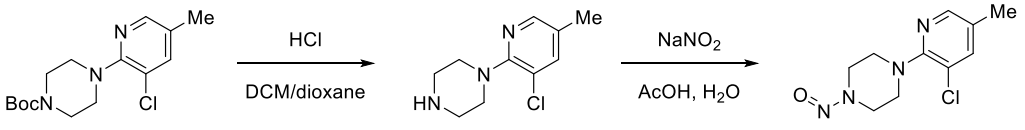
^{1}H NMR of compound 17



^{13}C NMR of compound 17



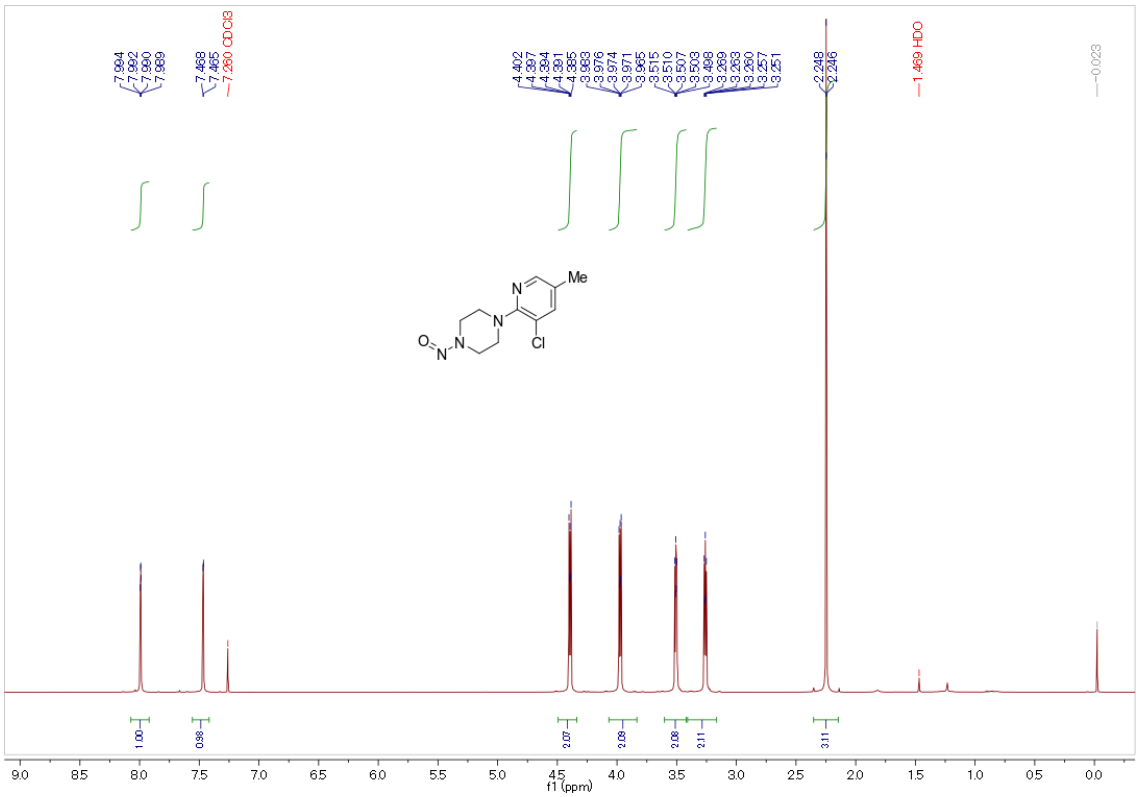
1-nitroso-4-{3-chloro-5-(methyl)pyridin-2-yl}piperazine (19)



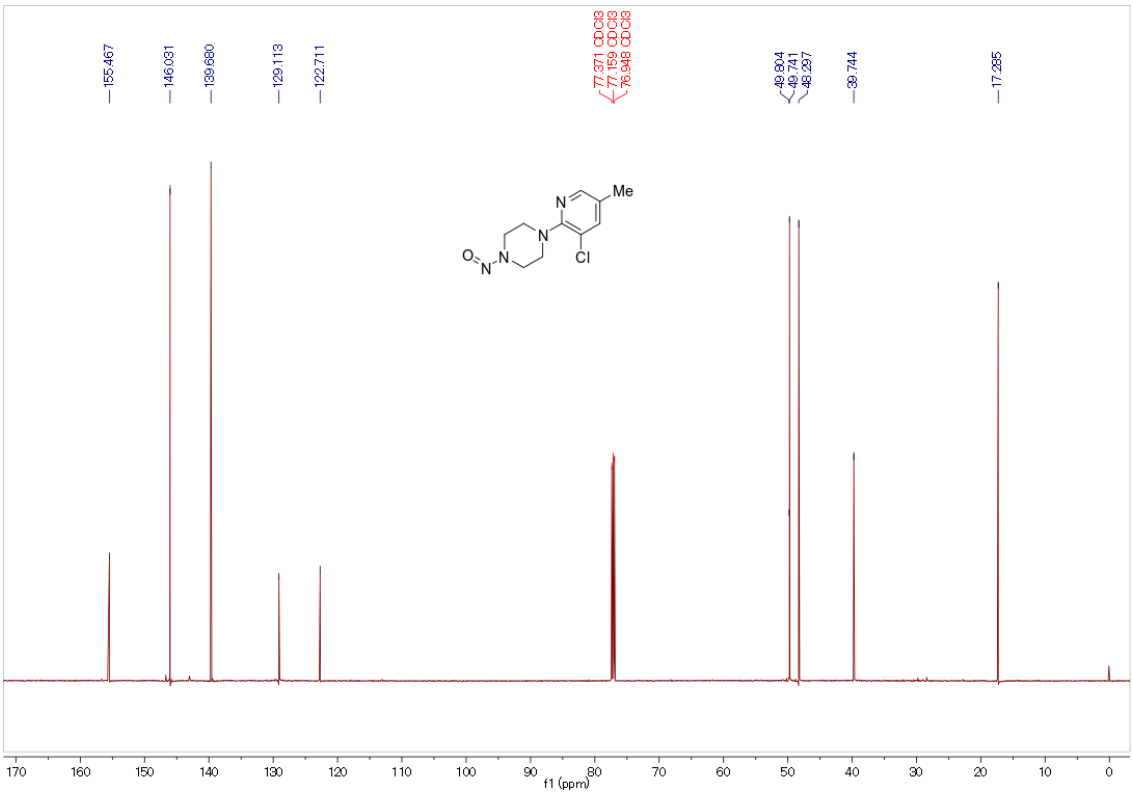
17 **18** **19**

To a solution of **17** (240 mg, 0.77 mmol) in DCM (3.0 mL) was added 4N HCl in dioxane (3.0 mL) in a dropwise fashion and stirred for 2 h at room temperature. The mixture was dried under N_2 stream. The residue was dissolved in acetic acid/water (2.4 mL/0.48 mL) and $NaNO_2$ (106.2 mg, 1.54 mmol) in water (1.83 mL) was added in a dropwise fashion on an ice bath. The mixture was stirred 2 h at room temperature and basified with sat.$NaHCO_3$. The mixture was extracted with DCM (X3). The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by flush column chromatography (hexane only then a gradient of ethylacetate:hexane = 1:10 to 1/3) to afford **19** (179 mg, 96% over 2 steps) as a pale yellow solid. ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.99 (dd, *J* = 2.1, 1.0 Hz, 1H), 7.47 (d, *J* = 2.0 Hz, 1H), 4.40 – 4.39 (m, 2H), 3.98 – 3.97 (m, 2H), 3.52 – 3.50 (m, 2H), 3.27 – 3.25 (m, 2H), 2.25 (s, 3H). ^{13}C NMR ($CDCl_3$, 150 MHz) 155.47, 146.03, 139.68, 129.11, 122.71, 49.80, 49.74, 48.30, 39.74, 17.28. HRMS calculated for $C_{10}H_{14}ClF_3N_4O$ $[M+H]^+$ 241.0856, found 241.0858.

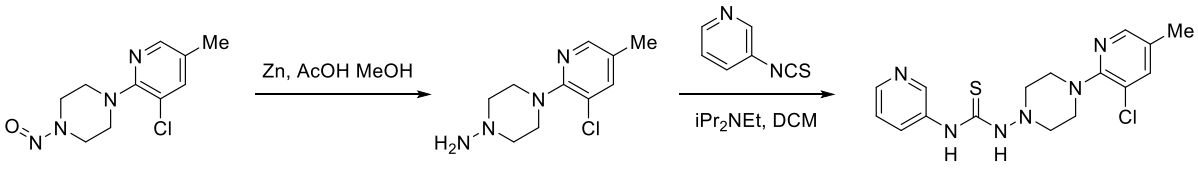
^{1}H NMR of compound 19



^{13}C NMR of compound 19



***N*-3-pyridyl-*N'*-[1-{3-chloro-5-(methyl)pyridin-2-yl}piperazine-4-yl]thiourea (DO127)**

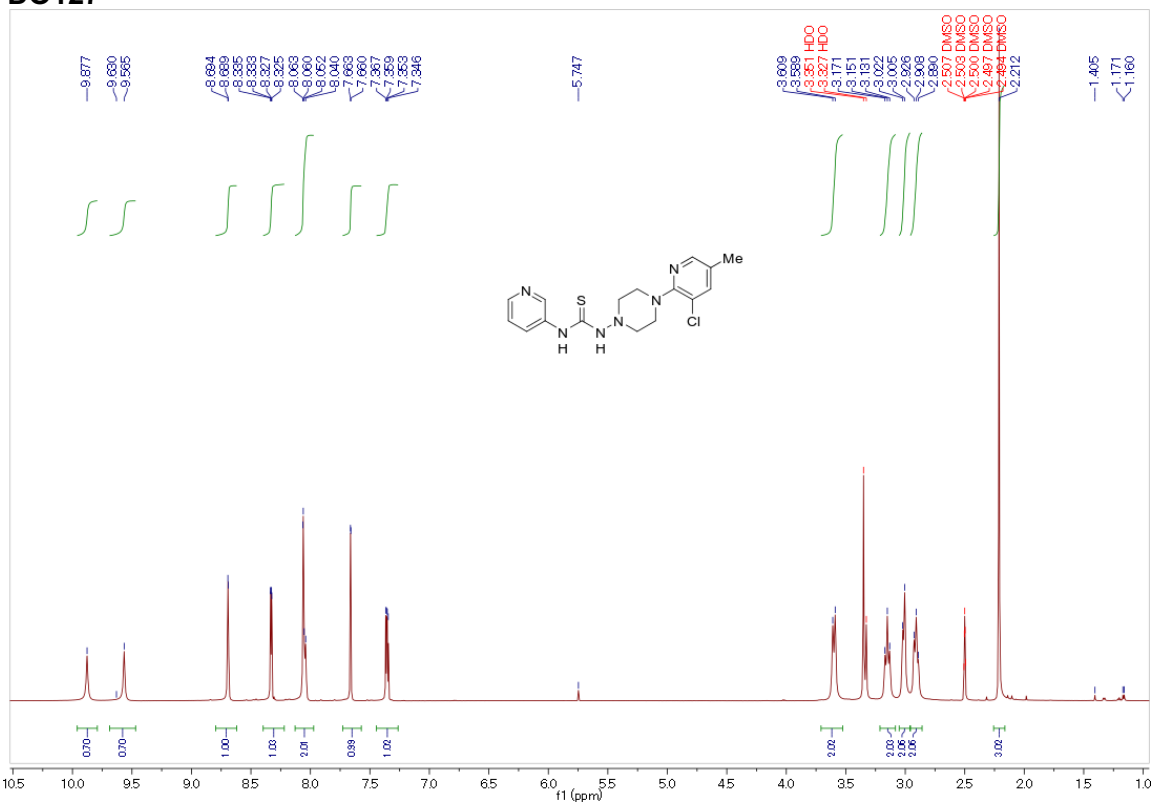


19 **20** **2 (DO127)**

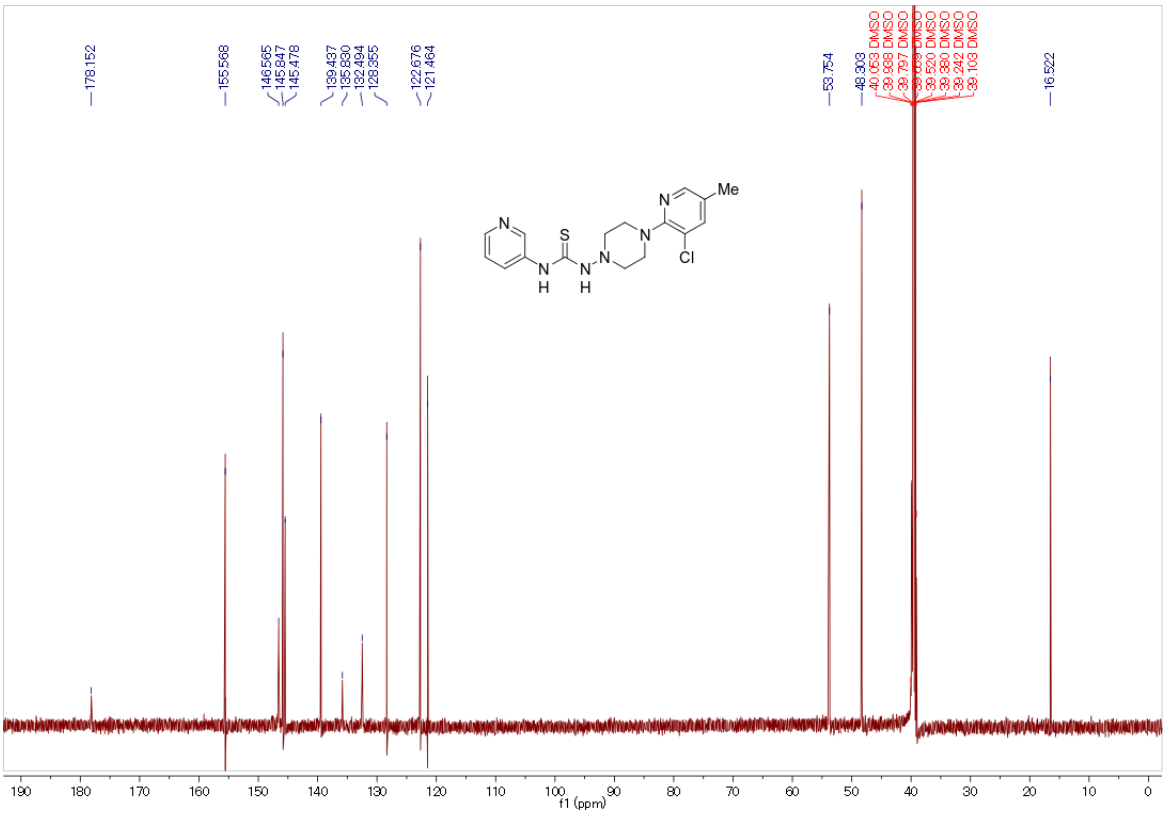
Step 1. To a solution of **19** (180 mg, 0.74 mmol) in MeOH/acetic acid (0.8 mL/0.8 mL) was added Zn powder (200 mg, 3.1 mmol) over 5 min on an ice bath and stirred for 3 h at room temperature. The mixture was basified with sat.$NaHCO_3$ and extracted with DCM (X3). The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was passed through a short flash column chromatography (DCM:MeOH = 10:1) to afford **20** (133 mg) as a crude brown amorphous.

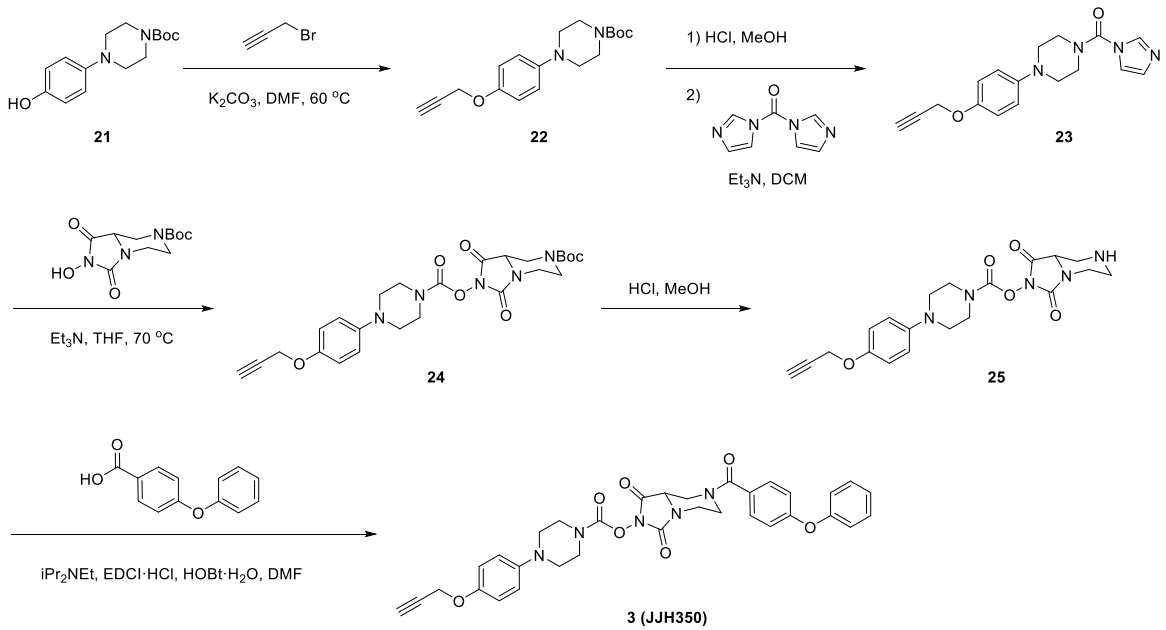
Step 2. To a solution of a crude compound **20** (80 mg) in DCM (1.6 mL) was added iPr2NEt (120 µL, 0.71 mmol) and 3-pyridineisothiocyanate (53 mg, 0.39 mmol) and stirred for overnight at room temperature. The mixture was diluted with DCM and washed with sat.NaHCO3. The organic layer was dried over Na2SO4 and concentrated under reduced pressure. The residue was purified by flash column chromatography (a gradient of ethylacetate:hexane = 1:1 to 5/1 and then ethylacetate only) to give a crude **DO127**, which was then suspended in ethylacetate:hexane = 1:1 at 50 °C. The solution was cooled to room temperature and the resulting precipitate was filtered to afford **DO127** (70 mg, 55% over 2 steps) as a white solid. ^{1}H NMR ($CDCl_3$, 600 MHz) δ 9.88 (s, 1H), 9.57 (s, 1H), 8.69 (d, *J* = 2.5 Hz, 1H), 8.33 (dd, *J* = 4.7, 1.4 Hz, 1H), 8.06 – 8.04 (m, 2H), 7.66 (d, *J* = 2.0 Hz, 1H), 7.36 (dd, *J* = 8.2, 4.7 Hz, 1H), 3.60 (d, *J* = 12.3 Hz, 2H), 3.15 (t, *J* = 12.0 Hz, 2H), 3.01 (d, *J* = 10.4 Hz, 2H), 2.91 (t, *J* = 11.1 Hz, 2H), 2.21 (s, 3H). ^{13}C NMR ($CDCl_3$, 150 MHz) 178.15, 155.57, 146.57, 145.85, 145.48, 139.44, 135.83, 132.49, 128.36, 122.68, 121.46, 53.75, 48.30, 16.52. HRMS calculated for $C_{16}H_{20}ClN_6S$ $[M+H]^+$ 363.1159, found 363.1160.

^{1}H NMR of DO127



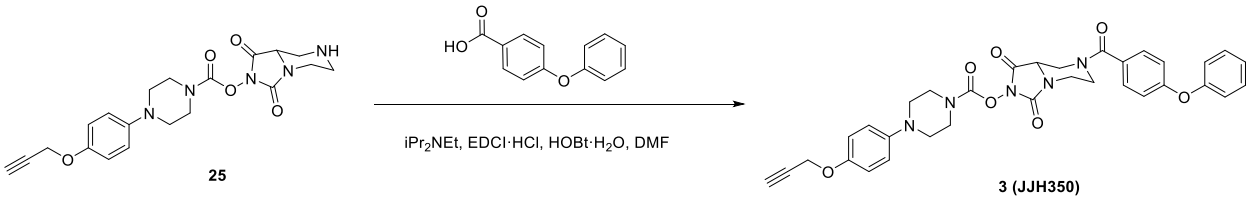
^{13}C NMR of DO127





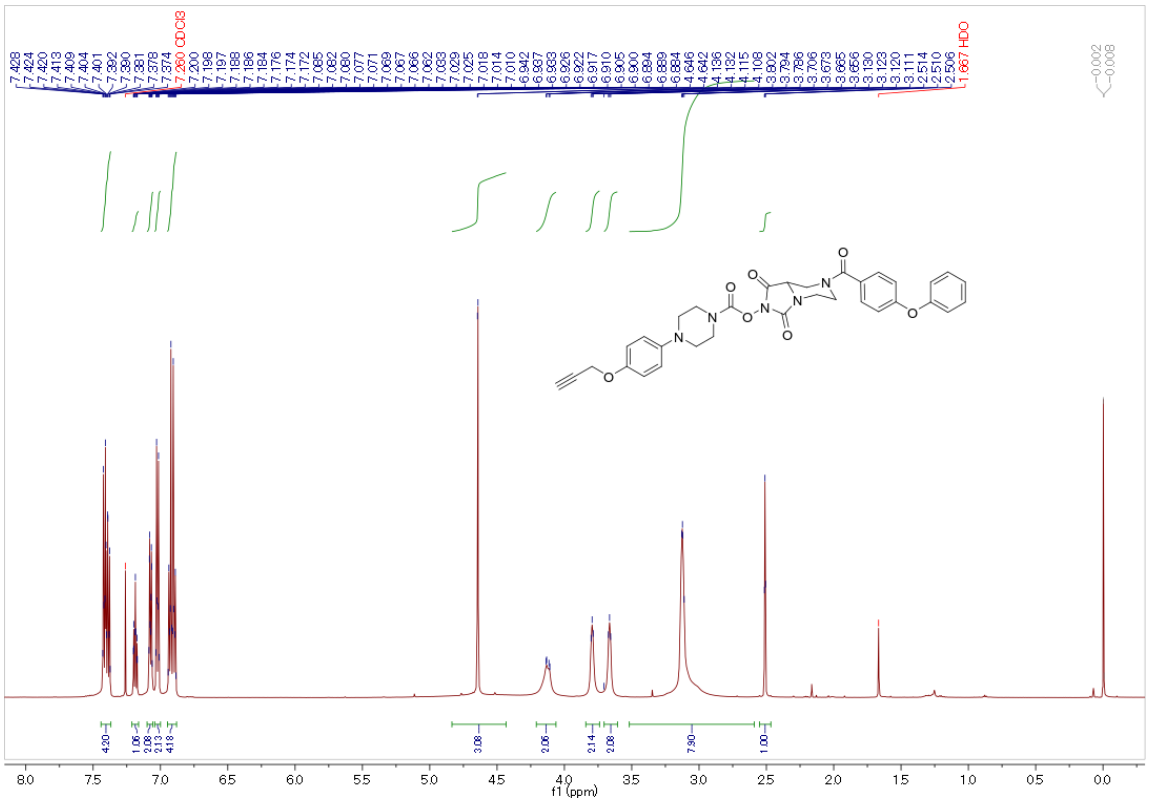
Synthesis of JJH350.

Synthesis of 1,3-dioxo-7-(4-phenoxybenzoyl)hexahydroimidazo[1,5-*a*]pyrazine-2(3H)-yl 4-(4-(prop-2-yn-1-yloxy)phenyl)piperazine-1-carboxylate (JJH350)

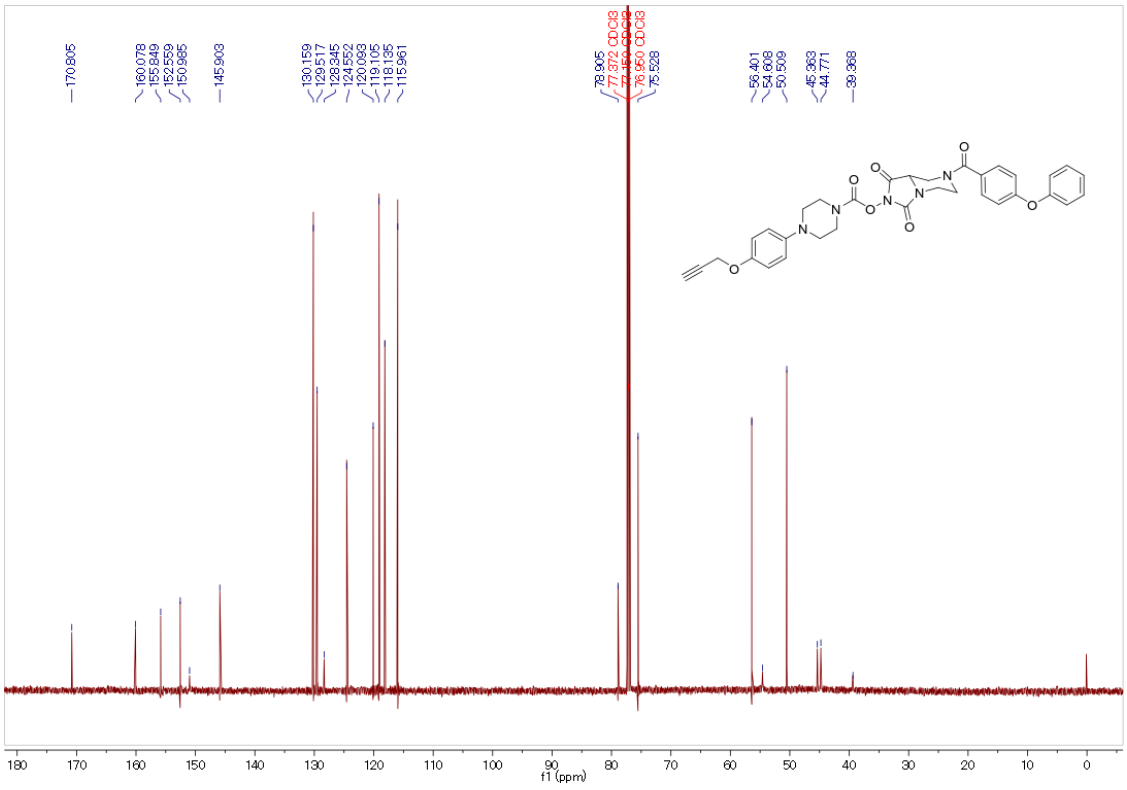


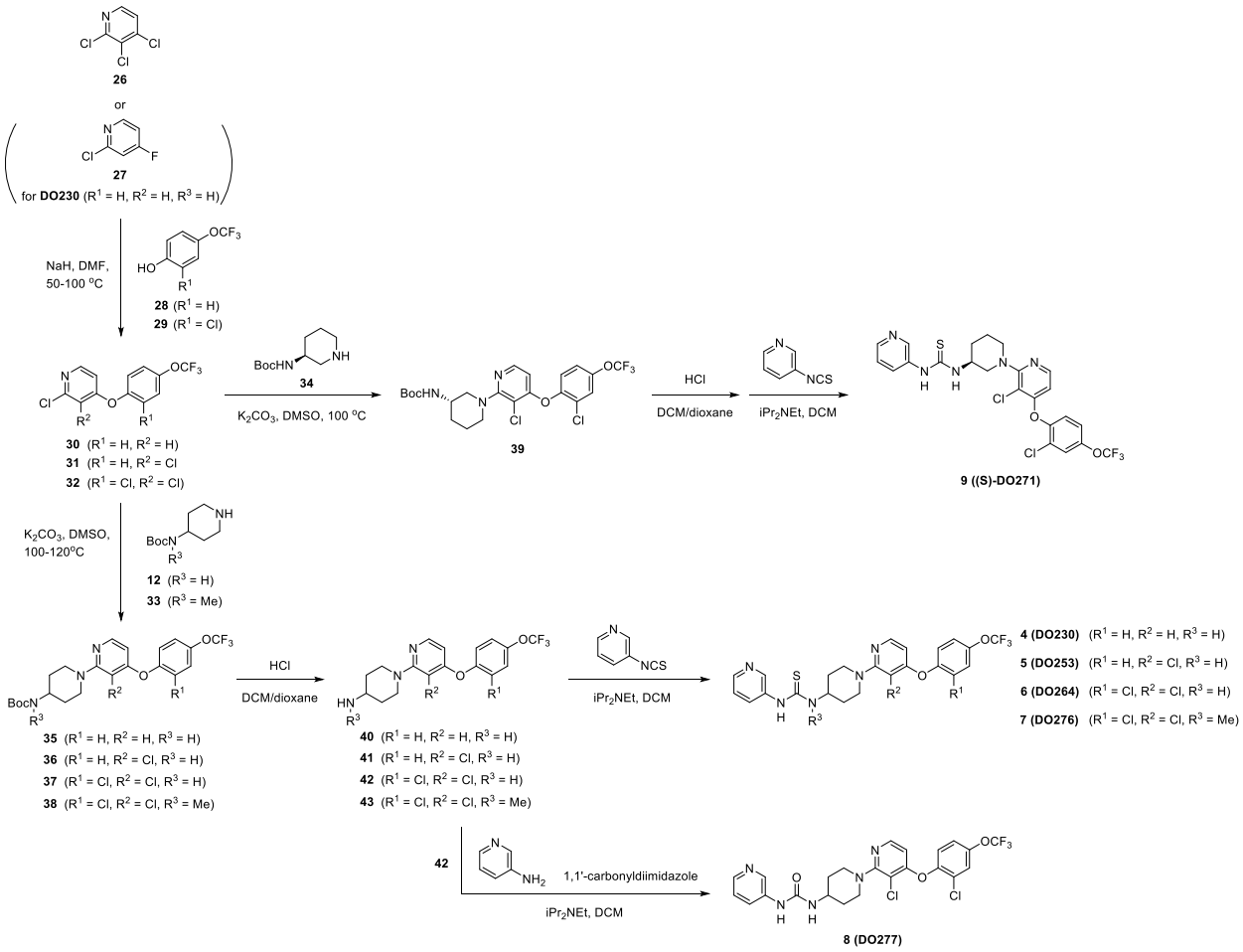
1,3-dioxohexahydroimidazo[1,5-a]pyrazin-2(3H)-yl 4-(4-(prop-2-yn-1-yloxy)phenyl)piperazine-1-carboxylate (compound **25**)[1] (23 mg, 54 µmol, 1 eq) was dissolved in 200 µL DMF, and 4-phenoxy benzoic acid (12 mg, 54 µmol, 1 eq), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI·HCl) (16 mg, 82 µmol, 1.5 eq), 1-hydroxybenzotriazole monohydrate (HOBt·H_2O) (13 mg, 82 µmol, 1.5 eq), *N*,*N*-diisopropylethylamine (19 µL, 110 µmol, 2 eq), were added. The resulting mixture was stirred at room temperature overnight and poured into saturated aqueous $NaHCO_3$ solution. The mixture was extracted with ethyl acetate (two times). The combined organic layer was washed with H_2O and brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by prep-TLC (ethyl acetate:hexane= 2:1) to afford (**JJH350**) (28 mg, 86**%**) as a white solid. ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.43 – 7.37 (m, 4H), 7.19 (tt, *J* = 7.4, 1.1 Hz, 1H), 7.09 – 7.06 (m, 2H), 7.03 – 7.01 (m, 2H), 6.94 – 6.88 (m, 4H), 4.64 (d, *J* = 2.5 Hz, 3H), 4.14 (brs, 1H), 4.12 (brs, 1H), 3.77 (s, 2H), 3.64 (s, 2H), 3.22 − 2.91 (m, 8H), 2.51 (t, *J* = 2.4 Hz, 1H). ^{13}C NMR ($CDCl_3$, 150 MHz) 170.81, 160.08, 155.85, 152.56, 150.99, 145.90, 130.16, 129.52, 128.35, 124.55, 120.09, 119.11, 118.14, 116.00, 78.91, 75.53, 56.40, 54.61, 50.51, 45.38, 44.77, 39.37. HRMS calculated for $C_{33}H_{31}N_5O_7$ $[M+H]^+$ 610.2296, found 610.2298.

^{1}H NMR of JJH350



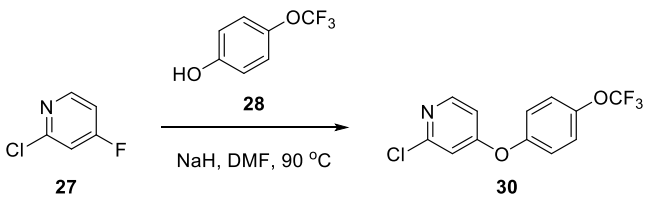
^{13}C NMR of JJH350





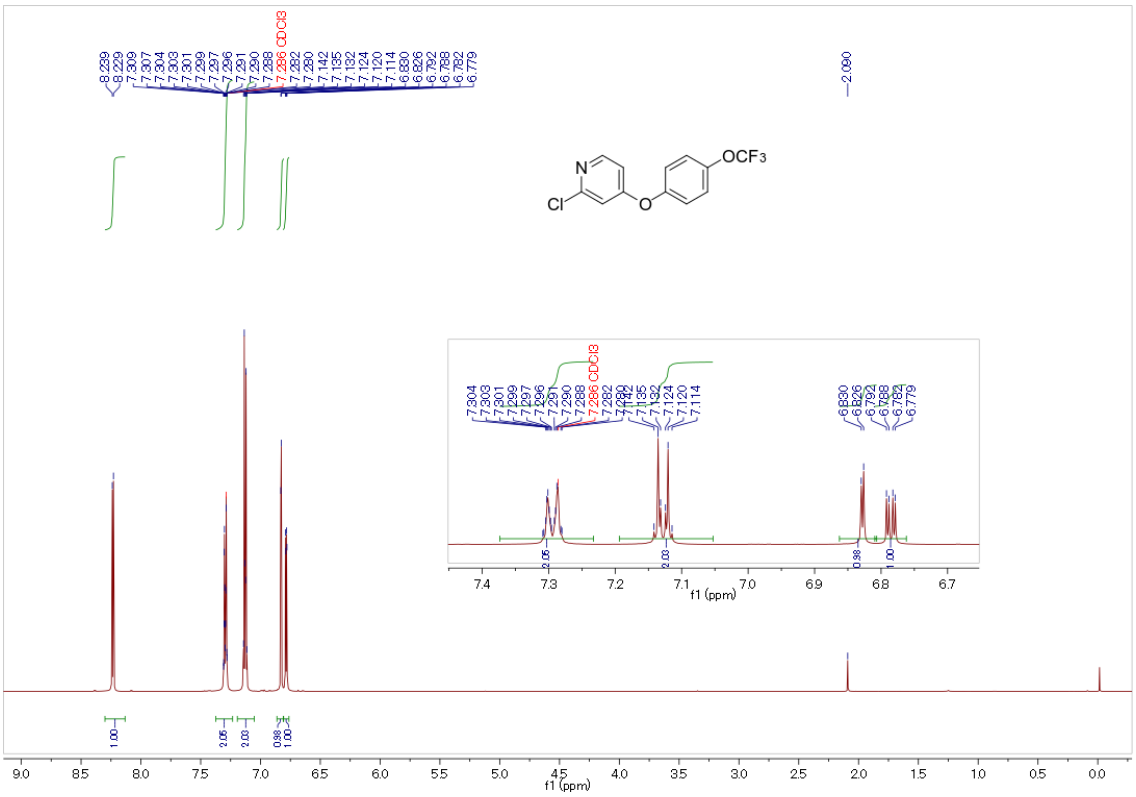
Synthesis of DO230, DO253, DO264, DO276, DO277 and (S)-DO271.

2-chrolo-4-{4-(trifluoromethoxy)phenoxy}pyridine (30)

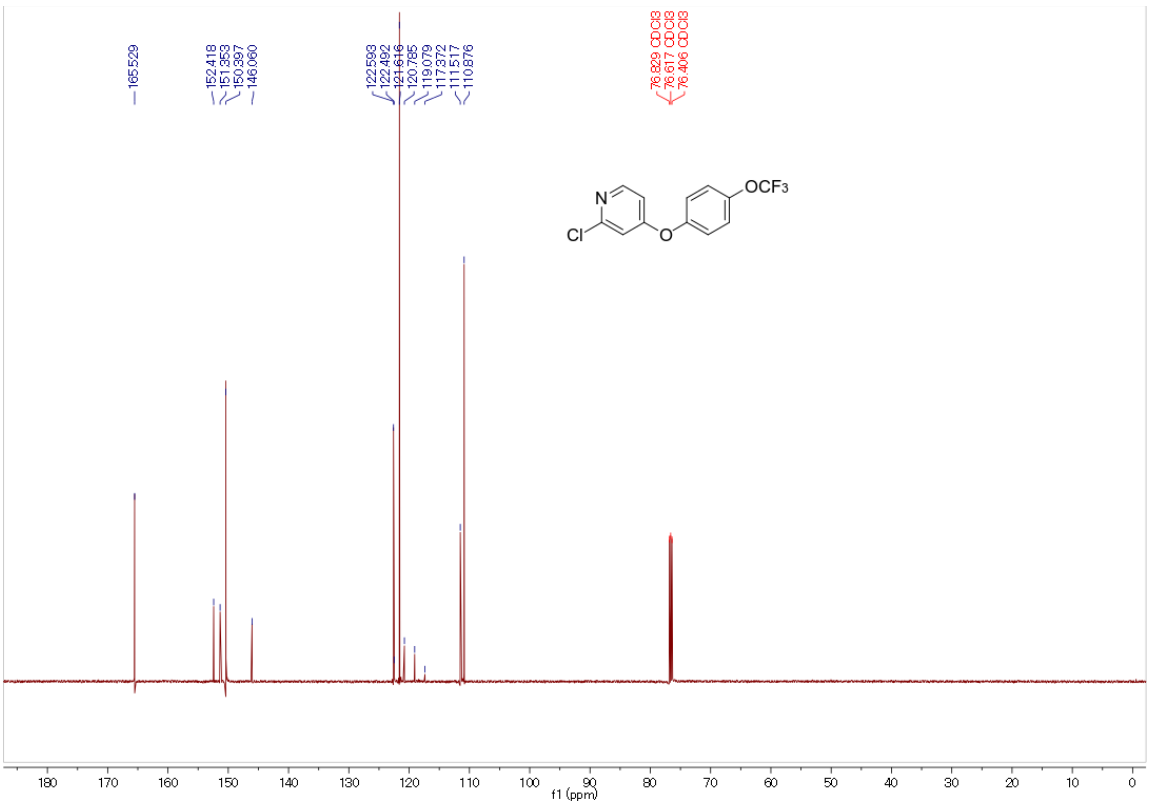


To a solution of 4-trifluoromethoxyphenol (**28**) (406 mg, 2.3 mmol) in dry DMF (1.0 mL) was slowly added 60% sodium hydride in mineral oil (91 mg, 2.3 mmol) at 0 °C. The mixture was warmed to room temperature and stirred for 20 min. The reaction mixture was cooled to 0 °C and added 2-chloro-4-fluoropyridine (**27**) (300 mg, 2.3 mmol). The reaction mixture was stirred overnight at 90 °C and concentrated under reduced pressure. The residue was purified by flash column chromatography (hexane only to ethylacetate:hexane = 1:10 to 1:6) to afford **30** (580 mg, 88%) as a colorless oil. ^{1}H NMR ($CDCl_3$, 600 MHz) δ 8.23 (d, 1H, J = 5.7 Hz), 7.31 − 7.28 (m, 2H), 7.14−7.11 (m, 2H), 6.83 (d, 1H, J = 2.2 Hz), 6.79 (dd, 1H, J = 5.7, 2.2 Hz). ^{13}C NMR ($CDCl_3$, 150 MHz) 166.07, 152.96, 151.90, 150.94, 146.60, 123.14, 122.16, 120.47 (q, J = 256.0 Hz, OCF_3), 112.06, 111.42. HRMS calculated for $C_{12}H_7ClF_3NO_2$ $[M+H]^+$ 290.0196, found 290.0198.

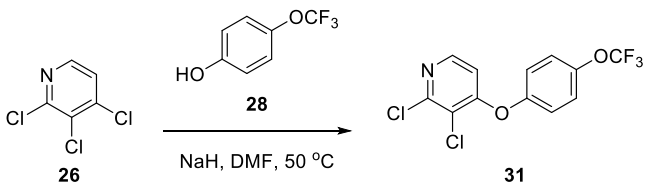
^{1}H NMR of compound 30



^{13}C NMR of compound 30

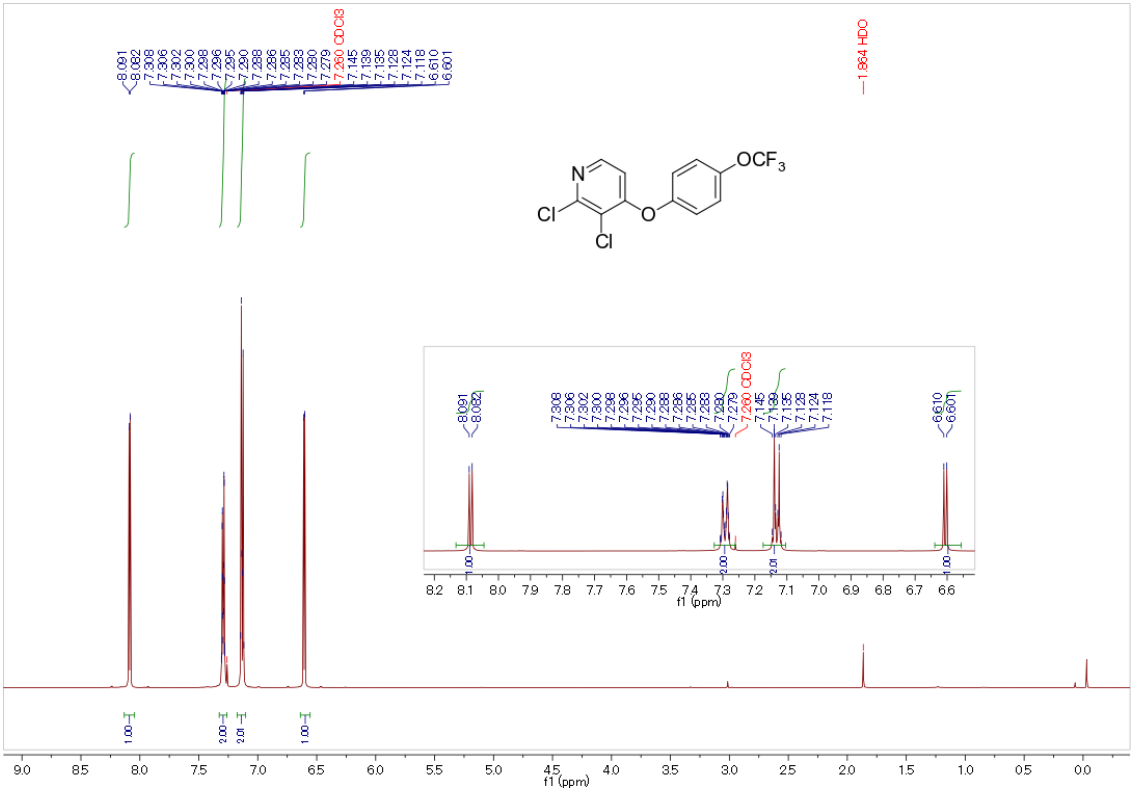


2,3-dichrolo-4-{4-(trifluoromethoxy)phenoxy}pyridine (31)

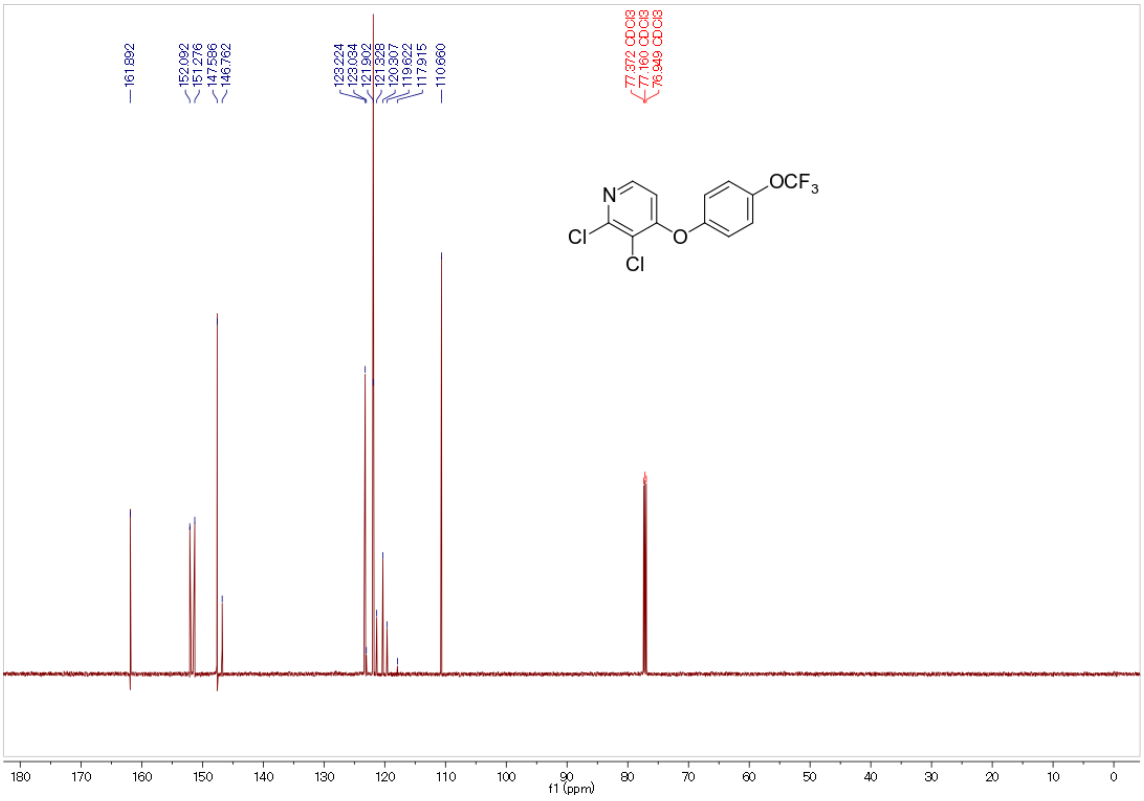


To a solution of 4-trifluoromethoxyphenol (**28**) (494 mg, 2.8 mmol) in dry DMF (1.8 mL) was slowly added 60% sodium hydride in mineral oil (110 mg, 2.8 mmol) at 0 °C. The mixture was warmed to room temperature and stirred for 20 min. The reaction mixture was cooled to 0 °C and added 2,3,4-trichloropyridine (**26**) (550 mg, 3.1 mmol). The reaction mixture was stirred for 2 h at room temperature and then 2 h at 50 °C. The mixture was concentrated under reduced pressure and the residue was purified by flash column chromatography (hexane only then gradient of ethylacetate:hexane = 1:100 to 1:10) to afford **31** (861 mg, 96%) as a colorless oil. The regiochemistry on the pyridine ring of **31** was confirmed by X-ray crystal structure of DO253, a final product from this intermediate (**see X-ray crystallographic Data for DO253**). ^{1}H NMR ($CDCl_3$, 600 MHz) δ 8.09 (d, 1H, *J* = 5.6 Hz), 7.31 − 7.28 (m, 2H), 7.15 − 7.12 (m, 2H), 6.61 (d, 1H, *J* = 5.5 Hz). ^{13}C NMR ($CDCl_3$, 150 MHz) 161.89, 152.09, 151.28, 147.59, 146.76, 123.22, 121.90, 120.47 (q, *J* = 256.0 Hz, OCF_3), 120.38, 110.66. HRMS calculated for $C_{12}H_6Cl_2F_3NO_2$ $[M+H]^+$ 323.9806, found 323.9813.

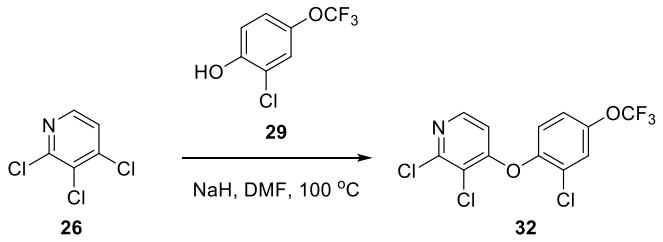
^{1}H NMR of compound 31



^{13}C NMR of compound 31

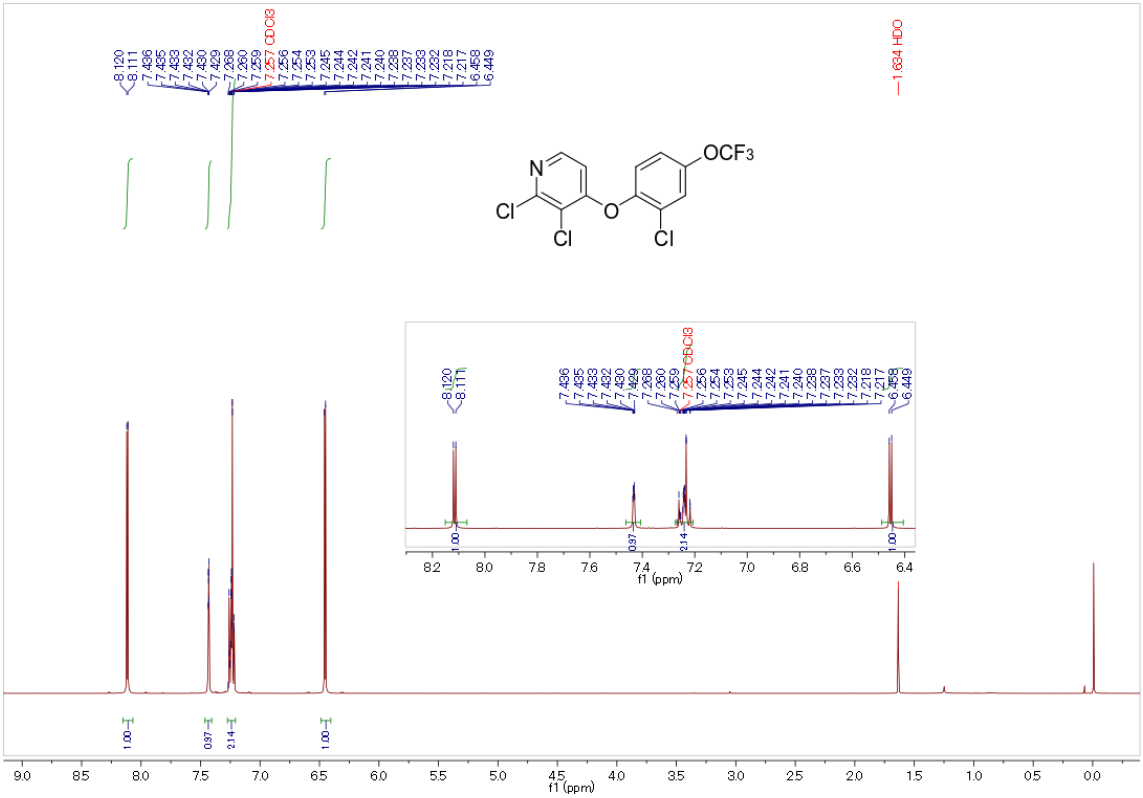


2,3-dichrolo-4-{2-chloro-4-(trifluoromethoxy)phenoxy}pyridine (32)

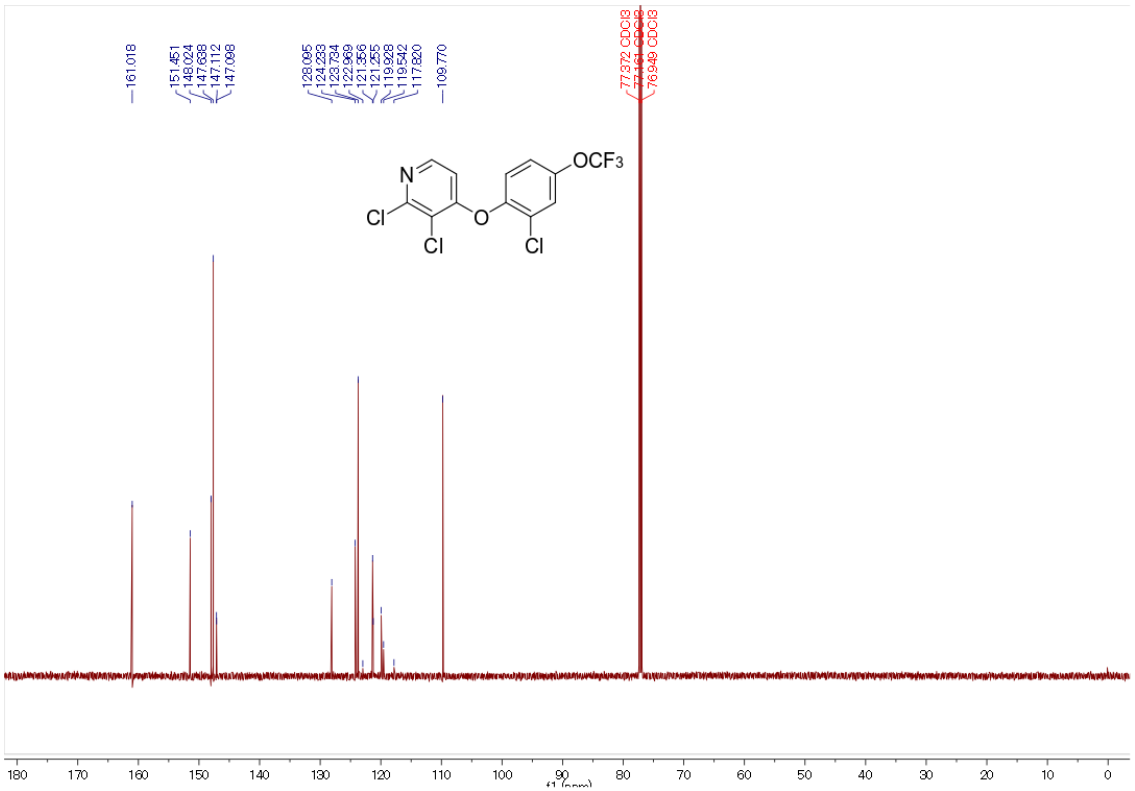


To a solution of 2-chloro-4-trifluoromethoxyphenol (**29**) (1.76 g, 8.3 mmol) in dry DMF (6.0 mL) was slowly added 60% sodium hydride in mineral oil (332 mg, 8.3 mmol) at 0 °C. The mixture was warmed to room temperature and stirred for 20 min. The reaction mixture was cooled to 0 °C and added 2,3,4-trichloropyridine (**26**) (1.5 g, 8.3 mmol). The reaction mixture was stirred for 2.5 h at 100 °C. The mixture was concentrated under reduced pressure and the residue was purified by flash column chromatography (hexane only then gradient of ethylacetate:hexane = 1:100 to 1:5) to afford **32** (2.4 g, 82%) as a white solid. ^{1}H NMR ($CDCl_3$, 600 MHz) δ 8.12 (d, 1H, *J* = 5.6 Hz), 7.43 (dt, 1H, *J* = 2.6, 0.8 Hz), 7.26 − 7.22 (m, 2H), 6.45 (d, 1H, *J* = 5.6 Hz). ^{13}C NMR ($CDCl_3$, 150 MHz) 161.02, 151.45, 148.02, 147.64, 147.09, 128.10, 124.23, 123.73, 121.36, 120.40 (q, *J* = 257.5 Hz, OCF_3), 119.93, 109.77. HRMS calculated for $C_{12}H_5Cl_3F_3NO_2$ $[M+H]^+$ 357.9416, found 357.9422.

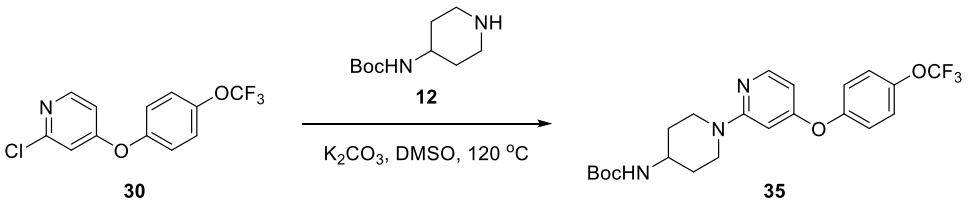
^{1}H NMR of compound 32



^{13}C NMR of compound 32

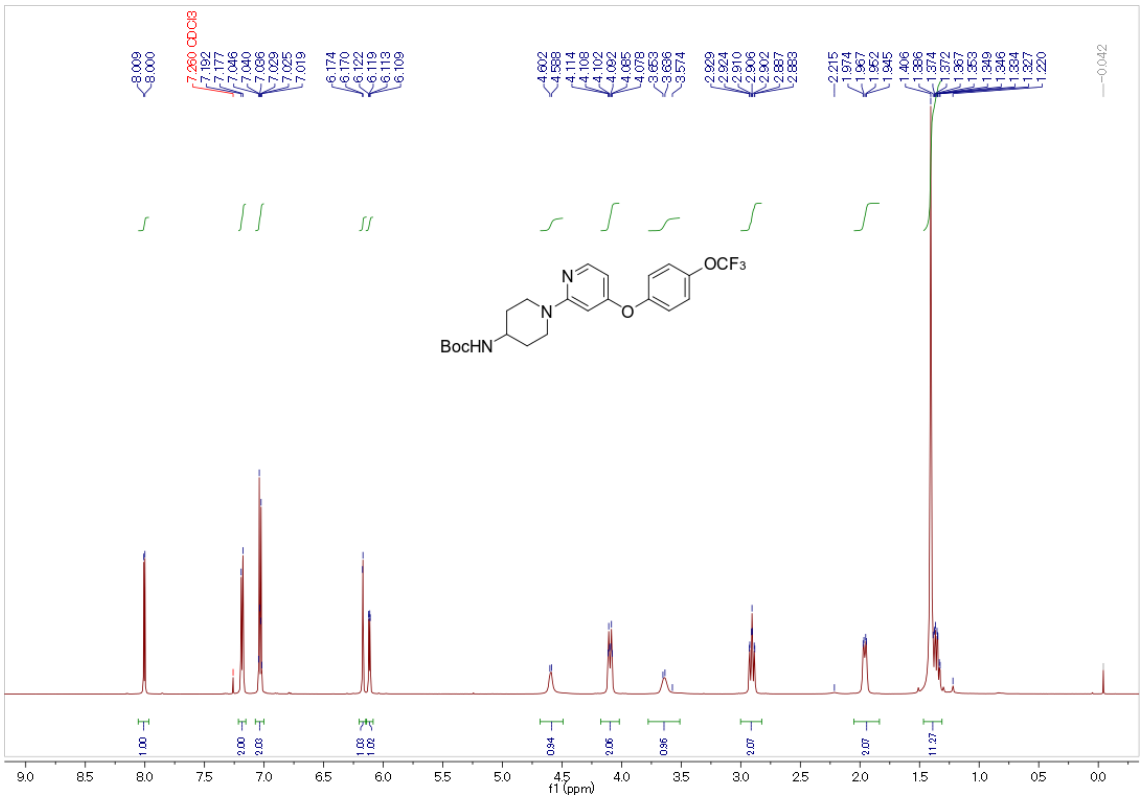


tert-butyl (1-[4-{4-(trifluoromethoxy)phenoxy}pyridine-2-yl]piperidin-4-yl)carbamate (35)

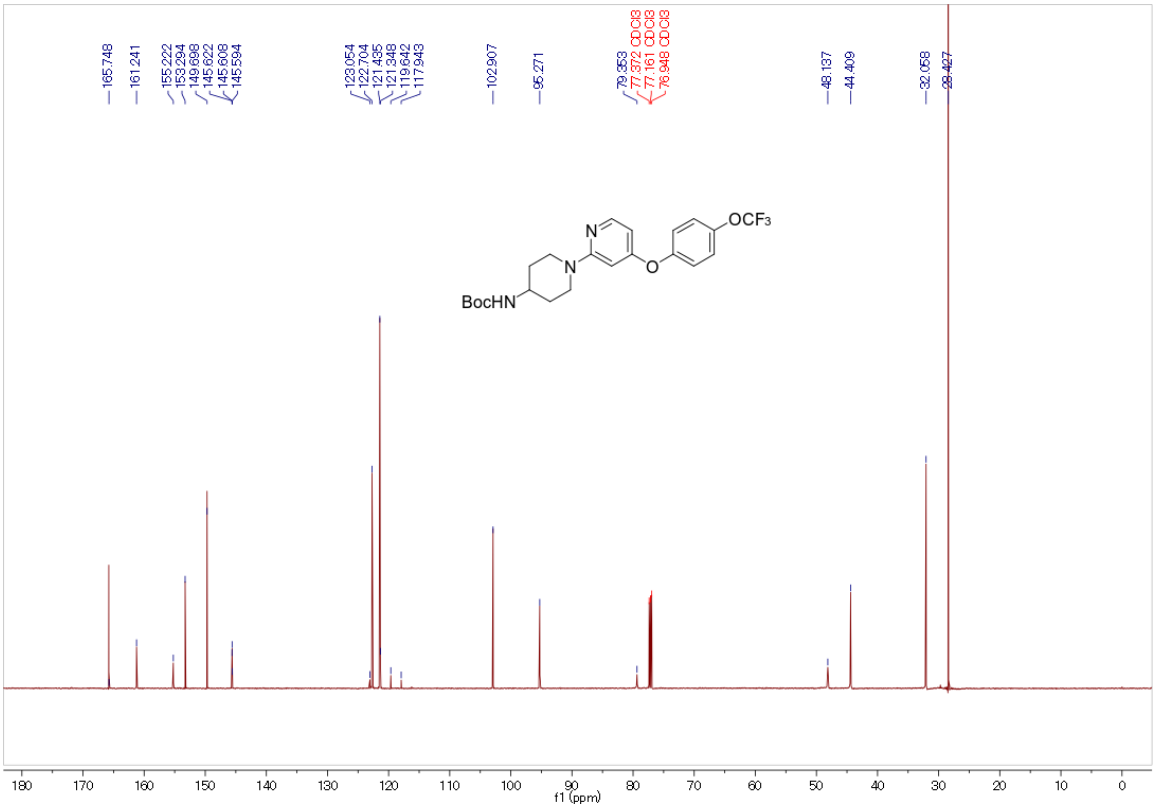


A solution of **30** (500 mg, 1.7 mmol), tert-butyl(piperidin-4-yl)carbamate (**12**) (1.38 g, 6.9 mmol) and potassium carbonate (360 mg, 2.6 mmol) in dry DMSO (0.85 mL) was stirred for 12 h at 120 °C. The mixture was dissolved in DCM and filtered through a pad of silica with ethylacetate. The eluent was concentrated under reduced pressure and the residue was purified by flash column chromatography (hexane only then gradient of ethylacetate:hexane = 1:10 to 1:2) to afford **35** (390 mg, 50%) as a white solid. ^{1}H NMR ($CDCl_3$, 600 MHz) δ 8.00 (d, *J* = 5.7 Hz, 1H), 7.18 (d, *J* = 8.7 Hz, 2H), 7.05 – 7.02 (m, 2H), 6.17 (d, *J* = 2.1 Hz, 1H), 6.12 (dd, *J* = 5.7, 2.0 Hz, 1H), 4.60 (d, *J* = 8.0 Hz, 1H), 4.10 (dt, *J* = 13.8, 3.9 Hz, 2H), 3.64 (d, *J* = 10.2 Hz, 1H), 2.91 (ddd, *J* = 13.9, 11.6, 2.8 Hz, 2H), 1.96 (d, *J* = 13.0 Hz, 2H), 1.41 (s, 9H), 1.40 – 1.33 (m, 2H). ^{13}C NMR ($CDCl_3$, 150 MHz) 165.75, 161.24, 155.22, 153.29, 149.70, 145.61, 122.70, 121.44, 120.50 (q, *J* = 255.6 Hz, OCF_3), 102.91, 95.27, 79.35, 48.14, 44.41, 32.06, 28.43. HRMS calculated for $C_{22}H_{26}F_3N_3O_4$ $[M+H]^+$ 454.1954, found 454.1957.

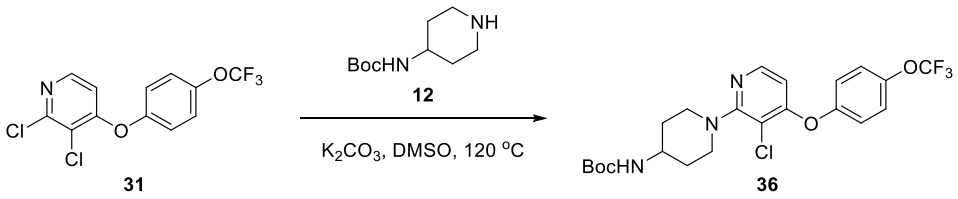
^{1}H NMR of compound 35



^{13}C NMR of compound 35

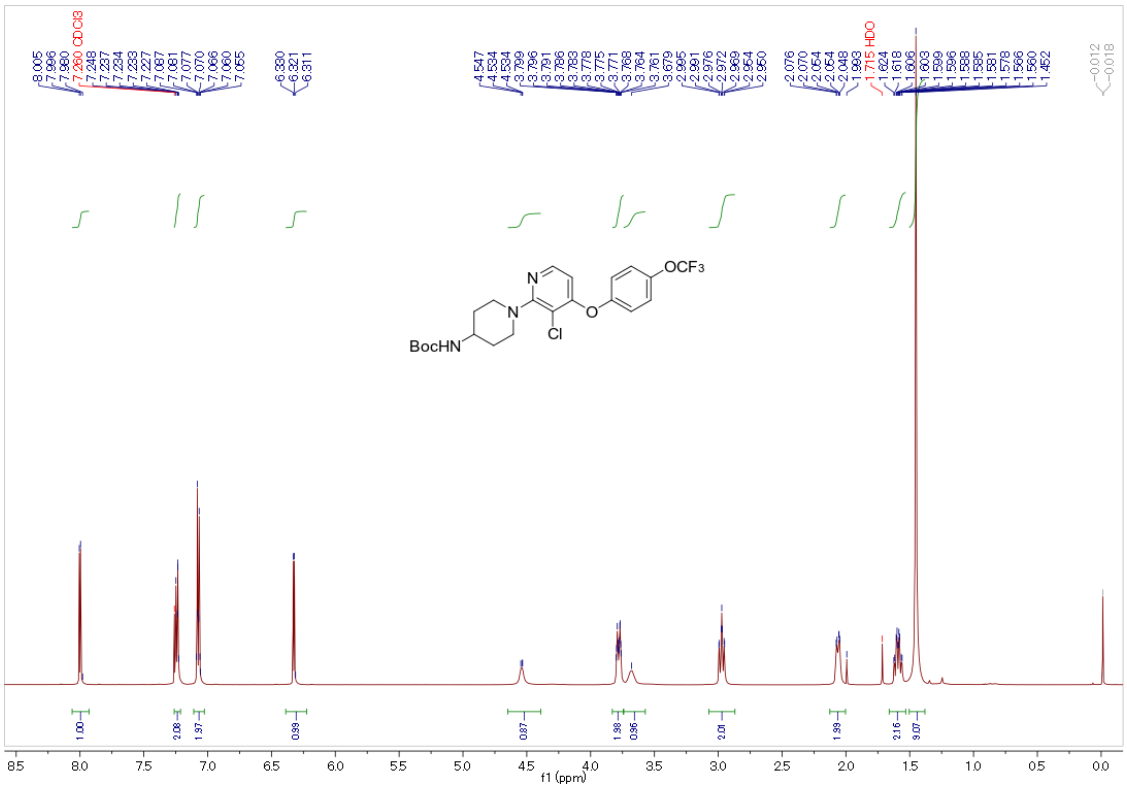


tert-butyl (1-[3-chrolo-4-{4-(trifluoromethoxy)phenoxy}pyridine-2-yl]piperidin-4-yl)carbamate (36)

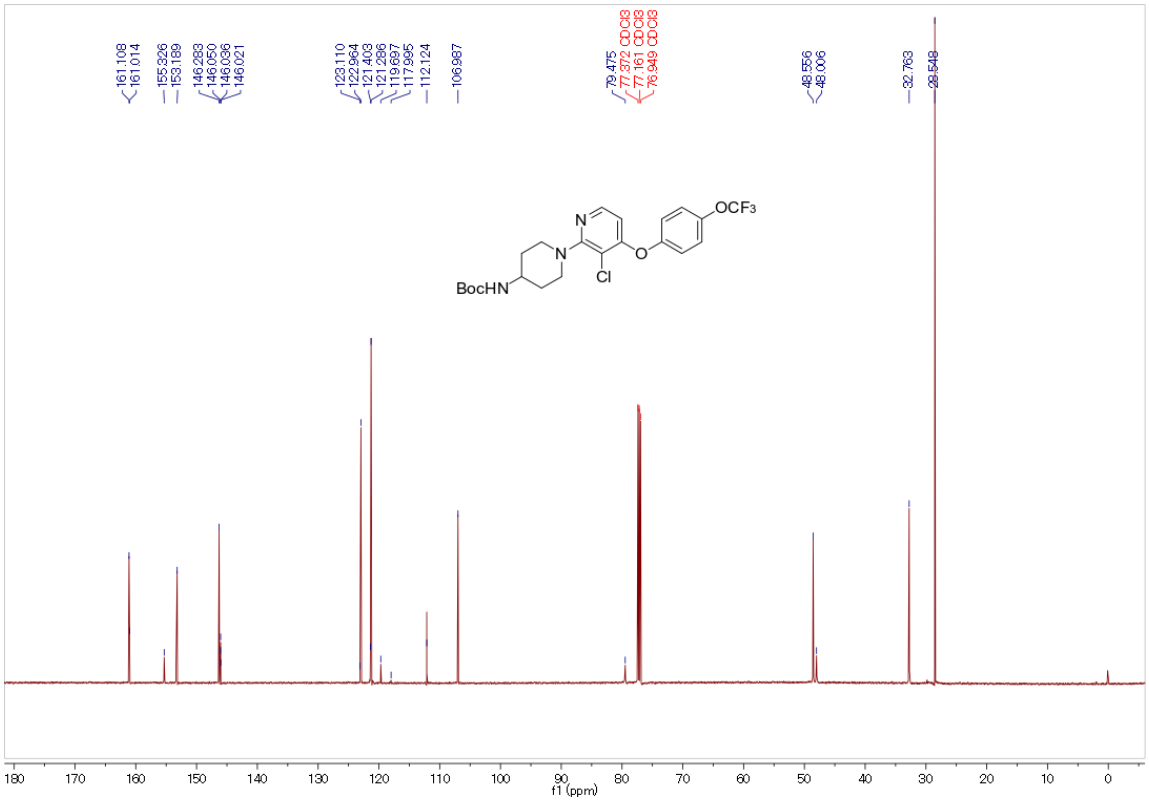


A solution of **31** (700 mg, 2.2 mmol), tert-butyl(piperidin-4-yl)carbamate (**12**) (1.3 g, 6.5 mmol) and potassium carbonate (450 mg, 3.2 mmol) in dry DMSO (1.5 mL) was stirred for 3 h at 120 °C. The mixture was dissolved in DCM and filtered through a pad of silica with ethylacetate:hexane = 1:1. The eluent was concentrated under reduced pressure and the residue was purified by flash column chromatography (hexane only then gradient of ethylacetate:hexane = 1:20 to 1:4) to afford a crude solid of **36** which was recrystallized hexane:ethylacetate to yield **36** (660 mg, 63%) as a white needle. ^{1}H NMR ($CDCl_3$, 600 MHz) δ 8.00 (d, *J* = 5.6 Hz, 1H), 7.24 (d, *J* = 8.5 Hz, 2H), 7.09 – 7.06 (m, 2H), 6.33 (d, J = 5.6 Hz, 1H), 4.56 – 4.51 (m, 1H), 3.78 (d, *J* = 13.0 Hz, 2H), 3.68 (s, 1H), 2.97 (ddd, *J* = 13.4, 11.3, 2.5 Hz, 2H), 2.06 (d, *J* = 12.1 Hz, 2H), 1.59 (dtd, *J* = 12.6, 11.0, 3.9 Hz, 2H), 1.45 (s, 9H). ^{13}C NMR ($CDCl_3$, 150 MHz) 161.11, 161.01, 155.33, 153.19, 146.28, 146.04, 122.96, 121.29, 120.55 (q, *J* = 255.8 Hz, OCF_3), 112.12, 106.99, 79.48, 48.56, 48.01, 32.76, 28.55. HRMS calculated for $C_{22}H_{25}ClF_3N_3O_4$ $[M+H]^+$ 488.1564, found 488.1571.

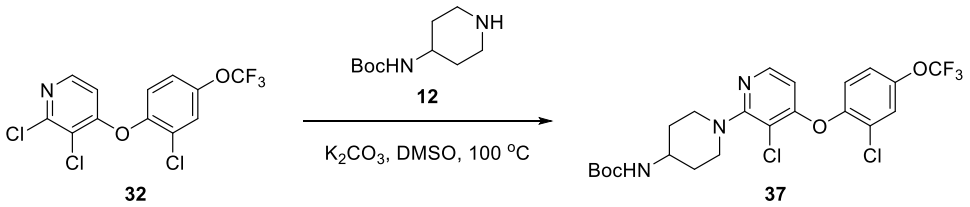
^{1}H NMR of compound 36



^{13}C NMR of compound 36

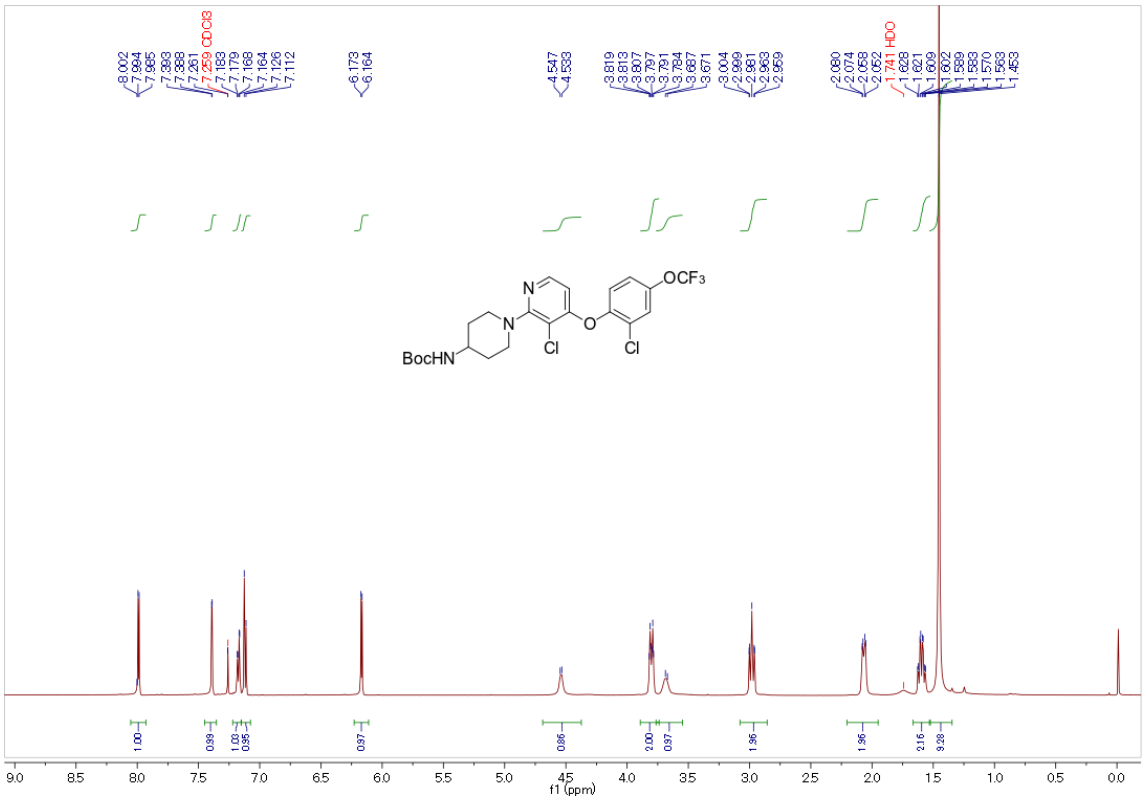


tert-butyl (1-[3-chrolo-4-{2-chloro-4-(trifluoromethoxy)phenoxy}pyridine-2-yl]piperidin-4-yl)carbamate (37)

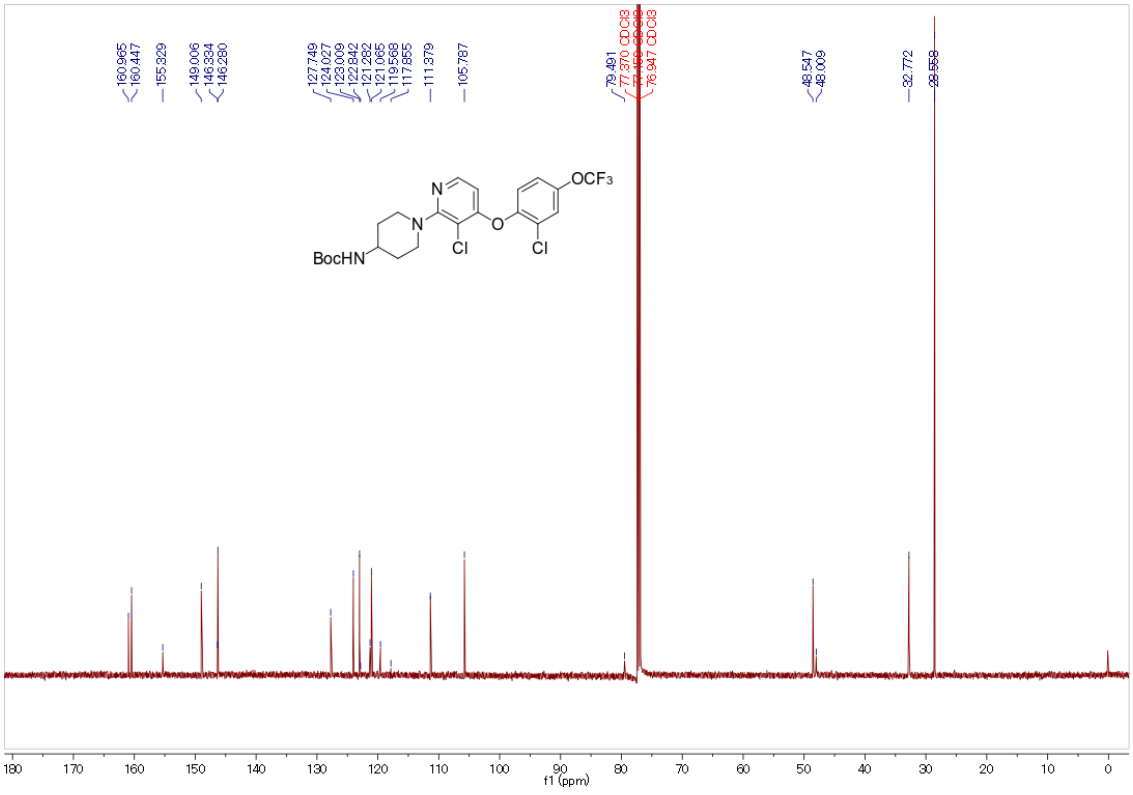


A solution of **32** (600 mg, 1.7 mmol), tert-butyl(piperidin-4-yl)carbamate (**12**) (600 mg, 3.0 mmol) and potassium carbonate (280 mg, 2.0 mmol) in dry DMSO (0.5 mL) was stirred for 3 h at 100 °C. The mixture was dissolved in DCM and filtered through a pad of silica with ethylacetate:hexane = 1:1. The eluent was concentrated under reduced pressure and the residue was purified by flash column chromatography (hexane only then gradient of ethylacetate:hexane = 1:50 to 1:5) to afford **37** (360 mg, 41%) as a white solid. ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.99 (d, *J* = 5.5 Hz, 1H), 7.39 (d, *J* = 2.7 Hz, 1H), 7.17 (dd, *J* = 8.9, 2.7 Hz, 1H), 7.12 (d, *J* = 8.8 Hz, 1H), 6.17 (d, *J* = 5.6 Hz, 1H), 4.54 (d, *J* = 8.0 Hz, 1H), 3.82 – 3.78 (m, 2H), 3.69 (s, 1H), 3.00 – 2.96 (m, 2H), 2.08 – 2.05 (m, 2H), 1.60 (qd, *J* = 11.5, 3.9 Hz, 2H), 1.45 (s, 9H). ^{13}C NMR ($CDCl_3$, 150 MHz) 160.97, 160.45, 155.33, 149.01, 146.33, 146.28, 127.75, 124.03, 123.01, 121.07, 120.42 (q, *J* = 255.3 Hz, OCF_3), 111.38, 105.79, 79.49, 48.55, 48.01, 32.77, 28.56. HRMS calculated for $C_{22}H_{24}Cl_2F_3N_3O_4$ $[M+H]^+$ 522.1174, found 522.1178.

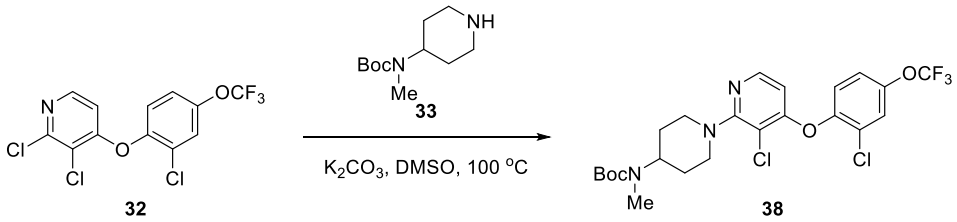
^{1}H NMR of compound 37



^{13}C NMR of compound 37

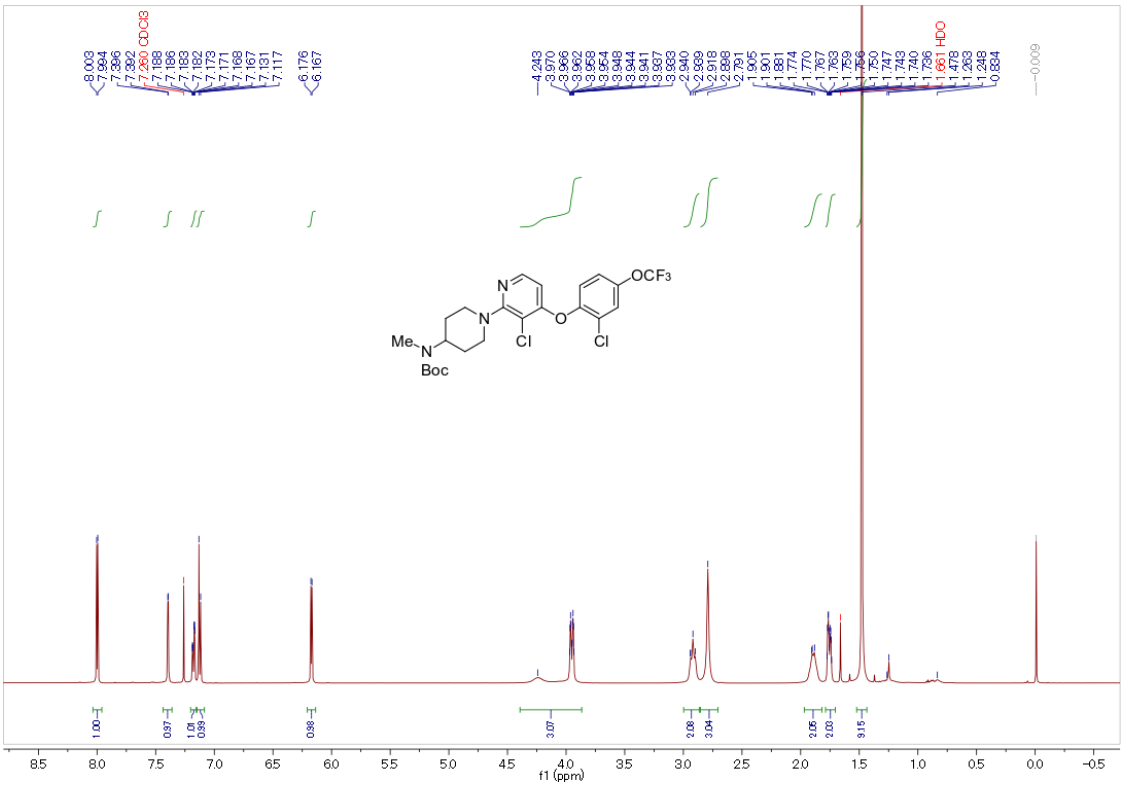


***N*-methyl-tert-butyl (1-[3-chrolo-4-{2-chloro-4-(trifluoromethoxy)phenoxy}pyridine-2-yl]piperidin-4-yl)carbamate (38)**

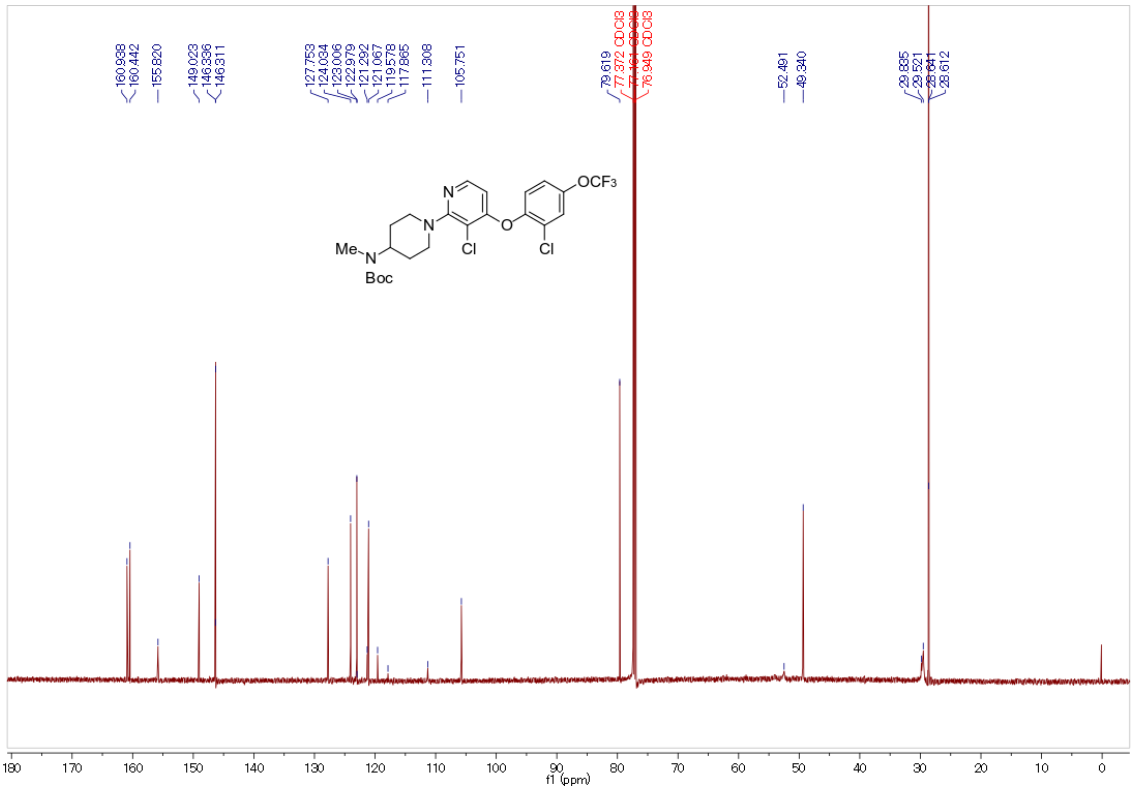


A solution of **32** (100 mg, 0.28 mmol), *N*-methyl-tert-butyl(piperidin-4-yl)carbamate (**33**) (120 mg, 0.56 mmol) and potassium carbonate (46 mg, 0.33 mmol) in dry DMSO (0.2 mL) was stirred for 2 h at 100 °C. The mixture was dissolved in DCM and filtered through a pad of silica with ethylacetate:hexane = 1:1. The eluent was concentrated under reduced pressure and the residue was purified by flash column chromatography (hexane only then gradient of ethylacetate:hexane = 1:50 to 1:3) to afford **38** (59 mg, 39%) as a white solid. ^{1}H NMR ($CDCl_3$, 600 MHz) δ 8.00 (d, *J* = 5.5 Hz, 1H), 7.39 (d, *J* = 2.7 Hz, 1H), 7.19 – 7.17 (m, 1H), 7.12 (d, *J* = 8.9 Hz, 1H), 6.17 (d, *J* = 5.6 Hz, 1H), 4.24 – 3.94 (m, 3H), 2.92 (t, *J* = 11.7 Hz, 2H), 2.79 (s, 3H), 1.92 – 1.86 (m, 2H), 1.76 (ddd, *J* = 11.7, 4.4, 2.0 Hz, 2H), 1.48 (s, 9H). ^{13}C NMR (CDCl3, 150 MHz) 160.94, 160.44, 155.82, 149.02, 146.34, 146.34, 127.75, 124.03, 123.01, 121.07, 120.43 (q, *J* = 255.7 Hz, OCF3), 111.31, 105.75, 79.62, 52.49, 49.34, 29.52, 28.64, 28.61. HRMS calculated for $C_{23}H_{26}Cl_2F_3N_3O_4$ $[M+H]^+$ 536.1331, found 536.1334.

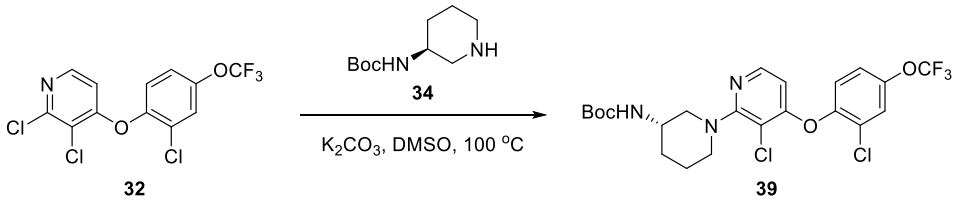
^{1}H NMR of compound 38



^{13}C NMR of compound 38

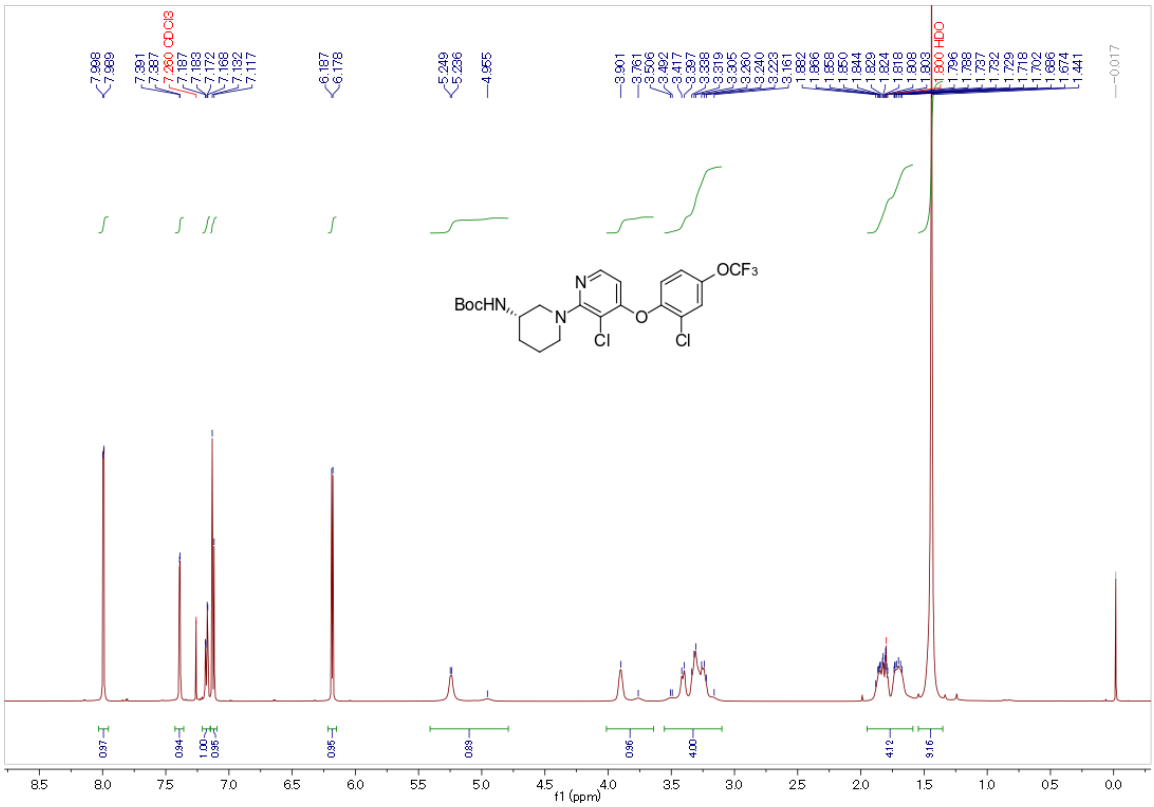


(*S*)-tert-butyl (1-[3-chrolo-4-{2-chloro-4-(trifluoromethoxy)phenoxy}pyridine-2-yl]piperidin-3-yl)carbamate (39)

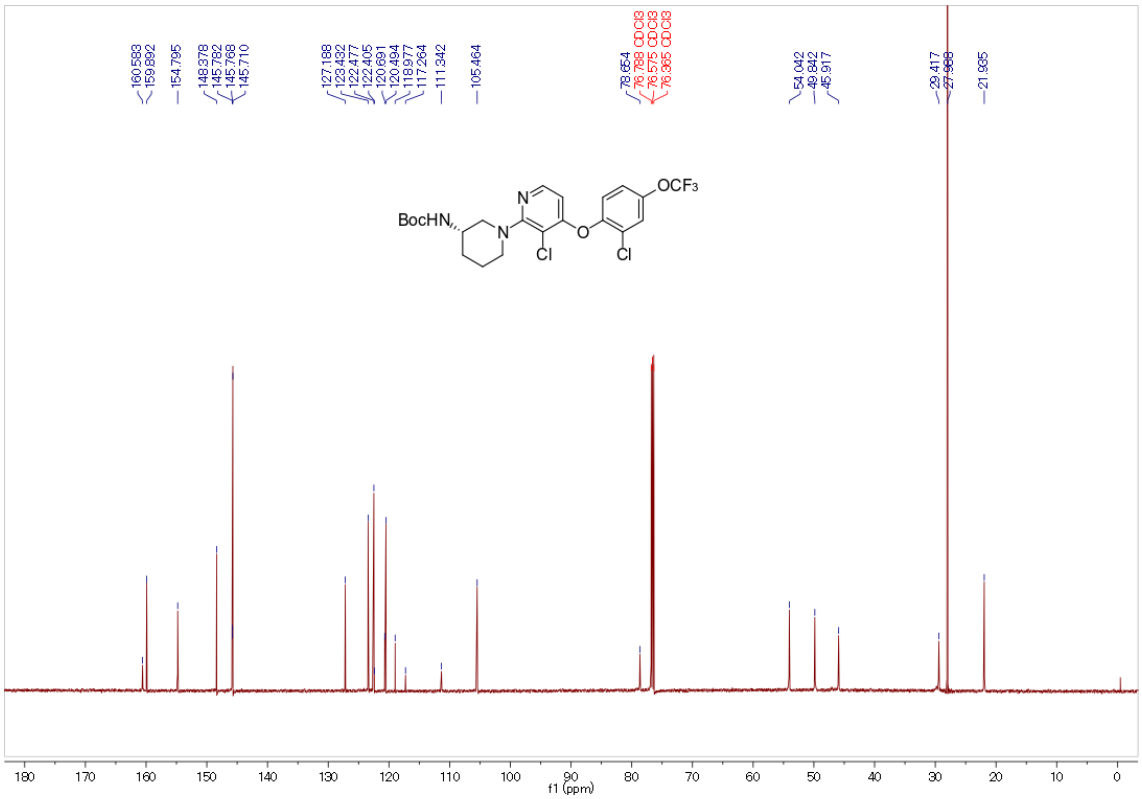


A solution of **32** (1.2 g, 3.4 mmol), (*S*)-tert-butyl(piperidin-3-yl)carbamate (**34**) (1.3 g, 6.1 mmol) and potassium carbonate (560 mg, 4.0 mmol) in dry DMSO (1.0 mL) was stirred for 2 h at 100 °C. The mixture was dissolved in DCM and filtered through a pad of silica with ethylacetate:hexane = 1:1. The eluent was concentrated under reduced pressure and the residue was purified by flash column chromatography (hexane only then gradient of ethylacetate:hexane = 1:50 to 1:3) to afford **39** (790 mg, 45%) as a white solid. ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.99 (d, *J* = 5.6 Hz, 1H), 7.39 (d, *J* = 2.7 Hz, 1H), 7.18 (dd, *J* = 8.9, 2.7 Hz, 1H), 7.12 (d, *J* = 8.9 Hz, 1H), 6.18 (d, *J* = 5.6 Hz, 1H), 5.24 (brs, 1H), 3.90 – 3.75 (s, 1H), 3.50 – 3.16 (m, 4H), 1.87 – 1.67 (m, 4H), 1.44 (s, 9H). ^{13}C NMR ($CDCl_3$, 150 MHz) 160.58, 159.89, 154.80, 148.38, 145.78, 145.70, 127.19, 123.43, 122.48, 120.49, 119.83 (q, *J* = 257.1 Hz, OCF3), 111.34, 105.46, 78.65, 54.04, 49.84, 45.92, 29.42, 27.99, 21.94. HRMS calculated for $C_{22}H_{24}Cl_2F_3N_3O_4$ $[M+H]^+$ 522.1174, found 522.1179.

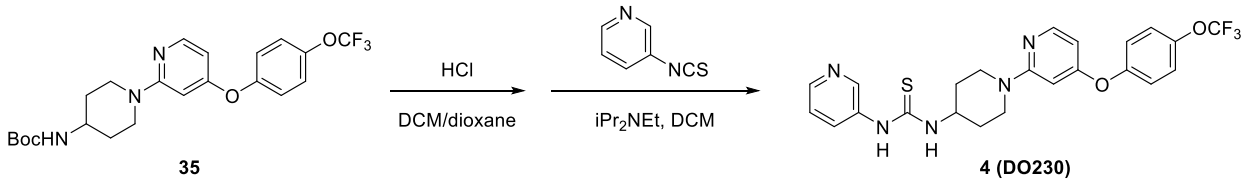
^{1}H NMR of compound 39



^{13}C NMR of compound 39



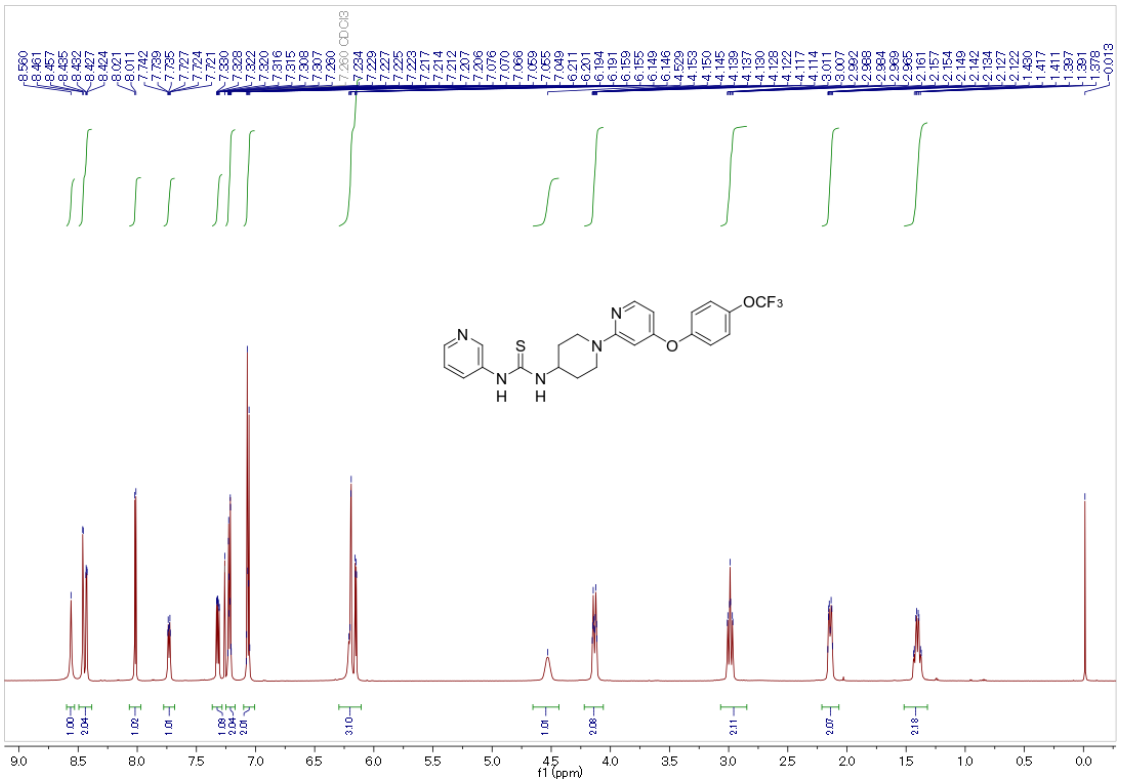
***N*-3-pyridyl-*N'*-(1-[4-{4-(trifluoromethoxy)phenoxy}pyridine-2-yl]piperidin-4-yl)thiourea (DO230)**



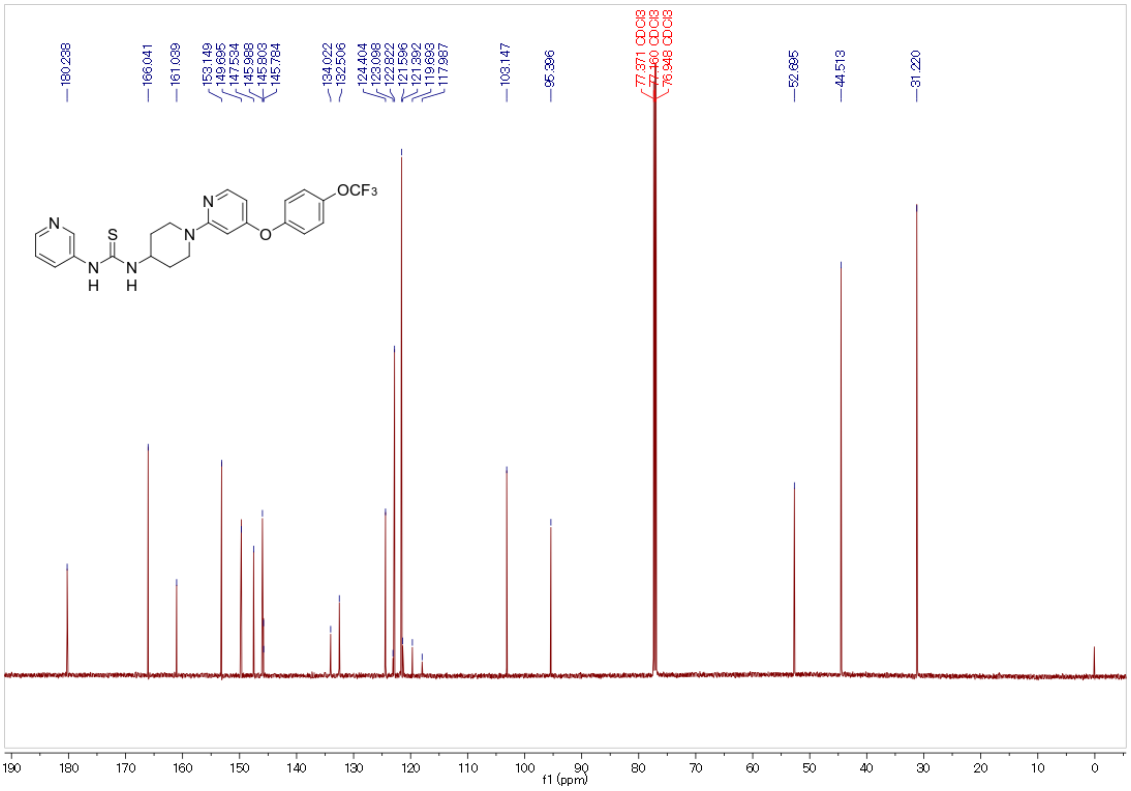
35 **4 (DO230)**

To a solution of **35** (390 mg, 0.86 mmol) in DCM (3.0 mL) was added 4N HCl in dioxane (4.0 mL) in a dropwise fashion and stirred for 1 h at room temperature. The mixture was dried under N_2 stream. The residue was dissolved in DCM (10 mL) with iPr_2NEt (600 µL, 3.5 mmol) and 3-pyridineisothiocyanate (130 mg, 0.95 mmol). The mixture was stirred overnight at room temperature. The mixture was diluted with DCM and washed with sat.$NaHCO_3$. The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by flash column chromatography (DCM only then a gradient of DCM:acetone = 10:1 to 1:1) to afford **DO230** (360 mg, 86%) as a white amorphous. ^{1}H NMR ($CDCl_3$, 600 MHz) δ 8.56 (s, 1H), 8.46 (d, *J* = 2.6 Hz, 1H), 8.43 (dd, *J* = 4.8, 1.5 Hz, 1H), 8.02 (d, *J* = 5.7 Hz, 1H), 7.73 (dt, *J* = 8.6, 1.8 Hz, 1H), 7.32 (ddd, *J* = 8.2, 4.8, 0.7 Hz, 1H), 7.23 – 7.21 (m, 2H), 7.08 – 7.05 (m, 2H), 6.21 (s, 1H), 6.19 (d, *J* = 2.1 Hz, 1H), 6.15 (dd, J = 5.7, 2.0 Hz, 1H), 4.53 (s, 1H), 4.15 – 4.11 (m, 2H), 2.99 (ddd, *J* = 13.9, 11.6, 2.7 Hz, 2H), 2.16 – 2.12 (m, 2H), 1.40 (qd, *J* = 11.7, 4.1 Hz, 2H). ^{13}C NMR ($CDCl_3$, 150 MHz) 180.24, 166.04, 161.04, 153.15, 149.70, 147.53, 145.99, 145.80, 134.02, 132.51, 124.40, 122.82, 121.60, 120.54 (q, *J* = 255.4 Hz, OCF3), 103.15, 95.40, 52.70, 44.51, 31.22. HRMS calculated for $C_{23}H_{22}F_3N_5O_2S$ $[M+H]^+$ 490.1525, found 490.1528.

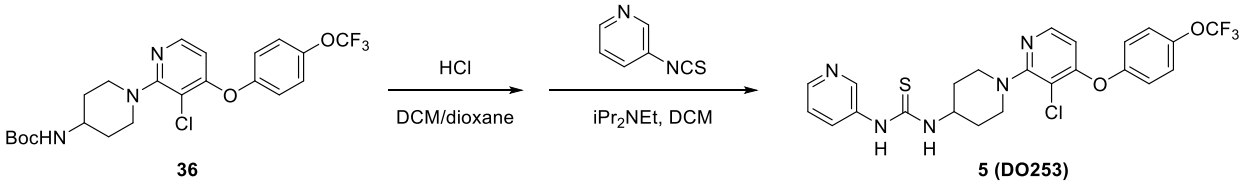
^{1}H NMR of DO230



^{13}C NMR of DO230

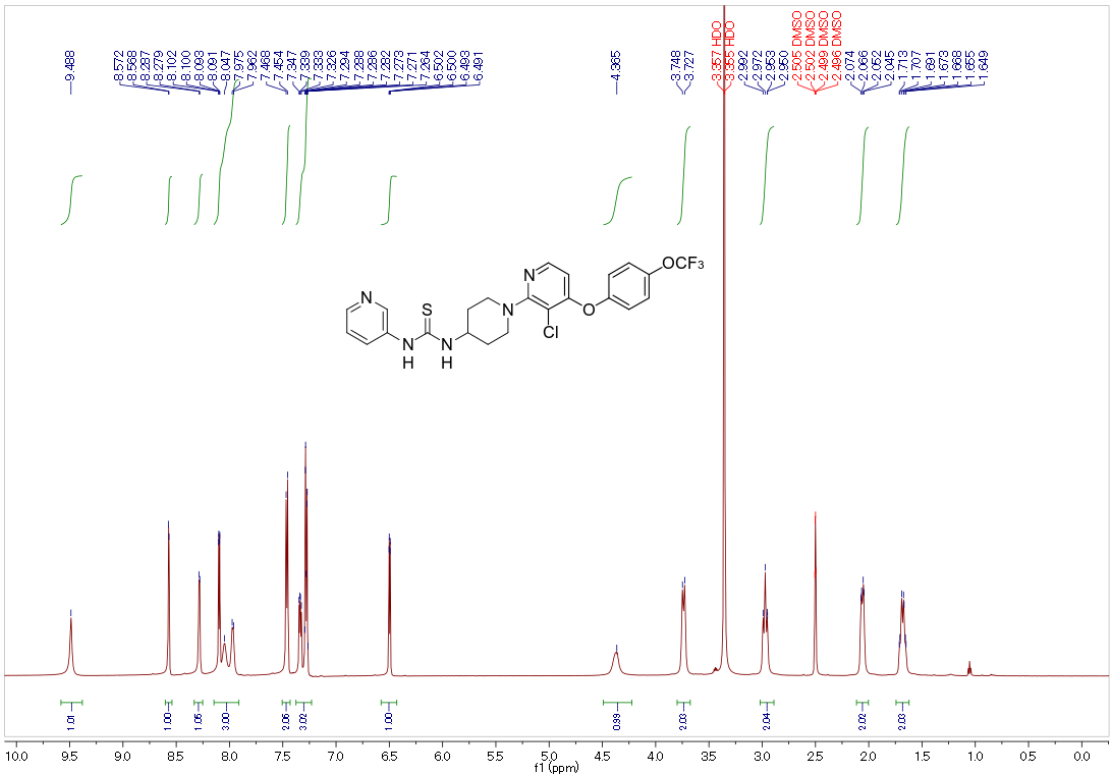


***N*-3-pyridyl-*N'*-(1-[3-chloro-4-{4-(trifluoromethoxy)phenoxy}pyridine-2-yl]piperidin-4-yl)thiourea (DO253)**

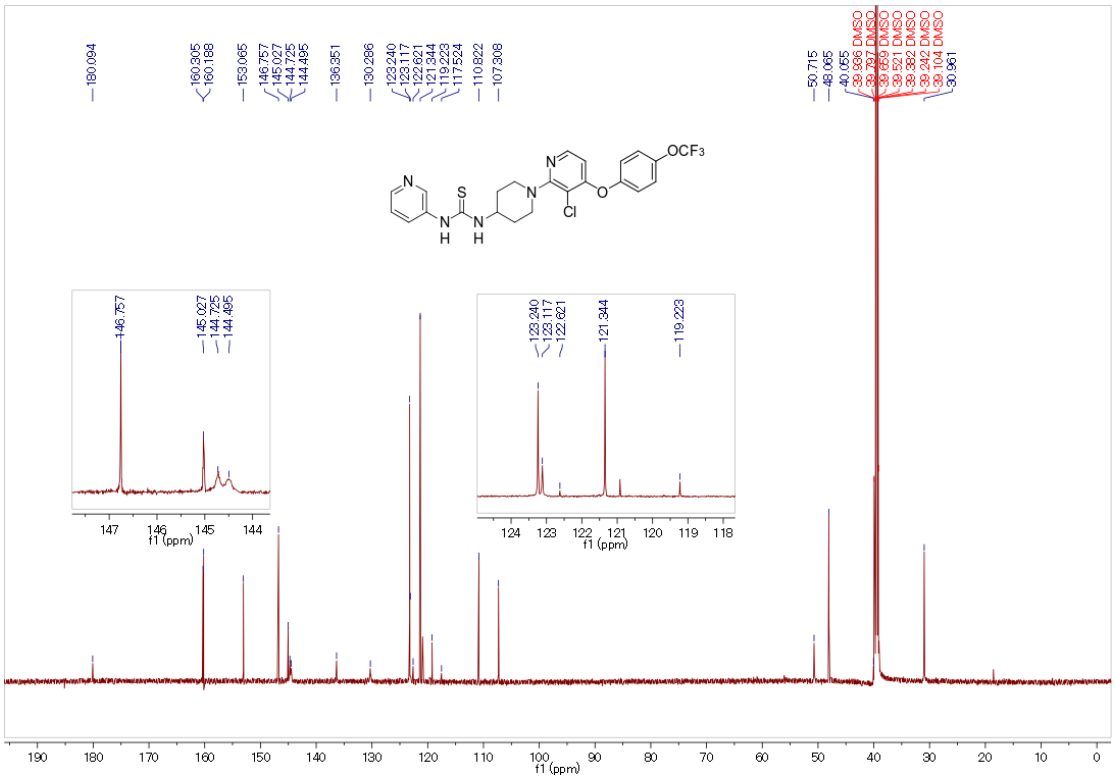


To a solution of **36** (600 mg, 1.2 mmol) in DCM (5.0 mL) was added 4N HCl in dioxane (5.0 mL) in a dropwise fashion and stirred for 1 h at room temperature. The mixture was dried under N_2 stream. The residue was dissolved in DCM (11 mL) with iPr_2NEt (860 µL, 4.9 mmol) and 3-pyridineisothiocyanate (180 mg, 1.35 mmol). The mixture was stirred overnight at room temperature. The mixture was diluted with DCM and washed with sat.$NaHCO_3$. The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by flash column chromatography (DCM only then a gradient of DCM:acetone = 10:1 to 2:1) to afford **DO253** (440 mg, 68%) as a white solid. ^{1}H NMR (DMSO-d6, 600 MHz) δ 9.49 (s, 1H), 8.57 (d, *J* = 2.5 Hz, 1H), 8.28 (d, *J* = 4.7 Hz, 1H), 8.10 (dd, *J* = 5.5, 1.1 Hz, 1H), 8.05 (s, 1H), 7.97 (d, *J* = 7.8 Hz, 1H), 7.46 (d, *J* = 8.5 Hz, 2H), 7.34 (dd, *J* = 8.3, 4.7 Hz, 1H), 7.29 – 7.26 (m, 2H), 6.50 (d, *J* = 5.5, 1H), 4.37 (s, 1H), 3.74 (d, *J* = 12.5 Hz, 2H), 2.97 (t, *J* = 11.8 Hz, 2H), 2.06 (d, *J* = 8.7 Hz, 2H), 1.68 (q, *J* = 10.6, 2H). ^{13}C NMR (DMSO-d6, 150 MHz) 180.09, 160.31, 160.19, 153.07, 146.76, 145.03, 144.72, 144.50, 136.35, 130.31, 123.24, 123.12, 121.34, 120.07 (q, *J* = 254.9 Hz, OCF3), 110.82, 107.31, 50.72, 48.07, 30.96. HRMS calculated for $C_{23}H_{21}ClF_3N_5O_2S$ $[M+H]^+$ 524.1135, found 524.1138.

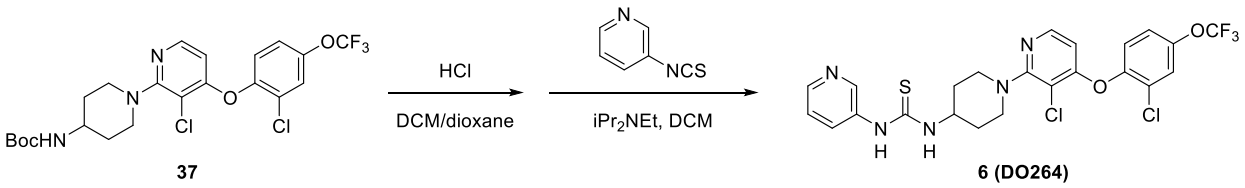
^{1}H NMR of DO253



^{13}C NMR of DO253



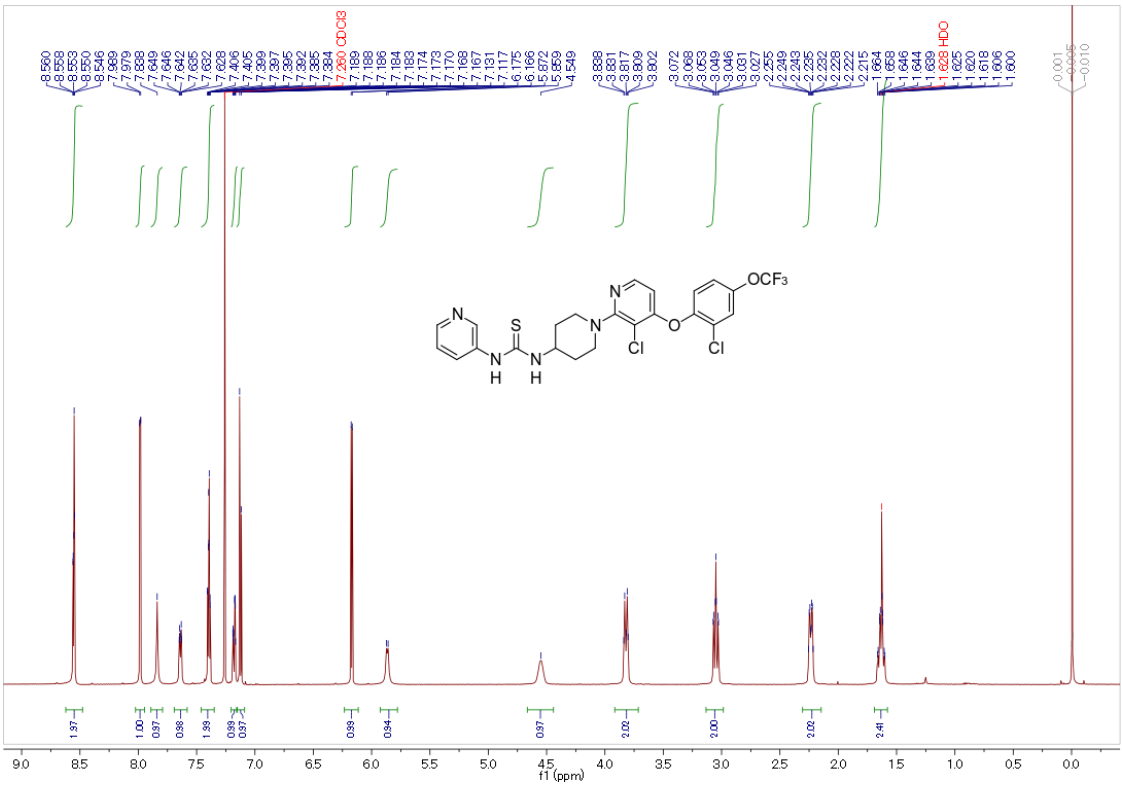
***N*-3-pyridyl-*N'*-(1-[3-chloro-4-{2-chloro-4-(trifluoromethoxy)phenoxy}pyridine-2-yl]piperidin-4-yl)thiourea (DO264)**



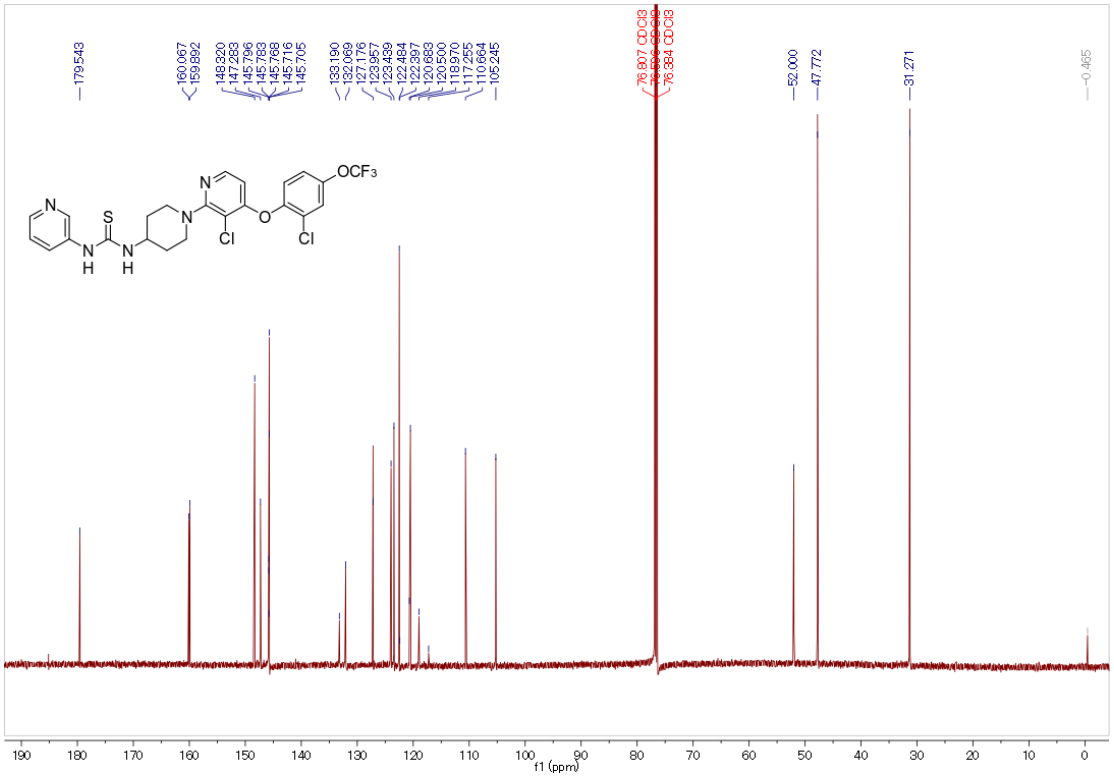
37 **6 (DO264)**

To a solution of **37** (240 mg, 0.46 mmol) in DCM (2.0 mL) was added 4N HCl in dioxane (2.0 mL) in a dropwise fashion and stirred for 1 h at room temperature. The mixture was dried under N_2 stream. The residue was dissolved in DCM (4.7 mL) with iPr_2NEt (320 µL, 1.8 mmol) and 3-pyridineisothiocyanate (69 mg, 0.51 mmol). The mixture was stirred overnight at room temperature. The mixture was diluted with DCM and washed with sat.$NaHCO_3$. The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by flash column chromatography (DCM only then a gradient of DCM:acetone = 50:1 to 1:1) and then recrystallized from MeCN/H_2O to afford **DO264** (195 mg, 76%) as a white solid. ^{1}H NMR ($CDCl_3$, 600 MHz) δ 8.56 – 8.55 (m, 2H), 7.98 (d, *J* = 5.6 Hz, 1H), 7.84 (s, 1H), 7.64 (d, *J* = 8.3 Hz, 0H), 7.41 – 7.38 (m, 2H), 7.18 (ddd, *J* = 9.0, 2.8, 0.9 Hz, 1H), 7.12 (d, *J* = 8.9 Hz, 1H), 6.17 (d, *J* = 5.6 Hz, 1H), 5.87 (d, *J* = 7.9 Hz, 1H), 4.55 (s, 1H), 3.82 (d, *J* = 12.9 Hz, 2H), 3.05 (ddd, *J* = 13.5, 11.4, 2.5 Hz, 2H), 2.26 – 2.22 (m, 2H), 1.66 – 1.60 (m, 2H). ^{13}C NMR ($CDCl_3$, 150 MHz) 179.54, 160.07, 159.89, 148.32, 147.28, 145.78, 145.72, 145.71, 133.19, 132.07, 127.18, 123.96, 123.44, 122.48, 120.50, 119.83 (q, *J* = 257.1 Hz, OCF3), 110.66, 105.25, 52.00, 47.77, 31.27. HRMS calculated for $C_{23}H_{20}Cl_2F_3N_5O_2S$ $[M+H]^+$ 558.0745, found 558.0751.

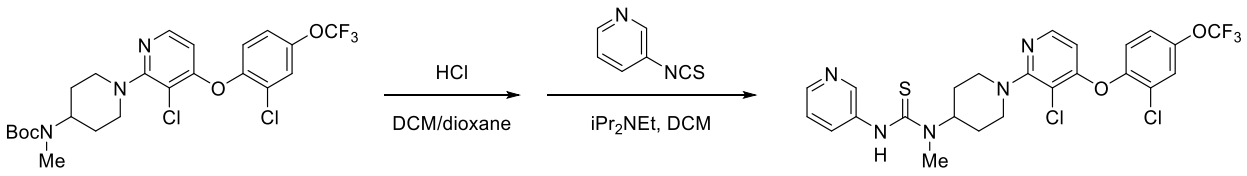
^{1}H NMR of DO264



^{13}C NMR of DO264



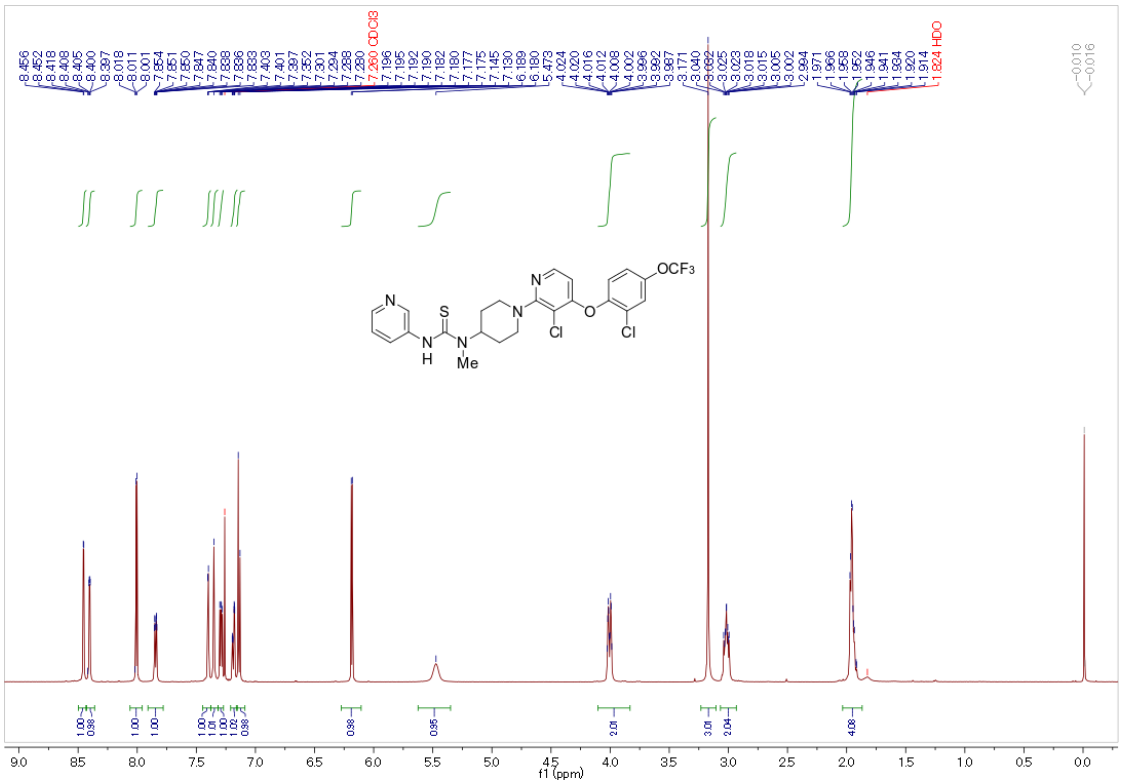
***N*-3-pyridyl-*N'*-methyl-*N'*-(1-[3-chloro-4-{2-chloro-4-(trifluoromethoxy)phenoxy}pyridine-2-yl]piperidin-4-yl)thiourea (DO276)**



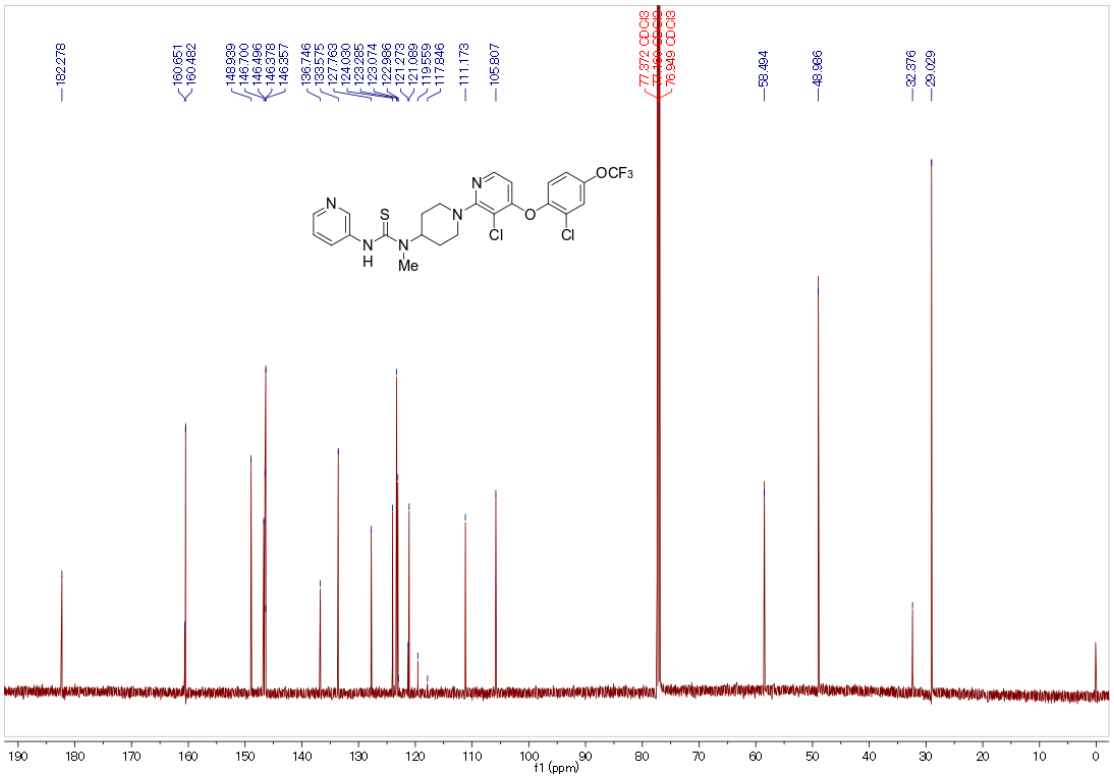
38 **7 (DO276)**

To a solution of **38** (59 mg, 0.11 mmol) in DCM (0.8 mL) was added 4N HCl in dioxane (0.8 mL) in a dropwise fashion and stirred for 1 h at room temperature. The mixture was dried under N_2 stream. The residue was dissolved in DCM (1.0 mL) with iPr_2NEt (76 µL, 0.44 mmol) and 3-pyridineisothiocyanate (16 mg, 0.12 mmol). The mixture was stirred overnight at room temperature. The mixture was diluted with DCM and washed with sat.$NaHCO_3$. The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by preparative TLC (ethylacetate:MeCN = 40:1) to afford **DO276** (33 mg, 52%) as a colorless amorphous. ^{1}H NMR ($CDCl_3$, 600 MHz) δ 8.45 (d, *J* = 2.6 Hz, 1H), 8.40 (dd, *J* = 4.7, 1.5 Hz, 1H), 8.01 (d, *J* = 5.6 Hz, 1H), 7.84 (ddd, *J* = 8.2, 2.6, 1.5 Hz, 1H), 7.40 (d, *J* = 2.6 Hz, 1H), 7.35 (s, 1H), 7.29 (dd, *J* = 8.2, 4.7 Hz, 1H), 7.19 (ddd, *J* = 8.9, 2.8, 1.0 Hz, 1H), 7.14 (d, *J* = 8.9 Hz, 1H), 6.18 (d, *J* = 5.6 Hz, 1H), 5.47 (s, 1H), 4.02 – 3.99 (m, 2H), 3.17 (s, 3H), 3.04 – 2.99 (m, 2H), 1.97 – 1.91 (m, 4H). ^{13}C NMR ($CDCl_3$, 150 MHz) 182.28, 160.65, 160.48, 148.94, 146.70, 146.50, 146.38, 146.36, 136.75, 133.58, 127.76, 124.03, 123.29, 123.07, 121.09, 120.42 (q, *J* = 257.0 Hz, OCF3), 111.17, 105.81, 58.49, 48.99, 33.38, 29.03. HRMS calculated for $C_{24}H_{22}Cl_2F_3N_5O_2S$ $[M+H]^+$ 572.0902, found 572.0906.

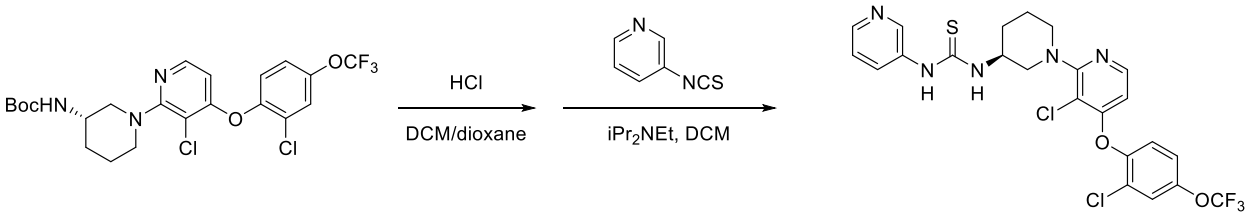
^{1}H NMR of DO276



^{13}C NMR of DO276



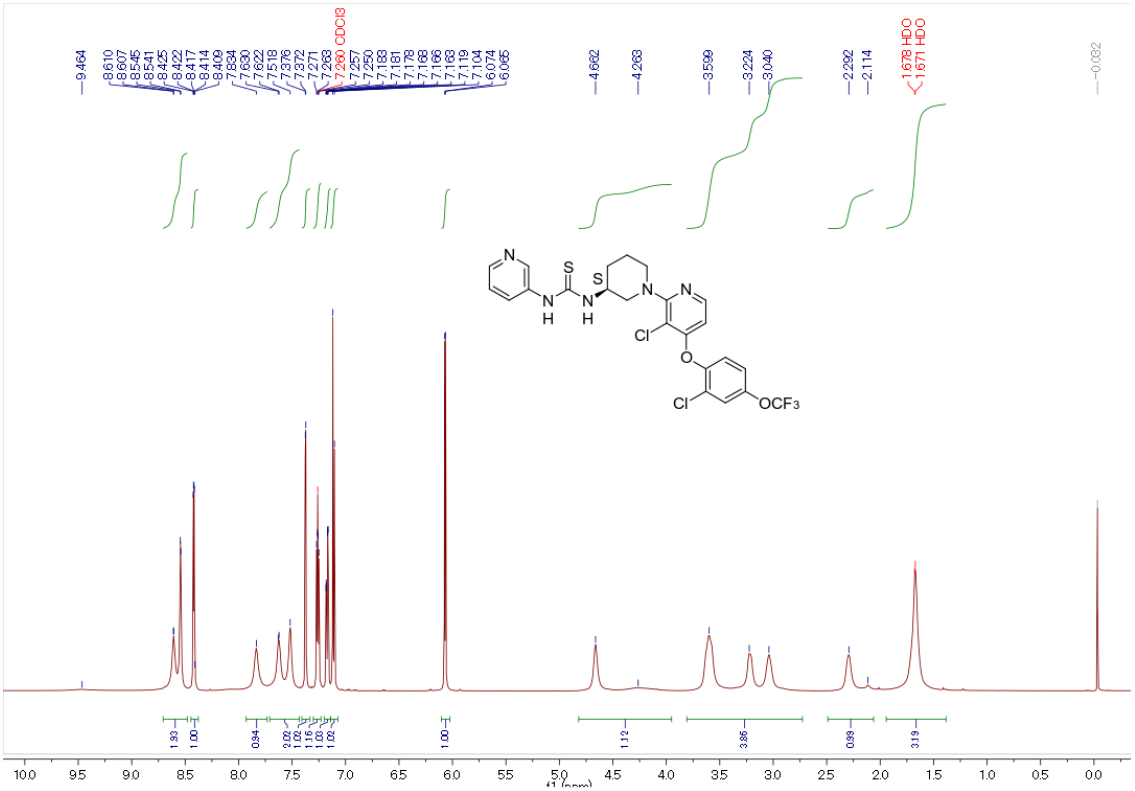
(*S*)-*N*-3-pyridyl-*N'*-(1-[3-chloro-4-{2-chloro-4-(trifluoromethoxy)phenoxy}pyridine-2-yl]piperidin-3-yl)thiourea ((S)-DO271)



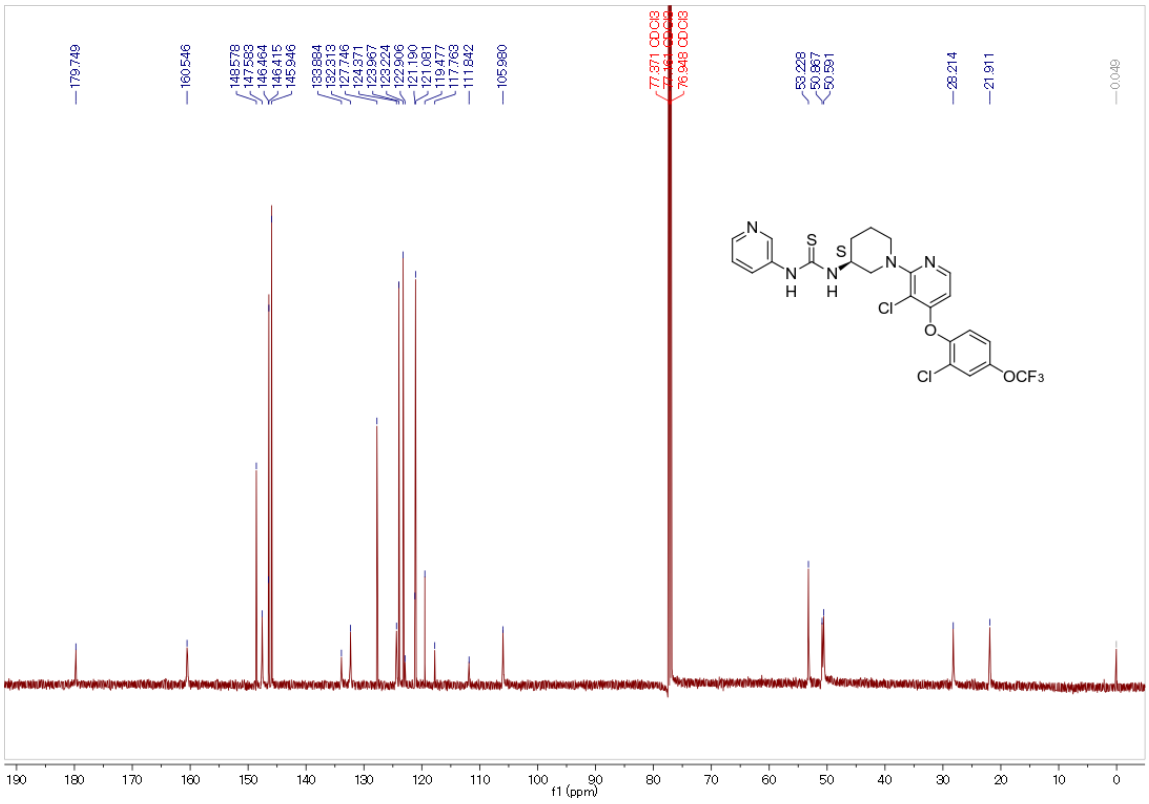
39 **9 ((S)-DO271)**

To a solution of **39** (520 mg, 1.0 mmol) in DCM (4.0 mL) was added 4N HCl in dioxane (4.0 mL) in a dropwise fashion and stirred for 1 h at room temperature. The mixture was dried under N_2 stream. The residue was dissolved in DCM (12.0 mL) with iPr_2NEt (700 µL, 4.0 mmol) and 3-pyridineisothiocyanate (150 mg, 1.1 mmol). The mixture was stirred overnight at room temperature. The mixture was diluted with DCM and washed with sat.$NaHCO_3$. The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by flash column chromatography (ethylacetate only then a gradient of ethylacetate:MeCN = 25:1 to 20:3) to afford **(S)-DO271** (480 mg, 86%, ee = 99.8% see HPLC analysis below) as a colorless amorphous. ^{1}H NMR ($CDCl_3$, 600 MHz) δ 8.61 (s, 1H), 8.55 (s, 1H), 8.42 (dd, *J* = 4.7, 1.5 Hz, 1H), 7.83 (s, 1H), 7.62 (s, 1H), 7.52 (s, 1H), 7.37 (d, *J* = 2.7 Hz, 1H), 7.26 (dd, *J* = 8.1, 4.8 Hz, 1H), 7.17 (dd, *J* = 8.9, 2.2 Hz, 1H), 7.11 (d, *J* = 8.9 Hz, 1H), 6.07 (d, *J* = 5.6 Hz, 1H), 4.66 – 4.20 (m, 1H), 3.60 – 3.04 (m, 4H), 2.29 – 2.11 (m, 1H), 1.67 (s, 3H). ^{13}C NMR ($CDCl_3$, 150 MHz) 179.75, 160.55, 160.44, 148.58, 147.58, 146.46, 146.42, 145.95, 133.88, 132.31, 127.75, 124.37, 124.00, 123.22, 121.08, 120.33 (q, *J* = 257.2 Hz, OCF3), 111.84, 105.98, 53.23, 50.87, 50.59, 28.21, 21.91. HRMS calculated for $C_{23}H_{20}Cl_2F_3N_5O_2S$ $[M+H]^+$ 558.0745, found 558.0757.

^{1}H NMR of (S)-DO271



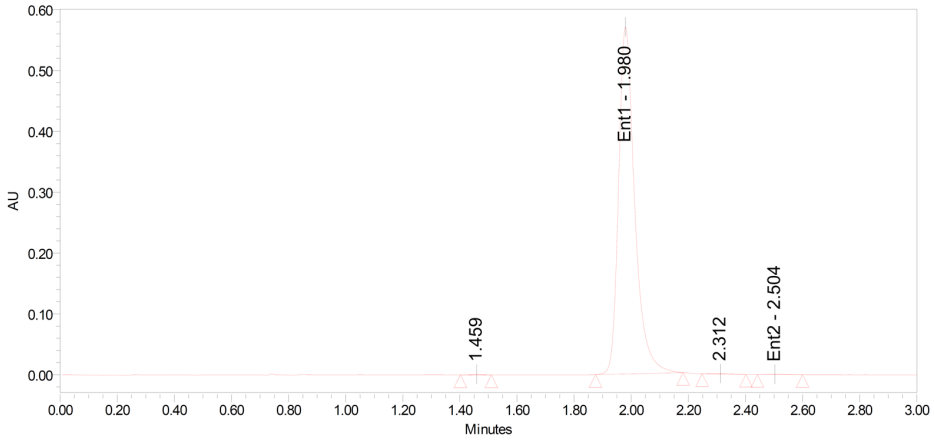
^{13}C NMR of (S)-DO271



HPLC analysis of enantiopurity of (S)-DO271

A separation of the enantiomes of DO271 were performed by chiral SFC on a Daicel IBN column (3 mm, 4.6x250 mm) under isocratic conditions [40% MeOH / CO2 (4 mL/min), 1600 psi backpressure] at 30 °C. The enantiomers were detected by UV light (257 nm).

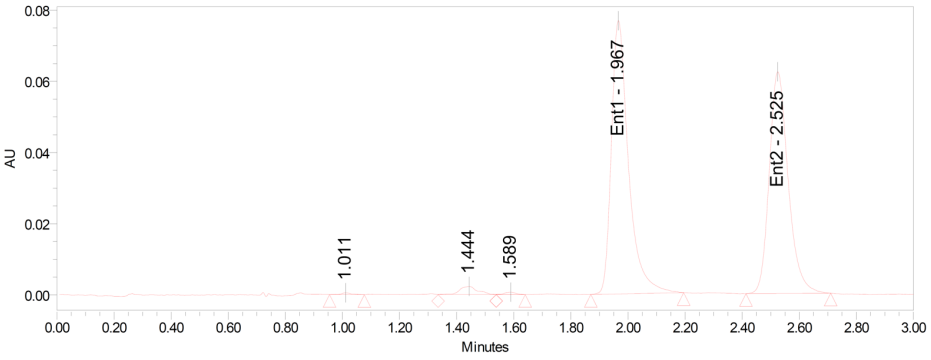
A. HPLC chromatogram of (S)-DO271



Area Summarized by Name

	SampleName	ent1	ent2	ee	Ent1	Ent2
1	(S)-DO271	99.89	0.11	99.77	2237686	2557

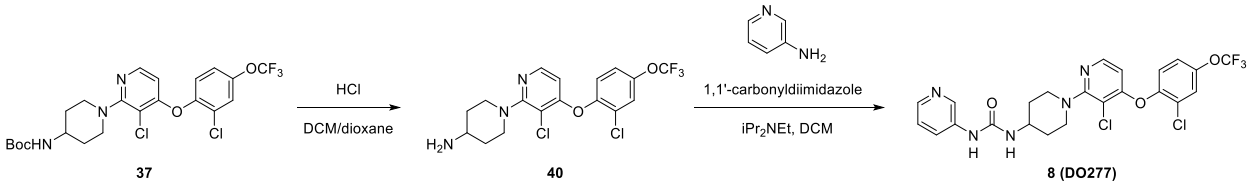
B. HPLC chromatogram of (S,R)-DO271



Area Summarized by Name

	SampleName	ent1	ent2	ee	Ent1	Ent2
1	(S,R)-DO271	52.68	47.32	5.36	319375	286903

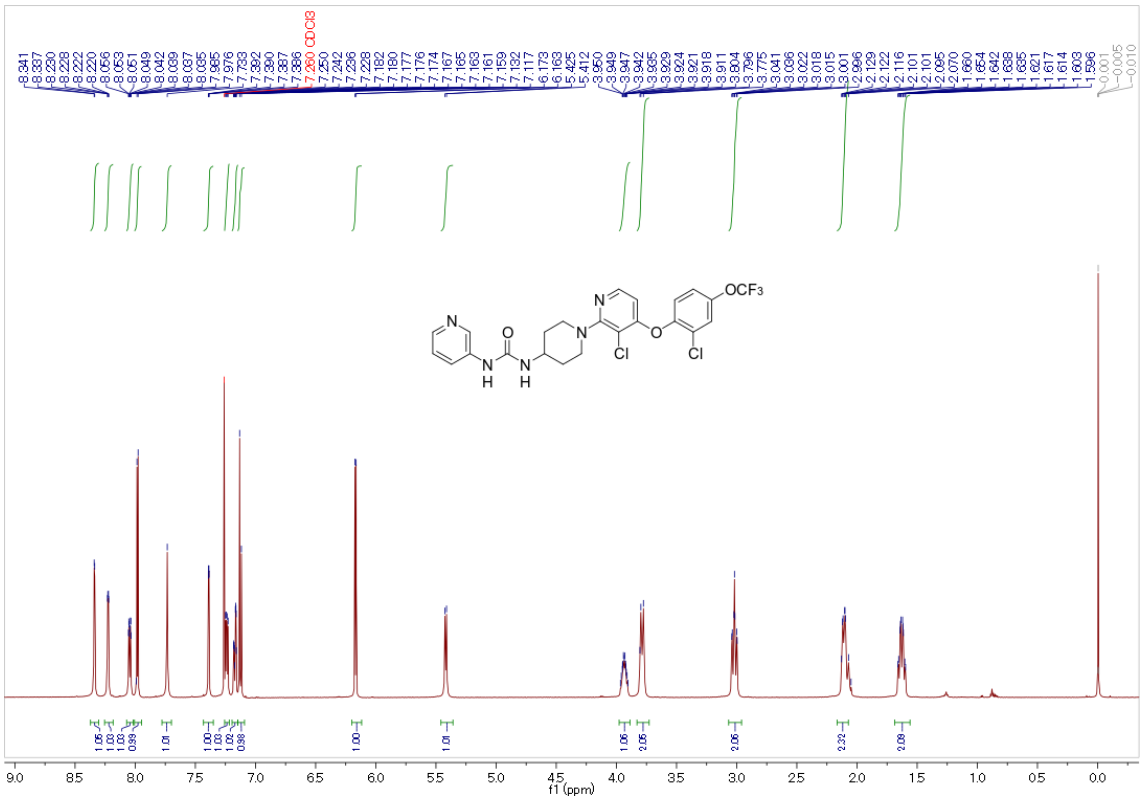
***N*-3-pyridyl-*N'*-(1-[3-chloro-4-{2-chloro-4-(trifluoromethoxy)phenoxy}pyridine-2-yl]piperidin-4-yl)urea (DO277)**



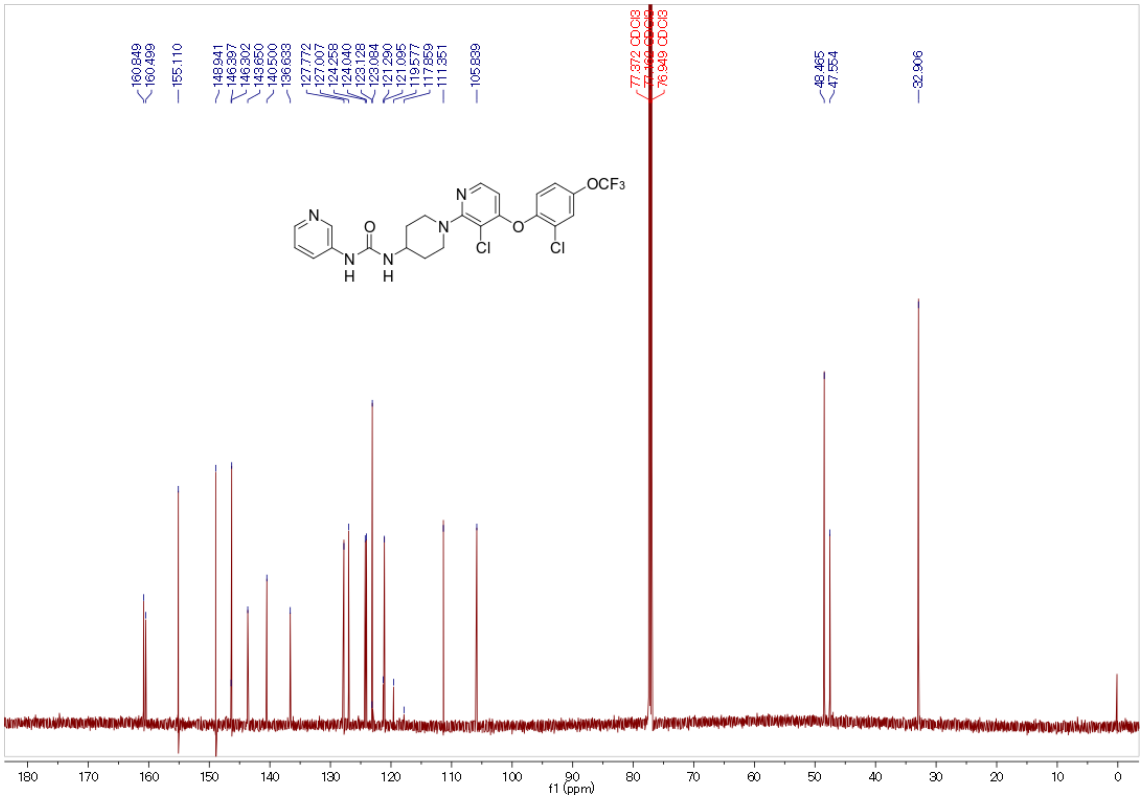
Step 1. To a solution of **37** (135 mg, 0.26 mmol) in DCM (1.0 mL) was added 4N HCl in dioxane (1.0 mL) in a dropwise fashion and stirred for 1 h at room temperature. The mixture was dried under N_2 stream. The residue was dissolved in DCM (12.0 mL) and washed with sat.$NaHCO_3$. The water layer was extracted with DCM (X2) and the combined organic layer was dried over Na_2SO_4 and concentrated under reduced pressure to afford **40** which was used in the next step without further purification.

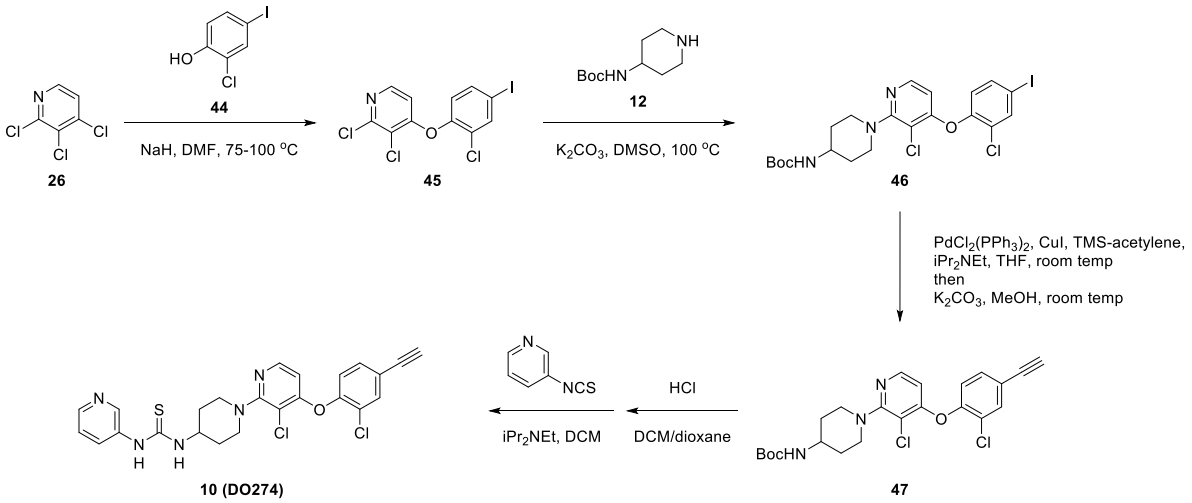
Step 2. To a solution of 3-aminopyridine (49 mg, 0.52 mmol) and iPr_2NEt (90 µL, 0.52 mmol) in DCM (0.5 mL) was added 1,1'-carbonyldiimidazole (100 mg, 0.62 mmol) and stirred for 3 h at room temperature. **40** (109 mg, 0.26 mmol) and iPr_2NEt (140 µL, 0.78 mmol) were added to the mixture and stirred for 17 h at room temperature. The mixture was diluted with DCM and washed with sat.$NaHCO_3$ and sat.NH_4Cl. The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by preparative TLC (DCM:MeOH = 15:1) to afford crude mixture of **DO277** which was recrystallized from hexane:ethylacetate to yield **DO277** (31 mg, 22%) as a white solid. ^{1}H NMR ($CDCl_3$, 600 MHz) δ 8.34 (d, *J* = 2.6 Hz, 1H), 8.23 (dd, *J* = 4.8, 1.5 Hz, 1H), 8.05 (ddd, *J* = 8.4, 2.7, 1.5 Hz, 1H), 7.98 (d, *J* = 5.6 Hz, 1H), 7.73 (s, 1H), 7.39 (d, *J* = 2.5 Hz, 1H), 7.24 (dd, *J* = 8.4, 4.7 Hz, 1H), 7.17 (ddd, *J* = 8.9, 2.7, 1.0 Hz, 1H), 7.12 (d, *J* = 8.9 Hz, 1H), 6.17 (d, *J* = 5.6 Hz, 1H), 5.42 (d, *J* = 7.8 Hz, 1H), 3.97 – 3.90 (m, 1H), 3.79 (d, *J* = 12.7 Hz, 2H), 3.02 (ddd, *J* = 13.3, 11.1, 2.5 Hz, 2H), 2.13 – 2.07 (m, 2H), 1.66 – 1.59 (m, 2H). ^{13}C NMR ($CDCl_3$, 150 MHz) 160.85, 160.50, 155.11, 148.94, 146.40, 146.30, 143.65, 140.50, 136.63, 127.77, 127.01, 124.26, 124.04, 123.08, 121.10, 120.43 (q, *J* = 257.3 Hz, OCF3), 111.35, 105.84, 48.47, 47.55, 32.91. HRMS calculated for $C_{23}H_{20}Cl_2F_3N_5O_3$ $[M+H]^+$ 542.0974, found 542.0984.

^{1}H NMR of DO277



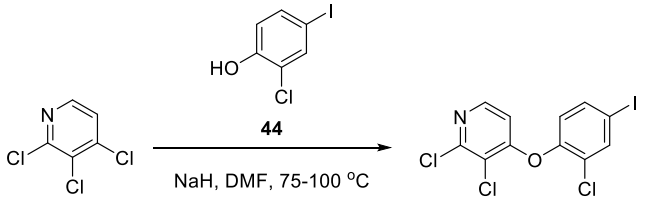
^{13}C NMR of DO277





Synthesis of DO274.

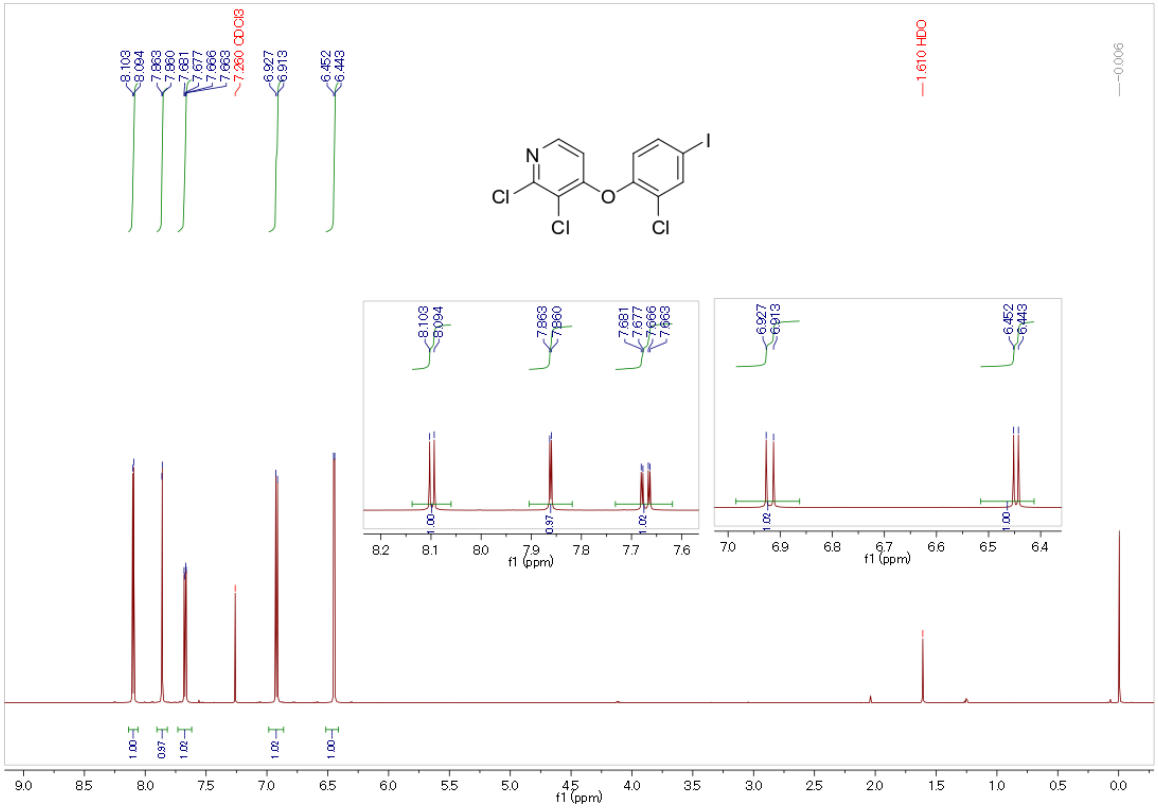
2,3-dichrolo-4-{2-chloro-4-(iodo)phenoxy}pyridine (45)



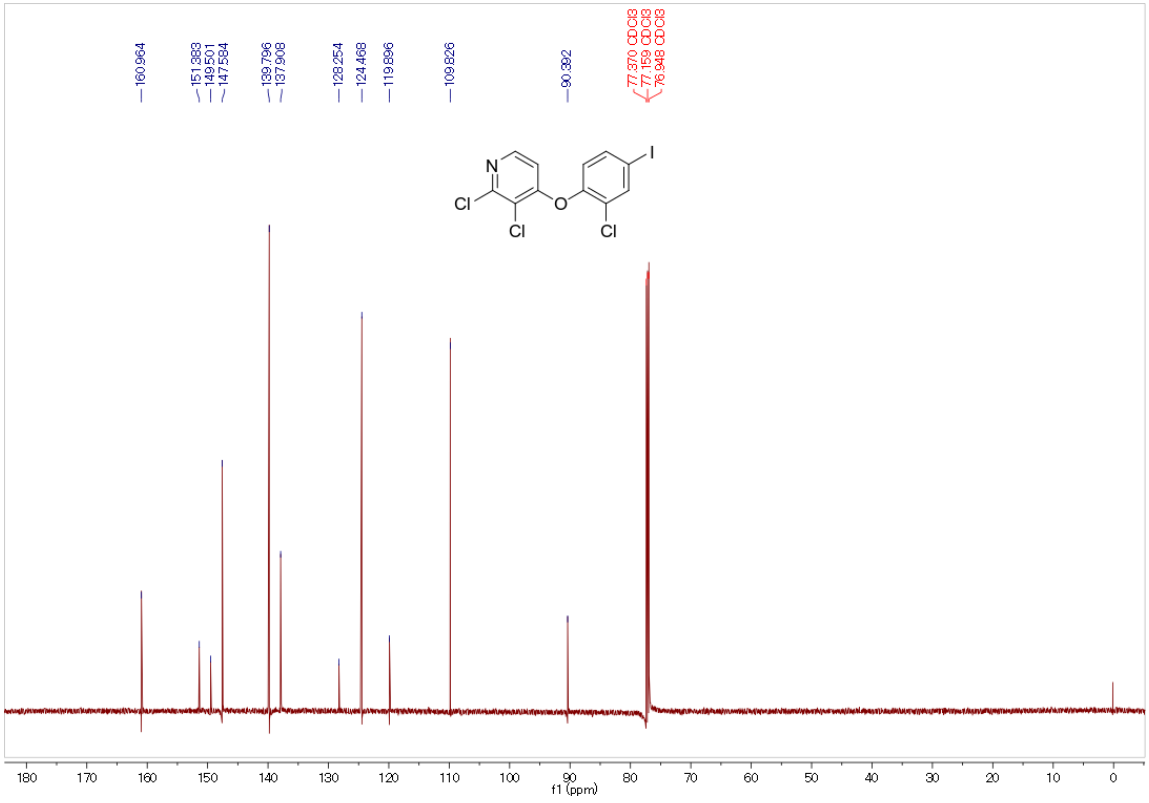
26 45

To a solution of 2-chloro-4-iodophenol (**44**) (710 mg, 2.8 mmol) in dry DMF (2.4 mL) was slowly added 60% sodium hydride in mineral oil (110 mg, 2.8 mmol) at 0 °C. The mixture was warmed to room temperature and stirred for 20 min. The reaction mixture was added 2,3,4-trichloropyridine (**26**) (500 mg, 2.8 mmol). The reaction mixture was stirred for 30 min at 75 °C and further stirred for 1.5 h at 100 °C. The mixture was concentrated under reduced pressure and the residue was purified by flash column chromatography (hexane only then gradient of ethylacetate:hexane = 1:100 to 1:5) to afford **45** (860 mg, 78%) as a colorless amorphous. ^{1}H NMR ($CDCl_3$, 600 MHz) δ 8.10 (d, *J* = 5.5 Hz, 1H), 7.86 (d, *J* = 2.1 Hz, 1H), 7.67 (dd, *J* = 8.5, 2.1 Hz, 1H), 6.92 (d, *J* = 8.5 Hz, 1H), 6.45 (d, *J* = 5.6 Hz, 1H). ^{13}C NMR ($CDCl_3$, 150 MHz) 160.96, 151.38, 149.50, 147.58, 139.80, 137.91, 128.25, 124.47, 119.90, 109.83, 90.39. HRMS calculated for $C_{11}H_6Cl_3INO$ $[M+H]^+$ 399.8560, found 399.8566.

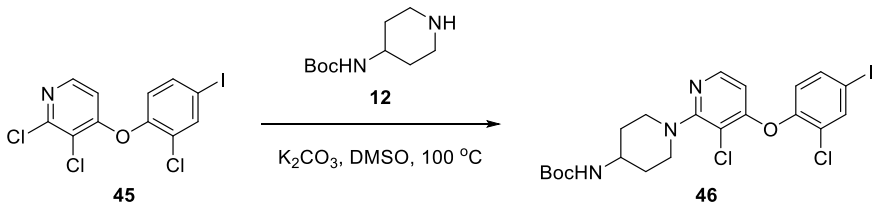
^{1}H NMR of compound 45



^{13}C NMR of compound 45



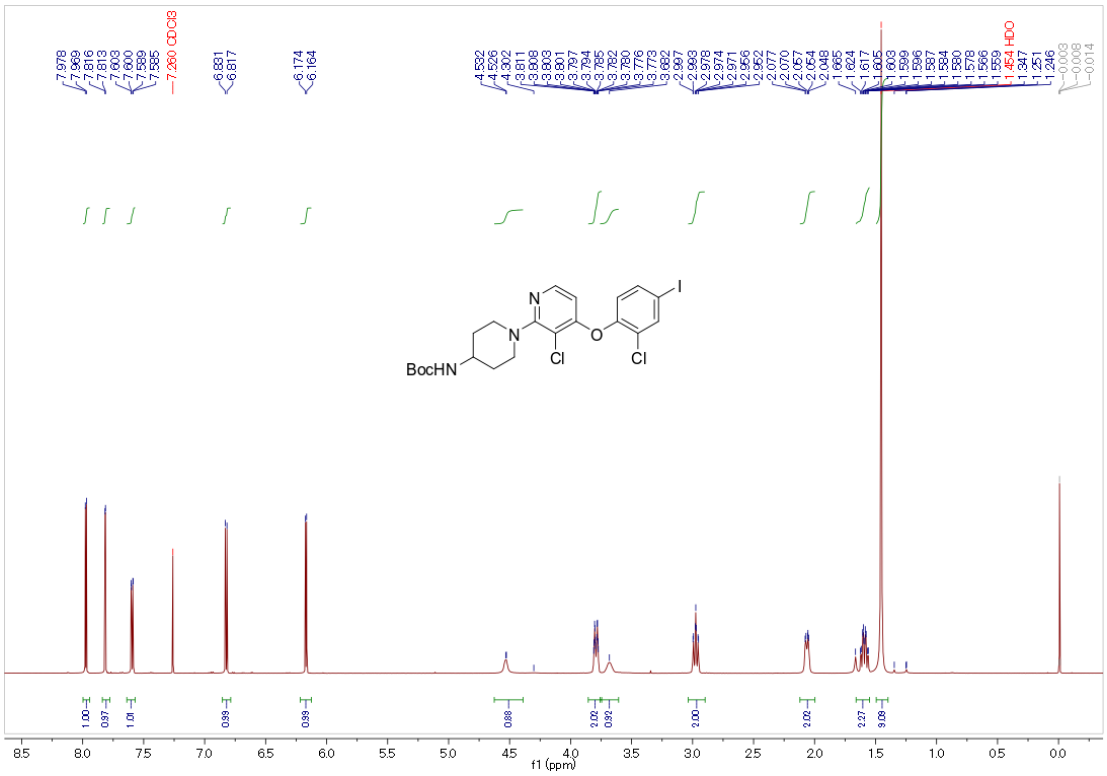
tert-butyl (1-[3-chrolo-4-{2-chloro-4-(iodo)phenoxy}pyridine-2-yl]piperidin-4-yl)carbamate (46)



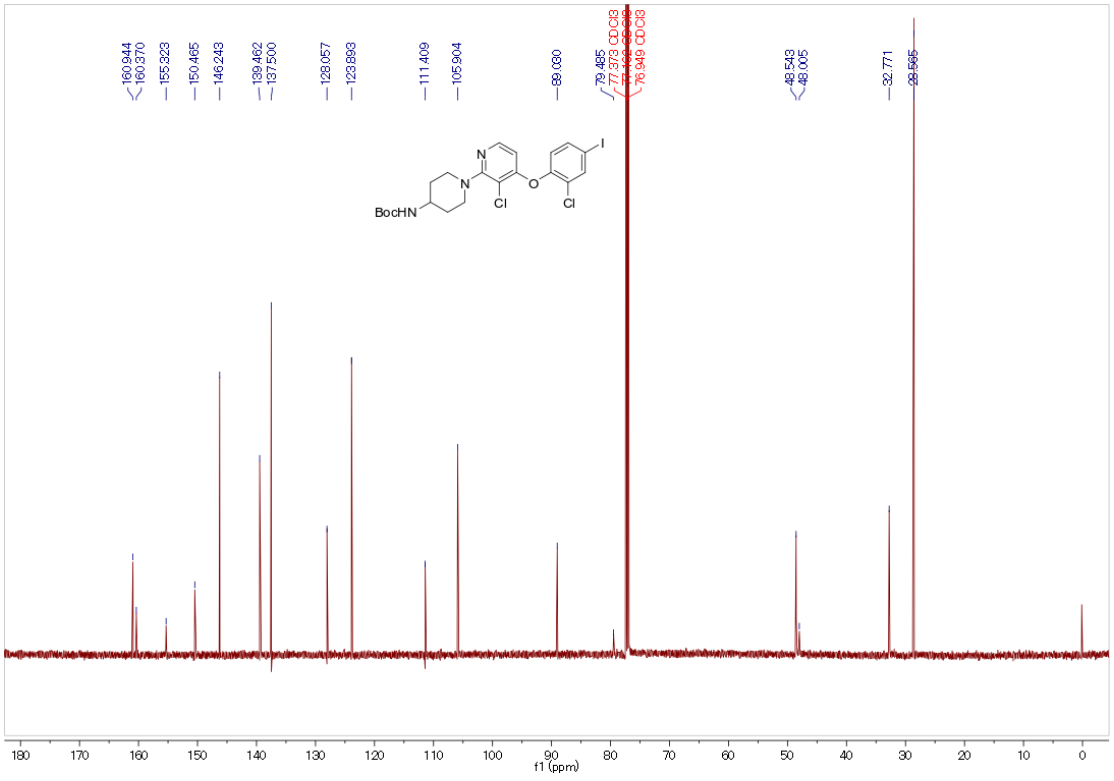
45 46

A solution of **45** (200 mg, 0.50 mmol), tert-butyl(piperidin-4-yl)carbamate (**12**) (200 mg, 1.0 mmol) and potassium carbonate (83 mg, 0.60 mmol) in dry DMSO (0.5 mL) was stirred for 3 h at 100 °C. The mixture was dissolved in DCM and filtered through a pad of silica with ethylacetate:hexane = 1:1. The eluent was concentrated under reduced pressure and the residue was purified by preparative-TLC (ethylacetate:hexane = 1:3) to afford **46** (100 mg, 36%) as a colorless amorphous. ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.97 (d, *J* = 5.6 Hz, 1H), 7.81 (d, *J* = 2.1 Hz, 1H), 7.59 (dd, *J* = 8.5, 2.1 Hz, 1H), 6.82 (d, *J* = 8.5 Hz, 1H), 6.17 (d, *J* = 5.6 Hz, 1H), 4.54 – 4.53 (m, 1H), 3.81 – 3.77 (m, 2H), 3.68 (s, 1H), 2.97 (ddd, *J* = 13.3, 11.4, 2.4 Hz, 2H), 2.08 – 2.05 (m, 2H), 1.59 (dtd, *J* = 12.7, 11.1, 3.9 Hz, 2H). ^{13}C NMR ($CDCl_3$, 150 MHz) 160.94, 160.37, 155.32, 150.47, 146.24, 139.46, 137.50, 128.06, 123.89, 111.41, 105.90, 89.03, 79.48, 48.54, 48.00, 32.77, 28.57. HRMS calculated for $C_{21}H_{25}Cl_2F_3IN_3O_3$ $[M+H]^+$ 564.0318, found 564.0322.

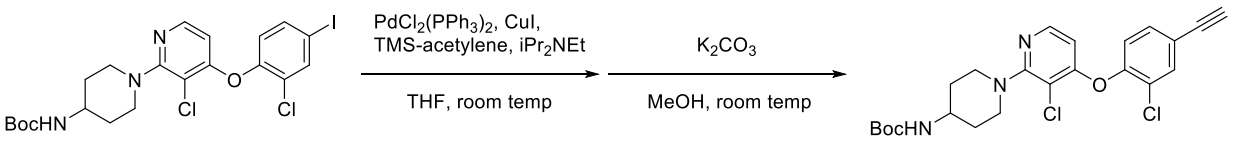
^{1}H NMR of compound 46



^{13}C NMR of compound 46



tert-butyl (1-[3-chrolo-4-{2-chloro-4-(ethynyl)phenoxy}pyridine-2-yl]piperidin-4-yl)carbamate (47)

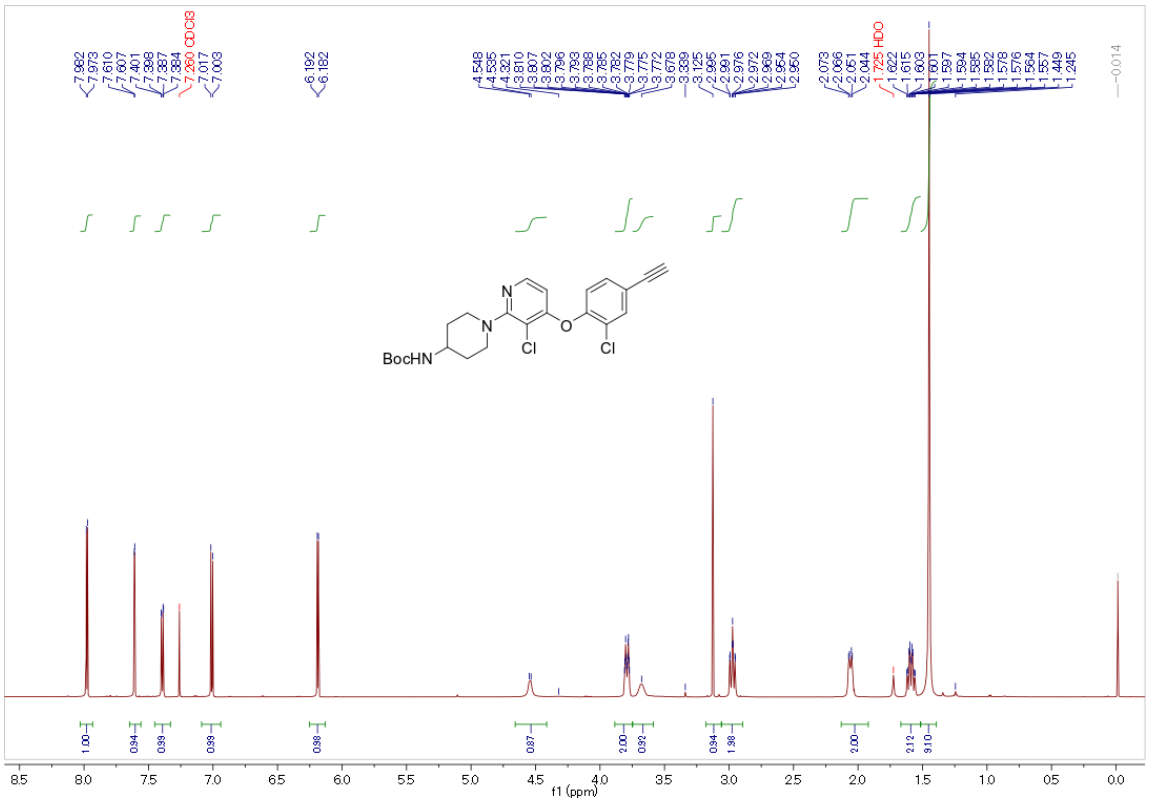


46 47

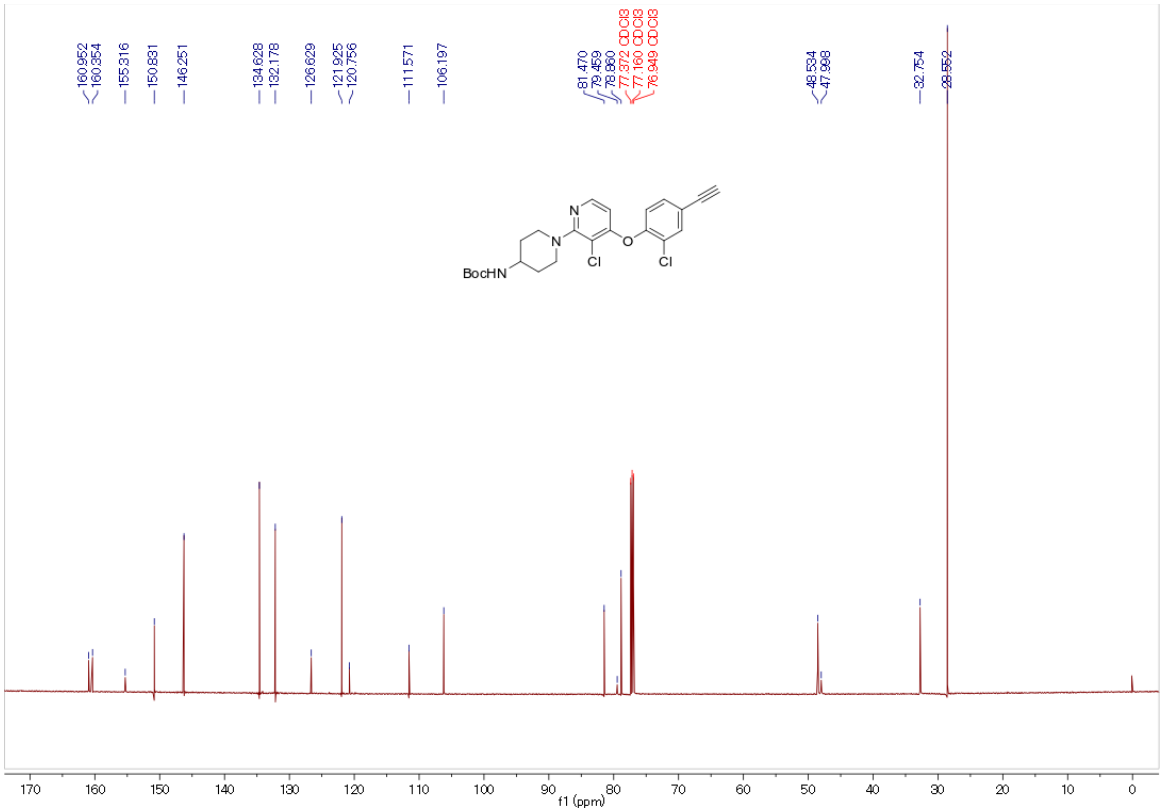
Step 1. To a solution of **46** (80 mg, 0.14 mmol) in THF (1.92 mL) was added $PdCl_2(PPh_3)_2$ (2.5 mg, 3.5 μmol), CuI (0.68 mg, 3.5 μmol), TMS-acetylene (42 μL, 0.16 mmol), iPr2NEt (130 μL, 0.72 mmol), and stirred for 2 h. The mixture was concentrated under reduced pressure, triturated with DCM and washed with sat.$NaHCO_3$. The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was passed through a short column chromatography (ethylacetate:hexane = 1:4) and used in the next step.

Step 2. To a solution of a mixture of the residue obtained in the step 1 in MeOH (3 mL) was added K_2CO_3 (39 mg, 0.28 mmol) and stirred for 2 h at room temperature. The mixture was concentrated under reduced pressure, triturated with DCM and purified by preparative-TLC (ethylacetate:hexane = 1:3) to afford **47** (61 mg, 93% over 2 steps) as a brown amorphous. ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.98 (d, *J* = 5.6 Hz, 1H), 7.61 (d, *J* = 2.0 Hz, 1H), 7.39 (dd, *J* = 8.4, 2.0 Hz, 1H), 7.01 (d, *J* = 8.4 Hz, 1H), 6.19 (d, *J* = 5.6 Hz, 1H), 4.54 (d, *J* = 7.8 Hz, 1H), 3.81 – 3.77 (m, 2H), 3.68 (s, 1H), 3.13 (s, 1H), 2.97 (ddd, *J* = 13.4, 11.3, 2.4 Hz, 2H), 2.06 (dd, *J* = 13.1, 4.0 Hz, 2H), 1.59 (dtd, *J* = 12.7, 11.0, 3.9 Hz, 2H), 1.45 (s, 9H). ^{13}C NMR ($CDCl_3$, 150 MHz) 160.95, 160.35, 155.32, 150.83, 146.25, 134.63, 132.18, 126.63, 121.93, 120.76, 111.57, 106.20, 81.47, 79.46, 78.86, 48.53, 48.00, 32.75, 28.55. HRMS calculated for $C_{23}H_{26}Cl_2N_3O_3$ $[M+H]^+$ 462.1351, found 462.1357.

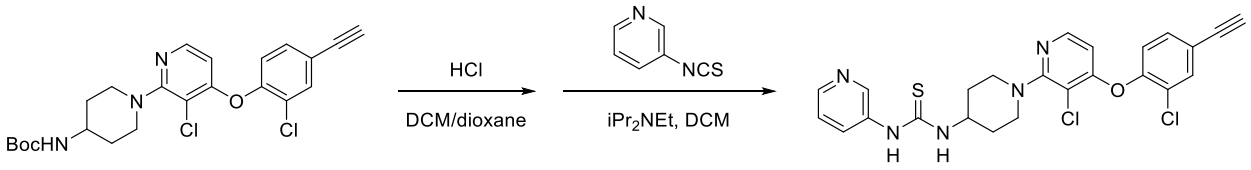
^{1}H NMR of compound 47



^{13}C NMR of compound 47



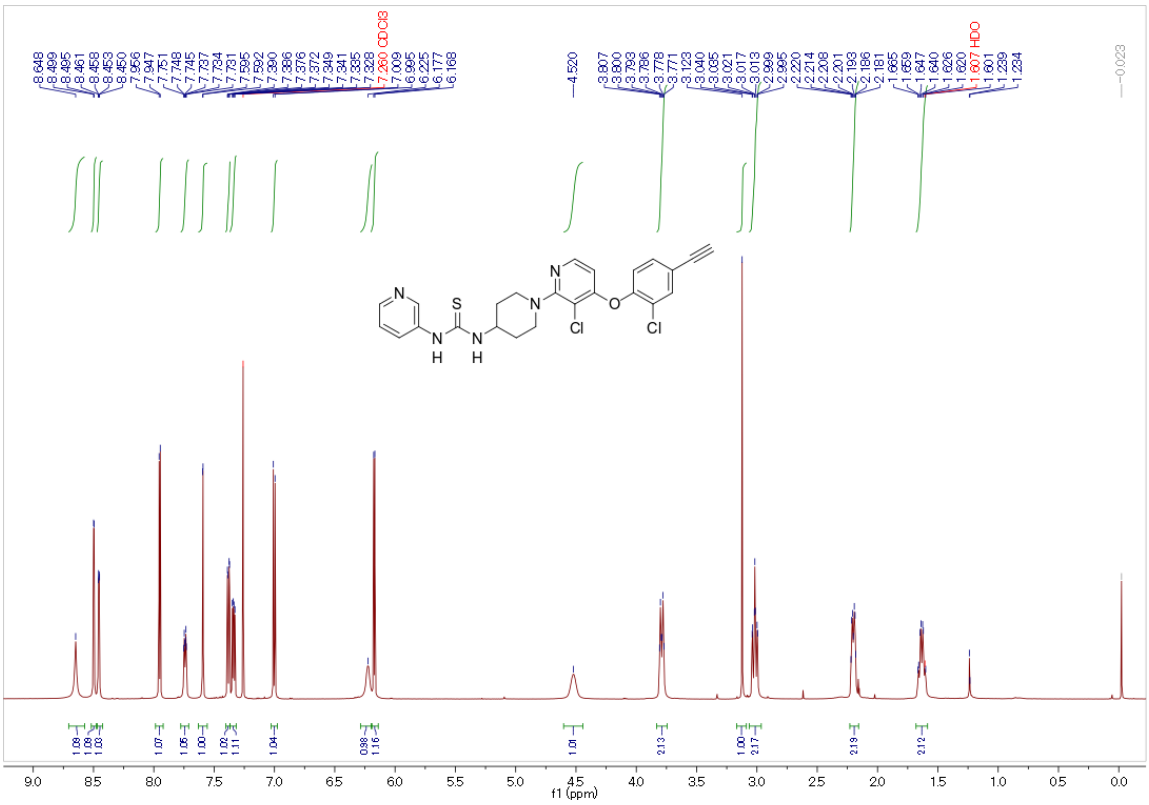
***N*-3-pyridyl-*N'*-(1-[3-chloro-4-{2-chloro-4-(ethynyl)phenoxy}pyridine-2-yl]piperidin-4-yl)thiourea (DO274)**



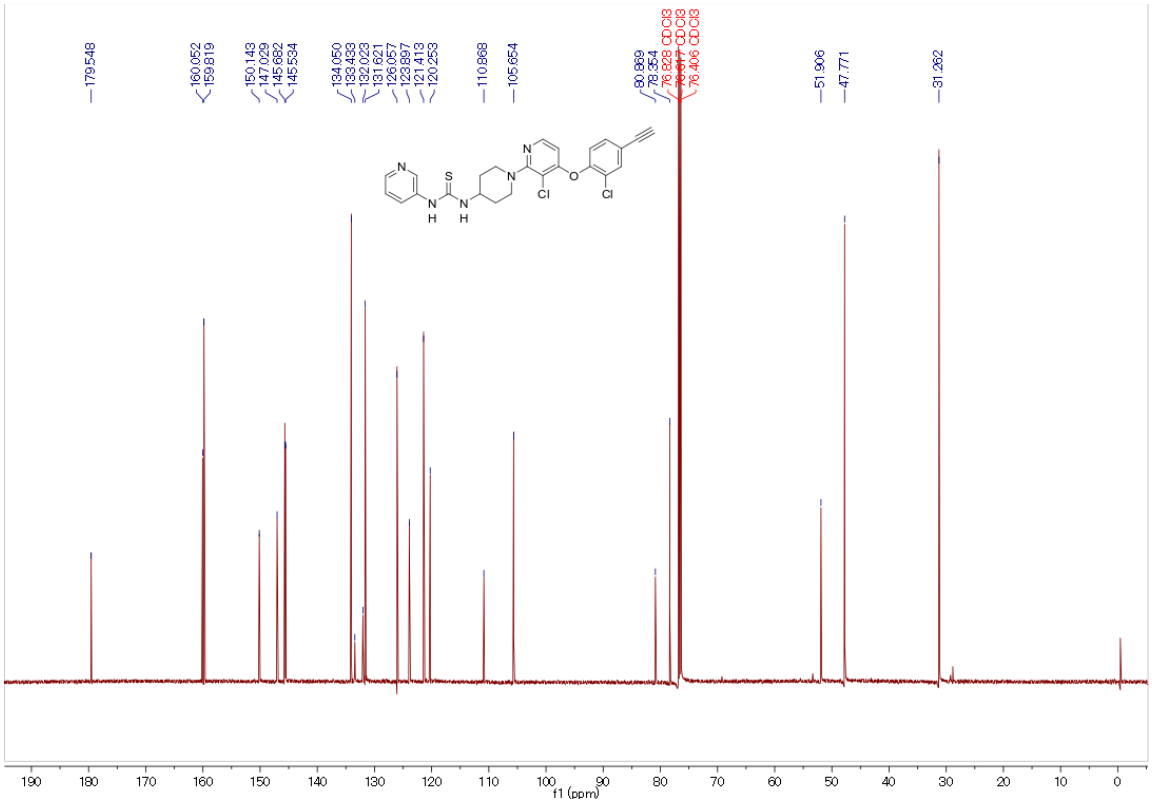
47 **10 (DO274)**

To a solution of **47** (56 mg, 0.12 mmol) in DCM (2.0 mL) was added 4N HCl in dioxane (2.0 mL) in a dropwise fashion and stirred for 1 h at room temperature. The mixture was dried under N_2 stream. The residue was dissolved in DCM (0.5 mL) with iPr_2NEt (84 µL, 0.49 mmol) and 3-pyridineisothiocyanate (18 mg, 0.13 mmol). The mixture was stirred overnight at room temperature. The mixture was diluted with DCM and washed with sat.$NaHCO_3$. The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by preparative-TLC (DCM:acetone = 3:1) to afford **DO274** (53 mg, 88%) as a white solid. ^{1}H NMR ($CDCl_3$, 600 MHz) δ 8.65 (s, 1H), 8.50 (d, *J* = 2.6 Hz, 1H), 8.46 (dd, *J* = 4.8, 1.5 Hz, 1H), 7.95 (d, *J* = 5.5 Hz, 1H), 7.74 (dt, *J* = 8.4, 2.0 Hz, 1H), 7.59 (d, *J* = 1.9 Hz, 1H), 7.38 (dd, *J* = 8.3, 2.0 Hz, 1H), 7.34 (dd, *J* = 8.2, 4.8 Hz, 1H), 7.00 (d, *J* = 8.4 Hz, 1H), 6.22 (s, 1H), 6.17 (d, *J* = 5.6 Hz, 1H), 4.52 (s, 1H), 3.79 (dd, *J* = 13.1, 4.4 Hz, 2H), 3.12 (s, 1H), 3.02 (ddd, *J* = 13.5, 11.1, 2.4 Hz, 2H), 2.22 – 2.18 (m, 2H), 1.64 (tt, *J* = 15.2, 7.7 Hz, 2H). ^{13}C NMR ($CDCl_3$, 150 MHz) 179.55, 160.05, 159.82, 150.14, 147.03, 145.68, 145.53, 134.05, 133.43, 132.02, 131.62, 126.06, 123.90, 121.41, 120.25, 110.87, 105.65, 80.87, 78.35, 51.91, 47.77, 31.26. HRMS calculated for $C_{24}H_{22}Cl_2N_5OS$ $[M+H]^+$ 498.0922, found 498.0925.

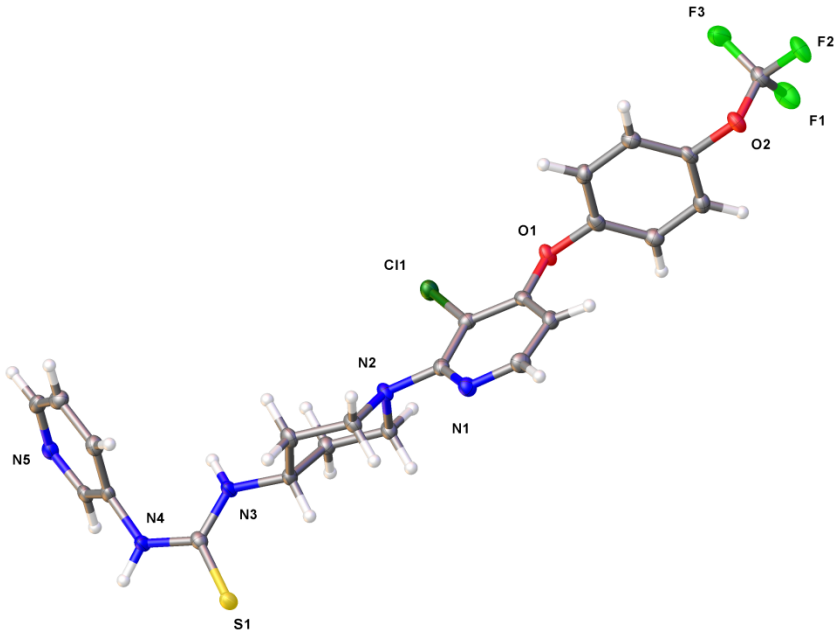
^{1}H NMR of DO274



^{13}C NMR of DO274



X-ray Crystallographic Data for DO253.



Supplementary Table 1. Crystal data and structure refinement for DO253.

Report date	2017-03-22	
Identification code	DO253	
Empirical formula	C23 H21 Cl F3 N5 O2 S	
Molecular formula	C23 H21 Cl F3 N5 O2 S	
Formula weight	523.96	
Temperature	100.0 K	
Wavelength	0.71073 Å	
Crystal system	Triclinic	
Space group	P-1	
Unit cell dimensions	a = 6.5765(3) Å	α= 102.7320(10)°.
	b = 10.4329(5) Å	β= 91.211(2)°.
	c = 17.1149(8) Å	γ = 101.1270(10)°.
Volume	1121.39(9) $Å^3$	
Z	2	
Density (calculated)	1.552 Mg/m^3	
Absorption coefficient	0.322 mm^{-1}	
F(000)	540	
Crystal size	0.067 x 0.01 x 0.005 mm^3	
Crystal color, habit	Colorless Needle	
Theta range for data collection	2.044 to 25.373°.	
Index ranges	-7<=h<=7, -12<=k<=12, -20<=l<=15	
Reflections collected	20166	

Independent reflections	4104 [R(int) = 0.0530, R(sigma) = 0.0529]
Completeness to theta = 25.000°	100.0 %
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	0.0916 and 0.0663
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	4104 / 2 / 324
Goodness-of-fit on F^2	1.016
Final R indices [I>2sigma(I)]	R1 = 0.0399, wR2 = 0.0729
R indices (all data)	R1 = 0.0696, wR2 = 0.0820
Extinction coefficient	n/a
Largest diff. peak and hole	0.314 and -0.247 e.$Å^{-3}$

Supplementary Table 2. Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters ($Å^2$x 10^3) for DO253. U(eq) is defined as one third of the trace of the orthogonalized U^{ij} tensor.

	x	y	z	U(eq)
Cl(1)	2879(1)	6026(1)	3534(1)	20(1)
S(1)	-2390(1)	10637(1)	943(1)	20(1)
F(1)	11322(2)	6118(2)	7845(1)	33(1)
F(2)	14615(2)	6543(2)	7717(1)	30(1)
F(3)	12580(2)	5063(1)	6817(1)	35(1)
O(1)	5833(2)	6838(2)	4910(1)	20(1)
O(2)	12668(2)	7248(2)	6954(1)	21(1)
N(1)	5744(3)	9795(2)	3636(1)	19(1)
N(2)	2937(3)	8410(2)	2789(1)	16(1)
N(3)	-1740(3)	8207(2)	1005(1)	19(1)
N(4)	-3956(3)	8286(2)	-28(1)	19(1)
N(5)	-6163(3)	4754(2)	-972(1)	19(1)
C(1)	4393(4)	8626(2)	3442(1)	16(1)
C(2)	4449(4)	7606(2)	3853(1)	16(1)
C(3)	5864(4)	7846(2)	4507(1)	16(1)
C(4)	7224(4)	9069(2)	4718(1)	19(1)
C(5)	7124(4)	9987(2)	4260(1)	21(1)
C(6)	7565(4)	6965(2)	5436(1)	19(1)
C(7)	7441(4)	7439(2)	6247(1)	20(1)
C(8)	9135(4)	7504(2)	6765(1)	20(1)
C(9)	10870(4)	7093(2)	6446(1)	18(1)

C(10)	11001(4)	6629(2)	5634(1)	19(1)
C(11)	9315(4)	6562(2)	5120(1)	20(1)
C(12)	12772(4)	6254(2)	7323(2)	22(1)
C(13)	3441(4)	9315(2)	2240(1)	20(1)
C(14)	1964(4)	8822(2)	1493(1)	20(1)
C(15)	-294(4)	8737(2)	1712(1)	18(1)
C(16)	-760(4)	7864(2)	2318(1)	19(1)
C(17)	796(3)	8373(2)	3040(1)	18(1)
C(18)	-2669(4)	8956(2)	629(1)	17(1)
C(19)	-4049(4)	6937(2)	-448(1)	17(1)
C(20)	-2309(4)	6526(2)	-778(1)	20(1)
C(21)	-2532(4)	5208(2)	-1202(1)	22(1)
C(22)	-4460(4)	4376(2)	-1290(1)	21(1)
C(23)	-5926(4)	6024(2)	-558(1)	18(1)

Supplementary Table 3. Bond lengths [Å] and angles [°] for DO253.

Cl(1)-C(2)	1.734(2)	N(5)-C(22)	1.344(3)
S(1)-C(18)	1.689(2)	N(5)-C(23)	1.336(3)
F(1)-C(12)	1.330(3)	C(1)-C(2)	1.404(3)
F(2)-C(12)	1.322(3)	C(2)-C(3)	1.387(3)
F(3)-C(12)	1.331(3)	C(3)-C(4)	1.379(3)
O(1)-C(3)	1.375(3)	C(4)-H(4A)	0.9500
O(1)-C(6)	1.404(3)	C(4)-C(5)	1.375(3)
O(2)-C(9)	1.414(3)	C(5)-H(5)	0.9500
O(2)-C(12)	1.339(3)	C(6)-C(7)	1.377(3)
N(1)-C(1)	1.333(3)	C(6)-C(11)	1.380(3)
N(1)-C(5)	1.341(3)	C(7)-H(7)	0.9500
N(2)-C(1)	1.407(3)	C(7)-C(8)	1.390(3)
N(2)-C(13)	1.473(3)	C(8)-H(8)	0.9500
N(2)-C(17)	1.476(3)	C(8)-C(9)	1.377(3)
N(3)-H(3)	0.901(16)	C(9)-C(10)	1.377(3)
N(3)-C(15)	1.459(3)	C(10)-H(10)	0.9500
N(3)-C(18)	1.336(3)	C(10)-C(11)	1.382(3)
N(4)-H(4)	0.921(17)	C(11)-H(11)	0.9500
N(4)-C(18)	1.361(3)	C(13)-H(13A)	0.9900
N(4)-C(19)	1.421(3)	C(13)-H(13B)	0.9900

C(13)-C(14)	1.519(3)	C(1)-C(2)-Cl(1)	121.57(18)
C(14)-H(14A)	0.9900	C(3)-C(2)-Cl(1)	119.02(17)
C(14)-H(14B)	0.9900	C(3)-C(2)-C(1)	119.3(2)
C(14)-C(15)	1.530(3)	O(1)-C(3)-C(2)	117.7(2)
C(15)-H(15)	1.0000	O(1)-C(3)-C(4)	123.5(2)
C(15)-C(16)	1.523(3)	C(4)-C(3)-C(2)	118.8(2)
C(16)-H(16A)	0.9900	C(3)-C(4)-H(4A)	121.0
C(16)-H(16B)	0.9900	C(5)-C(4)-C(3)	118.0(2)
C(16)-C(17)	1.518(3)	C(5)-C(4)-H(4A)	121.0
C(17)-H(17A)	0.9900	N(1)-C(5)-C(4)	124.5(2)
C(17)-H(17B)	0.9900	N(1)-C(5)-H(5)	117.8
C(19)-C(20)	1.386(3)	C(4)-C(5)-H(5)	117.8
C(19)-C(23)	1.388(3)	C(7)-C(6)-O(1)	119.3(2)
C(20)-H(20)	0.9500	C(7)-C(6)-C(11)	122.3(2)
C(20)-C(21)	1.385(3)	C(11)-C(6)-O(1)	118.4(2)
C(21)-H(21)	0.9500	C(6)-C(7)-H(7)	120.7
C(21)-C(22)	1.376(3)	C(6)-C(7)-C(8)	118.7(2)
C(22)-H(22)	0.9500	C(8)-C(7)-H(7)	120.7
C(23)-H(23)	0.9500	C(7)-C(8)-H(8)	120.6
		C(9)-C(8)-C(7)	118.7(2)
C(3)-O(1)-C(6)	116.49(17)	C(9)-C(8)-H(8)	120.6
C(12)-O(2)-C(9)	116.91(19)	C(8)-C(9)-O(2)	119.6(2)
C(1)-N(1)-C(5)	117.6(2)	C(8)-C(9)-C(10)	122.6(2)
C(1)-N(2)-C(13)	115.29(19)	C(10)-C(9)-O(2)	117.6(2)
C(1)-N(2)-C(17)	112.54(18)	C(9)-C(10)-H(10)	120.7
C(13)-N(2)-C(17)	110.92(17)	C(9)-C(10)-C(11)	118.6(2)
C(15)-N(3)-H(3)	116.1(15)	C(11)-C(10)-H(10)	120.7
C(18)-N(3)-H(3)	119.1(15)	C(6)-C(11)-C(10)	119.1(2)
C(18)-N(3)-C(15)	124.6(2)	C(6)-C(11)-H(11)	120.5
C(18)-N(4)-H(4)	118.2(17)	C(10)-C(11)-H(11)	120.5
C(18)-N(4)-C(19)	125.81(19)	F(1)-C(12)-F(3)	106.6(2)
C(19)-N(4)-H(4)	115.9(17)	F(1)-C(12)-O(2)	112.9(2)
C(23)-N(5)-C(22)	116.5(2)	F(2)-C(12)-F(1)	108.41(19)
N(1)-C(1)-N(2)	118.2(2)	F(2)-C(12)-F(3)	108.1(2)
N(1)-C(1)-C(2)	121.7(2)	F(2)-C(12)-O(2)	107.4(2)
C(2)-C(1)-N(2)	120.1(2)	F(3)-C(12)-O(2)	113.2(2)

N(2)-C(13)-H(13A)	109.7	C(19)-C(20)-H(20)	121.0
N(2)-C(13)-H(13B)	109.7	C(21)-C(20)-C(19)	118.0(2)
N(2)-C(13)-C(14)	109.68(19)	C(21)-C(20)-H(20)	121.0
H(13A)-C(13)-H(13B)	108.2	C(20)-C(21)-H(21)	120.5
C(14)-C(13)-H(13A)	109.7	C(22)-C(21)-C(20)	119.0(2)
C(14)-C(13)-H(13B)	109.7	C(22)-C(21)-H(21)	120.5
C(13)-C(14)-H(14A)	109.5	N(5)-C(22)-C(21)	124.0(2)
C(13)-C(14)-H(14B)	109.5	N(5)-C(22)-H(22)	118.0
C(13)-C(14)-C(15)	110.93(19)	C(21)-C(22)-H(22)	118.0
H(14A)-C(14)-H(14B)	108.0	N(5)-C(23)-C(19)	123.4(2)
C(15)-C(14)-H(14A)	109.5	N(5)-C(23)-H(23)	118.3
C(15)-C(14)-H(14B)	109.5	C(19)-C(23)-H(23)	118.3
N(3)-C(15)-C(14)	111.67(19)		
N(3)-C(15)-H(15)	108.4		
N(3)-C(15)-C(16)	110.12(19)		
C(14)-C(15)-H(15)	108.4		
C(16)-C(15)-C(14)	109.78(19)		
C(16)-C(15)-H(15)	108.4		
C(15)-C(16)-H(16A)	109.6		
C(15)-C(16)-H(16B)	109.6		
H(16A)-C(16)-H(16B)	108.1		
C(17)-C(16)-C(15)	110.4(2)		
C(17)-C(16)-H(16A)	109.6		
C(17)-C(16)-H(16B)	109.6		
N(2)-C(17)-C(16)	110.69(19)		
N(2)-C(17)-H(17A)	109.5		
N(2)-C(17)-H(17B)	109.5		
C(16)-C(17)-H(17A)	109.5		
C(16)-C(17)-H(17B)	109.5		
H(17A)-C(17)-H(17B)	108.1		
N(3)-C(18)-S(1)	123.80(18)		
N(3)-C(18)-N(4)	116.4(2)		
N(4)-C(18)-S(1)	119.81(17)		
C(20)-C(19)-N(4)	121.1(2)		
C(20)-C(19)-C(23)	119.1(2)		
C(23)-C(19)-N(4)	119.7(2)		

Supplementary Table 4. Anisotropic displacement parameters ($Å^2 x\ 10^3$) for DO253. The anisotropic displacement factor exponent takes the form: $-2\pi^2[\ h^2\ a^{*2}U^{11} + ...\ + 2\ h\ k\ a^*\ b^*\ U^{12}\ ]$

	U^{11}	U^{22}	U^{33}	U^{23}	U^{13}	U^{12}
Cl(1)	20(1)	17(1)	22(1)	4(1)	-5(1)	-1(1)
S(1)	22(1)	16(1)	21(1)	4(1)	-5(1)	4(1)
F(1)	26(1)	49(1)	33(1)	24(1)	8(1)	8(1)
F(2)	22(1)	43(1)	27(1)	16(1)	-7(1)	4(1)
F(3)	43(1)	26(1)	37(1)	2(1)	-12(1)	13(1)
O(1)	17(1)	21(1)	20(1)	8(1)	-6(1)	-2(1)
O(2)	19(1)	25(1)	21(1)	11(1)	-4(1)	3(1)
N(1)	17(1)	20(1)	21(1)	4(1)	-2(1)	2(1)
N(2)	13(1)	22(1)	15(1)	7(1)	-1(1)	4(1)
N(3)	21(1)	15(1)	19(1)	2(1)	-7(1)	5(1)
N(4)	21(1)	16(1)	18(1)	1(1)	-6(1)	6(1)
N(5)	23(1)	17(1)	17(1)	4(1)	-3(1)	3(1)
C(1)	14(1)	19(1)	14(1)	2(1)	1(1)	4(1)
C(2)	15(1)	15(1)	18(1)	2(1)	2(1)	2(1)
C(3)	15(1)	19(1)	16(1)	5(1)	2(1)	7(1)
C(4)	16(1)	22(1)	18(1)	2(1)	-2(1)	4(1)
C(5)	18(1)	17(1)	26(1)	4(1)	-2(1)	-1(1)
C(6)	20(1)	16(1)	22(1)	8(1)	-3(1)	1(1)
C(7)	17(1)	21(1)	23(1)	7(1)	2(1)	4(1)
C(8)	23(1)	21(1)	17(1)	5(1)	-1(1)	2(1)
C(9)	17(1)	17(1)	21(1)	8(1)	-4(1)	3(1)
C(10)	19(1)	19(1)	22(1)	6(1)	2(1)	4(1)
C(11)	23(1)	20(1)	16(1)	4(1)	1(1)	4(1)
C(12)	20(1)	26(2)	22(1)	8(1)	-3(1)	4(1)
C(13)	18(1)	24(1)	18(1)	9(1)	0(1)	4(1)
C(14)	23(1)	22(1)	17(1)	8(1)	-1(1)	4(1)
C(15)	19(1)	17(1)	16(1)	1(1)	-5(1)	4(1)
C(16)	18(1)	22(1)	18(1)	4(1)	-1(1)	6(1)
C(17)	16(1)	22(1)	16(1)	4(1)	-1(1)	4(1)

C(18)	14(1)	21(1)	17(1)	6(1)	0(1)	4(1)
C(19)	24(1)	15(1)	13(1)	4(1)	-3(1)	4(1)
C(20)	19(1)	22(1)	18(1)	5(1)	-1(1)	2(1)
C(21)	25(2)	22(1)	19(1)	6(1)	4(1)	7(1)
C(22)	31(2)	16(1)	16(1)	3(1)	-1(1)	6(1)
C(23)	20(1)	20(1)	15(1)	6(1)	-3(1)	5(1)

Supplementary Table 5. Hydrogen coordinates (x 10^4) and isotropic displacement parameters ($Å^2$x 10^3) for DO253.

	x	y	z	U(eq)
H(3)	-2080(40)	7307(17)	840(13)	22(7)
H(4)	-4790(40)	8750(20)	-244(15)	37(8)
H(4A)	8200	9271	5167	22
H(5)	8100	10815	4394	25
H(7)	6223	7717	6449	24
H(8)	9095	7825	7327	24
H(10)	12225	6360	5431	23
H(11)	9359	6243	4557	24
H(13A)	4890	9341	2087	24
H(13B)	3318	10235	2512	24
H(14A)	2156	7925	1205	24
H(14B)	2289	9443	1130	24
H(15)	-486	9661	1966	22
H(16A)	-2181	7877	2495	23
H(16B)	-696	6926	2062	23
H(17A)	654	9285	3321	21
H(17B)	504	7778	3419	21
H(20)	-1005	7130	-715	24
H(21)	-1372	4883	-1428	26
H(22)	-4596	3481	-1593	25
H(23)	-7105	6317	-326	21

Supplementary Table 6. Hydrogen bonds for DO253 [Å and °].

D-H...A	d(D-H)	d(H...A)	d(D...A)	<(DHA)
N(3)-H(3)...N(5)#1	0.901(16)	2.296(19)	3.118(3)	152(2)
N(4)-H(4)...S(1)#2	0.921(17)	2.444(18)	3.340(2)	164(2)

Symmetry transformations used to generate equivalent atoms:

#1 -x-1,-y+1,-z #2 -x-1,-y+2,-z

Reference

1 Cognetta, A. B., 3rd *et al.* Selective N-Hydroxyhydantoin Carbamate Inhibitors of Mammalian Serine Hydrolases. *Chem. Biol.* **22**, 928-937, doi:10.1016/j.chembiol.2015.05.018 (2015).