**Supporting Information for:**

# Optimization of 1,4-Oxazine β-Secretase 1 (BACE1) Inhibitors Towards a Clinical Candidate

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## Pharmacological and physicochemical profiling protocols

**hERG Patch-Clamp Assay**. Experiments were performed using HEK293 cells stably expressing the hERG potassium channel. Cells were grown at 37 °C and 5% CO2 in culture flasks in MEM medium supplemented with 10% heat-inactivated fetal bovine serum, 1% L-glutamine−penicillin−streptomycin solution, 1% nonessential amino acids (100×), 1% sodium pyruvate (100 mM), and 0.8% Geneticin (50 mg/mL). Before use, the cells were subcultured in MEM medium in the absence of 5 mL of L-glutamine−penicillin−streptomycin. For use in the automated patch-clamp system PatchXpress 7000A (Axon Instruments), cells were harvested to obtain cell suspension of single cells. The extracellular solution contained the following (mM): 150 NaCl, 4 KCl, 1 MgCl2, 1.8 CaCl2, 10 HEPES, 5 glucose (pH 7.4 with NaOH). The pipet solution contained the following (mM): 120 KCl, 10 HEPES, 5 EGTA, 4 ATP-Mg2+, 2 MgCl2, 0.5 CaCl2 (pH 7.2 with KOH). Patch-clamp experiments were performed in the voltage clamp mode, and whole-cell currents were recorded with an automated patch-clamp assay utilizing the PatchXpress 7000A system (Axon Instruments). Current signals were amplified and digitized by a multiclamp amplifier, stored, and analyzed by using the PatchXpress and DataXpress software and Igor 5.0 (Wavemetrics). The holding potential was −80 mV. The hERG current (K+-selective outward current) was determined as the maximal tail current at −40 mV after a 2 s depolarization to +60 mV. The pulse cycling rate was 15 s. Before each test pulse, a short pulse (0.5 s) from the holding potential to −60 mV was given to determine (linear) leak current. After establishment of whole-cell configuration and a stability period, the vehicle was applied for 5 min followed by the test substance at increasing concentrations of 10−7, 3 × 10−7, and 3 × 10−6 M. Each concentration of the test substance was applied twice. The effect of each concentration was determined after 5 min as an average current of three sequential voltage pulses. To determine the extent of block, the residual current was compared with vehicle pretreatment.

**Microsomal metabolic stability assay.** Compounds (1 μM) were incubated at 37 °C with mouse, rat, dog, and human liver microsomes at a protein concentration of 1 mg protein/mL microsomal protein, 1 mM NADPH, 1 mM $MgCl_2$, and 0.1 M phosphate buffer, pH 7.4. DMSO stock solutions (5 mM) of each compound were diluted with acetonitrile:water (1:1) to provide a working stock solution at 0.1 mM. The total incubation volume was 0.5 mL with a final total solvent content of 0.01% (v/v) DMSO and 0.5% (v/v) acetonitrile. The reaction was initiated by the addition of 100 μL pre-warmed NADPH solution. The incubation mixture was sampled at 15 min, and the sample quenched with 200 μL acetonitrile, centrifuged and analyzed using a specific HPLC-MS/MS technique. The percentage metabolized was taken as the disappearance of test compound at 15 min.

**Hepatocyte metabolic stability assay.** Compounds (1 μM) were incubated at 37 °C with cryopreserved mouse, rat, dog, and human liver hepatocytes at a cell density of $1x10^6$ hepatocytes/mL, incubation volume 40μL. The final solvent concentration in the incubation was 0.24% acetonitrile and 0.02 % DMSO. Incubations for the different time points 0, 5, 10, 20, 40, 60 and 120 min were performed in separate plates, with on-deck incubation. The incubations were terminated by quenching with 80 μL DMSO. Prior to centrifugation at 5000 rpm for 10 min (4°C) all samples were further diluted with 180 μL acetonitrile/water (2/1 v/v) and mixed. After centrifugation 200μL supernatant was transferred and further diluted with an equal amount of acetonitrile/water (2/1 v/v). All incubations were performed in triplicate and samples were analyzed for compound levels using a specific HPLC-MS/MS technique. Calculations were performed using the Simplified Well-Stirred model. All calculations remain uncorrected for binding to plasma or hepatocytes and blood / plasma ratio is assumed to be 1.

**CYP450 Inhibition Assay.** The potential to reversibly inhibit the major human P450 isoforms (CYPs 1A2, 2C9, 2C19, 2D6, and 3A4) was determined using recombinantly expressed human CYPs. Specific probe substrates were used for each CYP isoform which were known to be selectively metabolized to defined fluorescent metabolites. Each test compound was incubated with individual CYPs over a concentration range up to 10 μM. At the end of the incubation, the level of fluorescence was measured on a plate reader. The level of fluorescence in the presence and absence of test compound was used to determine the $IC_{50}$ against each CYP isoform.

**Plasma protein binding assay.** The free fraction in mouse, rat and human plasma was determined by Rapid Equilibrium Dialysis (RED Device, Thermo Fisher Scientific, Geel, Belgium). The RED device consists of a 48 well plate containing disposable inserts bisected by a semi-permeable membrane creating two chambers. A 300 μL aliquot of plasma containing test compound at 5 μM was placed one side and 500 μL of phosphate buffered saline (PBS) the other. The plate was sealed and incubated at approximately 37 °C for 4.5 h. After 4.5 h samples were removed and both the plasma and buffer compartment and analyzed for test compound using a specific HPLC-MS/MS method to estimate free and bound concentrations.

**Non-specific binding to brain tissue assay.** The *in vitro* non-specific binding of compounds to rat brain homogenate was determined using the RED Device (see above). Each test compound was diluted with rat brain homogenate, prepared following a 1:10 dilution with PBS, to achieve a final concentration of 5 μM. The plate was incubated at approximately 37 °C for 5 h. After 5 h samples were removed from both the brain homogenate and buffer compartment and analyzed for test compound using a specific HPLC-MS/MS method to estimate free and bound concentrations.

***In vitro* permeability/P-gp efflux assay.** The in vitro permeability and potential to be transported by P-glycoprotein (P-gp) was determined using an LLC cell line transfected with human MDR1 (P-glycoprotein). Each test compound (5 μM) was added to either the apical (A) side of a confluent monolayer of LLC-MDR1 cells and permeability towards the basolateral (B) direction measured by monitoring the appearance of the test compound on the opposite side of the membrane using a specific LC-MS/MS method. Permeability was assessed in with and without elacridar (GF 120918, CAS 143851-98-3), a well-known P-gp inhibitor. The A>B+elacridar/A>B-elacridar ratio ($P_{app}$ ratio) was calculated and used to determine if the test compound was subject to efflux by P-gp.

**pKa assay.** Dissociation constants were determined at 25 °C by potentiometric titration of a solution of the compound of interest using a Sirius GlpKa apparatus, and values were calculated using the Henderson-Hasselbach equation. For poorly soluble compounds, titrations were performed with MeOH as co-solvent. In this case pKa was calculated via Yasuda-Shedlovsky extrapolation.

**LogD assay.** The LogD of compounds was determined chromatographically at Sirius Analytical Ltd.[1]

**High content screen cytotoxicity assay with HepG2-cells.** Subconfluent HepG2 cells (human hepatocarcinoma cell line) growing in Poly-D-Lysine coated 96-well plates are treated with 9 concentrations of a test compound: 100, 46.5, 21.6, 10, 4.65, 2.16, 1.00, 0.47 and 0.22μM (the final DMSO concentration is 1%). For each compound, triplicate treatment cultures are set up. After 72 h of treatment, 6 key cell health indicators are simultaneously measured by means of automated fluorescence microscopy. For this, cells are stained with a mixture of 3 fluorescent probes: Hoechst, Tetramethylrhodamine ethylester (TMRE) and SYTOX ® Green. The 6 endpoints include cell proliferation determined by nuclear count (NC), nuclear size (NS), mitochondrial membrane potential (MMP (I, intensity) and MMP (N, number of cells)), mitochondrial area (MA) and finally the plasma membrane permeability (PMP). Nuclear Hoechst stain is used to measure cell number and nuclear size. TMRE is a mitochondrial stain to quantify the mitochondrial membrane potential and the mitochondrial area. To discriminate

between total cell population effects or effects on individual cells, the mitochondrial membrane potential is measured in 2 ways: (1) as the mean TMRE-fluorescence intensity (background corrected) (MMP (I)) and (2) as the number of cells stained with TMRE (MMP (N)). Finally, SYTOX®Green stains cells with a damaged plasma membrane. Curve fitting is performed using XL-fit software (IDBS) and $EC_{20}$-values are calculated by means of the best fitting curve. After automated image analysis, concentration response curves and EC20-values (calculated concentration showing 20% effect compared to solvent controls) are generated for each of the 6 endpoints. The final score is given based on the Lowest Toxic Concentration:
Lowest Toxic Concentration (LTC) = The lowest of the 6 calculated $EC_{20}$-values. Compounds are flagged non-cytotoxic with a LTC > 30µM and cytotoxic at a LTC <30µM.

**In vitro phospholipidosis assay.**[2] To determine both the cytotoxicity and the phospholipidosis-inducing potential, cell cultures from a THP-1 cell line (human monocytes) are exposed to the compounds in dose response using 8 concentrations: 200 µM, 100 µM, 50 µM, 25 µM, 12.5 µM, 6.3 µM, 3.1 µM and 1.6 µM. Concurrent negative, vehicle and positive controls are included in all tests. To analyse the cytotoxicity, cells are incubated with the compounds for 48 h and stained with an Ethidiumbromide analogue. To analyse the phospholipidogenic potential, cells are loaded with fluorescent phospholipids for a first incubation period of 24 h. Fluorescent phospholipids are then removed from the incubation medium followed by a degradation period of 24 hours in which the fluorescent phospholipids are degraded by lysosomal phospholipases. At the 48 h time point, the fluorescent phospholipids are degraded in the vehicle treated cells while treatment with phospholipidosis-inducing drugs results in the lysosomal accumulation of undegraded fluorescent phospholipids. The fluorescence of the treated cells, compared to the vehicle treated cells, is the measurement of the phospholipidosis-inducing potential of the test substances. Overall cytotoxicity of the compound is taken into account to exclude false positives due to cell death. The phospholipidosis-inducing potential of the test substances is determined by calculation of the 2-fold fluorescence increase values. Absolute fluorescence values are converted to a percentage increase of fluorescence, relative to the vehicle controls (incubated with the fluorescent phospholipids only). Dose response curves and the concentrations causing a 2-fold fluorescence increase in the fluorescence are calculated with XL-fit, according to the sigmoidal model, (no 600) or the exponential model (no 500). A 2-fold increase is the first significant signal above background fluorescence of phospholipidosis-induction (STDEV = 30%) and the 2-fold increase value (µM) is verified to induce lamellated bodies in the cells, as determined in the validation study of the assay.[2]
Concentrations causing more than a 20% reduction in the viability (calculated in the cytotoxicity assay) are not taken into account for analyzing the phospholipidosis-induction potential, as cytotoxicity influences the fluorescence of the cells. Relevance of an *in vitro* signal for *in vivo* phospholipidosis-induction is determined based on the 2-fold increase value of the test compound. Compounds are normally ranked into three categories
1) STRONG PHOSPHOLIPIDOSIS-INDUCER: 2-fold increase value ≤ 10 µM.

2) WEAK PHOSPHOLIPIDOSIS-INDUCER: 10 μM < 2-fold increase value ≤ 20 μM. Phospholipidosis occurs at therapeutic less relevant concentrations *in vitro*. No risk for phospholipidosis *in vivo* at therapeutic concentrations, but phospholipidosis might occur at high doses in preclinical studies.
3) NON PHOSPHOLIPIDOSIS-INDUCER: 25 μM < 2-fold increase value.

### In vitro Selectivity Profile (CEREP)

Compound **3** was screened at CEREP for inhibition of the aspartate proteases Cathepsins D and E. In a concentration-response curve, the compound showed less than 25% effect at the highest dose tested (10 μM), indicating an $IC_{50}$ for Cathepsins D and E above 10 μM.**Error! Reference source not found.**

To examine the binding selectivity profile, the compound was evaluated on 50 different receptor, ion channel or transporter targets at CEREP (Le bois l'Evêque, 86600 Celle l'Evescault, France). Radioligand binding and functional assays at CEREP were conducted using standard protocols (details can be found under http://www.cerep.fr/cerep/users/pages/catalog/General/catalog.asp). All tested targets showed <50% inhibition, except for kappa and mu opiate receptors (Table xx, 76% and 91% inhibition at 10 μM).

Table xx: Interaction of **3** with a broad selection of other targets

| Species | Target | CEREP %inh at 10 µM** (IC50) |
|---|---|---|
| human | $A_1$ | - |
| human | $A_{2A}$ | 10 |
| human | $A_3$ | **-** |
| rat | alpha1 (non-selective) | - |
| rat | alpha2 (non-selective) | - |
| human | beta1 | - |
| human | $AT_1$ | - |
| rat | BZD (central) | - |
| human | $B_2$ | - |
| human | $CCK_1$ (CCKA) | 16 |
| human | $D_1$ | - |
| human | $D_{2S}$ | - |
| human | ETA | - |
| rat | GABA (non-selective) | - |
| human | $GAL_2$ | - |
| human | CXCR2 (IL-8B) | - |
| human | $CCR_1$ | - |
| human | $H_1$ | - |
| human | $H_2$ | 13 |
| human | $MC_4$ | - |
| human | $MT_1$ ($ML_{1A}$) | - |
| human | $M_1$ | - |
| human | $M_2$ | - |
| human | $M_3$ | 21 |
| human | $NK_2$ | - |
| human | $NK_3$ | - |
| human | $Y_1$ | - |
| human | $Y_2$ | - |
| human | $NTS_1$ ($NT_1$) | - |
| human | delta 2 (DOP) | - |
| guinea pig | kappa (KOP) | **76** |
| human | mu (MOP) | **91** |
| human | NOP ($ORL_1$) | 16 |
| human | $5\text{-}HT_{1A}$ | - |
| rat | $5\text{-}HT_{1B}$ | - |
| human | $5\text{-}HT_{2A}$ | 15 |
| human | $5\text{-}HT_{2B}$ | - |
| human | $5\text{-}HT_3$ | - |
| human | $5\text{-}HT_{5a}$ | - |
| human | $5\text{-}HT_6$ | - |
| human | $5\text{-}HT_7$ | **-** |
| rat | sst (non-selective) | - |
| human | $VPAC_1$ ($VIP_1$) | - |
| human | V1a | - |
| rat | $Ca_{2+}$ channel | 40 |
| rat | KV channel | - |
| rat | SKCa | - |
| rat | Cl- channel (GABA-gated) | 29 |
| human | norepinephrine transporter | - |
| human | dopamine transporter | 36 |
| human | 5-HT transporter | 23 |

** For the test compounds, the results are expressed as a percent inhibition of control specific binding (mean value, n = 2). The symbol – indicates an inhibition of less than 10%.

Genotoxicity assays: AMES II and in vitro micronucleus test (MNT)

The mutagenic potential of **3** and/or its metabolites was assessed by its ability to induce reverse mutations in two strains of *Salmonella typhimurium*. The bacterial reverse gene mutation test (Ames II) was carried out using the Xenometrics strains of *Salmonella typhimurium* TA98 and TA mixed, in the absence and in the presence of an exogenous mammalian metabolic activation system, rat liver S9-mix. Compound **3** had no mutagenic properties towards the various *S. typhimurium* strains at concentrations up to 500 µg/mL.
The aniline metabolite **16** was evaluated separately in the same assay, and also had no mutagenic properties towards the various *S. typhimurium* strains at concentrations up to 500 µg/mL.

The ability of **3** and/or its metabolites to induce structural and/or numerical chromosome aberrations was assessed in an *in vitro* micronucleus test (MNT) with the human lymphoblastoid TK6 cell line in the absence and presence of metabolic activation (rat liver S9-mix). Evaluation of micronucleus induction was performed with flow cytometry. In a continuous treatment schedule without metabolic activation and in a short treatment schedule in the presence of metabolic activation (both with extended recovery incubation), **3** did not induce a biologically significant and/or concentration-related increase in the frequency of micronuclei when tested up to the required cytotoxicity level (80 µg/mL [24 h, -S9] and 188 µg/mL [4h, +S9]). The same experiment was run with aniline **16**, and **16** did not induce a biologically significant and/or concentration-related increase in the frequency of micronuclei up to 132 (24 h, -S9) and 500 µg/mL (4 h, +S9).

In vivo PK and toxicology studies

All in vivo experimental procedures were performed according to the applicable Directive 2010/63/EU of the European Parliament and of the Council of September 22, 2010 on the protection of animals used for scientific purposes, and approved by the local ethical committee.

PK studies in mouse, rat, and dog (Table 5 main manuscript)

The pharmacokinetics (PK) and oral bioavailability of **3** were investigated in male Swiss mouse, male Sprague Dawley rat, and male Beagle dog following single intravenous (i.v.) and oral (p.o.) administration at the doses indicated in table 5, main manuscript. For each arm, three animals were used. Animals had free access to food and water through each study. For both i.v. and p.o. dosing, **3** was dissolved in 20% (w/v) aqueous hydroxyl-propyl-beta-cyclodextrin (HP-β-CD) adjusted to approximately pH 4 and administered as a bolus. Blood samples were taken at 0.12, 0.33, 1, 2, 4, 7, and 24h. Plasma was prepared by protein precipitation with acetonitrile and the supernatant was analyzed for concentrations of **3** using a qualified liquid chromatography - tandem mass spectrometry (LC-MS/MS) method.

Cardio-hemodynamic and cardio-electrophysiological effects in anesthetized guinea-pigs[3]

The characteristics of the surface electrocardiogram (ECG), heart rate and mean arterial blood pressure were measured in two groups of experiments. In the first group (n = 7), increasing doses of compound were administered i.v. over a period of 5 min at 15-min intervals to pentobarbital-anesthetized female guinea-pigs. In the second group (n = 7), corresponding volumes of vehicle (20% PEG400 in 20% hydroxypropyl-β-cyclodextrin with HCl, pH 2.5) were administered according to the same protocol. This experimental model is known to readily detect ECG effects induced by compounds which produce cardiac electrophysiological changes, including prolongation of the QTc interval, similar to those expected in man. After preparation of the anesthetized animals by cannulation of two carotid arteries and two jugular veins for the monitoring of arterial blood pressure, collection of blood samples, intravenous infusion of pentobarbital, and intravenous infusion of the compound or vehicle, needle electrodes were attached for the recording of the surface ECG and instrumentation. One arterial catheter was connected to a pressure transducer for the measurement of arterial blood pressure. The ECG electrodes were connected to an ECG amplifier. All signals were fed into a computer, converted from analog to digital, recorded and saved continuously. Mean, systolic and diastolic arterial pressure, heart rate and ECG intervals were analyzed on-line with Notocord® software and reported at 1-min intervals. The duration of the QTc interval, i.e. the QT interval corrected for heart rate, was calculated using Bazett's formula1[4] (QTc= QT/ RR ). Detailed analyses in earlier studies with anesthetized guinea-pigs had shown that Bazett's formula is a valid approach for correcting changes in QT with respect to changes in heart rate. Following a stabilization period (usually 30 min), the baseline control values were recorded for at least 10 min. Thereafter, intravenous infusions of compound were administered over 5 min, at 15-min intervals to the same animal, delivering doses of 0.32, 0.64, 1.25, 2.5, 5, 10 mg/kg (total dose = 19.71 mg/kg i.v.; n = 7), or its vehicle (0.5, 0.5, 0.5, 0.5, 1 and 2 ml/kg; n = 7). In the vehicle-dosed animals, dofetilide (0.02 mg/kg i.v.) was infused over 1 minute at the end of the experiment, as a reference compound.
ECG tracings were inspected for the occurrence of cardiac conduction disturbances. Arterial blood samples (0.3 ml) were taken at the end of each infusion of compound or vehicle. The blood samples of the animals dosed with compound were collected in Eppendorf tubes containing 10 µl of heparin
(1000 I.U./ml), centrifuged (at 8000 rpm for 2 min) immediately after collection, and the plasma samples were stored in a freezer. At the end of the study, the plasma samples were analyzed for determination of the concentration of the compound.

One-month GLP toxicity studies

All activities were carried out in compliance with the current Good Laboratory Practice (GLP) principles of the OECD GLP guidelines. OECD Principles of GLP are accepted by Regulatory Authorities throughout the Member Countries of the OECD organization as described in the Mutual Acceptance of Data document (12 May 1981 -C(81)30/Final, Amended on 26 November 1997 - C(97) 186/Final). The design of the studies was based on international guidelines.

One-month Rat study:

A GMP-batch of **3** was administered orally via gavage to male and female SPF Sprague-Dawley rats (10/sex/group) at dosages of 25, 75 or 225 mg eq./kg body weight/day(mg eq./kg/day) during one month. Compound **3** was formulated as a suspension containing 0.5% w/v Methocel F4M (hydroxypropyl methylcellulose) and administered once daily by gavage at a volume of 10 ml/kg body weight. A similar group of rats received the vehicle only, according to the same regimen and served as avehicle control group. In addition, a recovery period of 1 month was added to investigate the reversibility of any possible toxic effect induced by repeated oral administration of **3**. For this purpose, extra rats for the vehicle and the high dose group were added to the main toxicity study (5/sex/group). The following parameters were studied: mortality, clinical and eye observations, body weight, weight gain, food consumption, haematology, coagulation, clinical chemistry, urinalysis, organ weights, gross pathology, histopathology and micronucleus evaluation. The toxicokinetic parameters were also determined.

One-month Dog Study

A GMP batch of compound **3** was administered to male and female beagle dogs (3/sex/group) at dosages of 20, 70 or 250 mg eq./kg body weight/day (mg eq./kg b.w./day) during 1 month. Compound **3** was formulated as an aqueous suspension, containing 0.5% w/v Methocel F4M Premium EP (hydroxypropyl methylcellulose) and administered once daily by gavage at a volume of 2 ml/kg b.w./day. A vehicle group was also included. A recovery period of 1 month was included to investigate the reversibility of any possible toxic effects induced by repeated oral dosing of **3**. For this purpose, extra dogs for the vehicle and high dose group were added to the study (2/sex/group). The following parameters were studied: mortality, clinical and eye observations, body weight, weight gain, ECG and heart rate, food consumption, haematology, coagulation, clinical chemistry, urinalysis, organ weights, gross pathology and histopathology. The toxicokinetic parameters of were also determined. Dogs were deprived of food overnight before dosing. During the first 4 days (Days 0-3) of dosing, dogs received their food shortly after dosing. However, because of CNS symptoms (ataxia, decreased general activity and/or tremors) at 70 and 250 mg/kg b.w./day, most likely related to high plasma peak exposures (Cmax), it was decided from Day 4 (5th dose) onwards, to keep the dogs deprived of food up to approximately 4 hours after dosing and afterwards allow them to eat during at least 3 hours.

**$^{1}$H-NOESY and $^{19}$F-$^{1}$H NOESY data for 25**

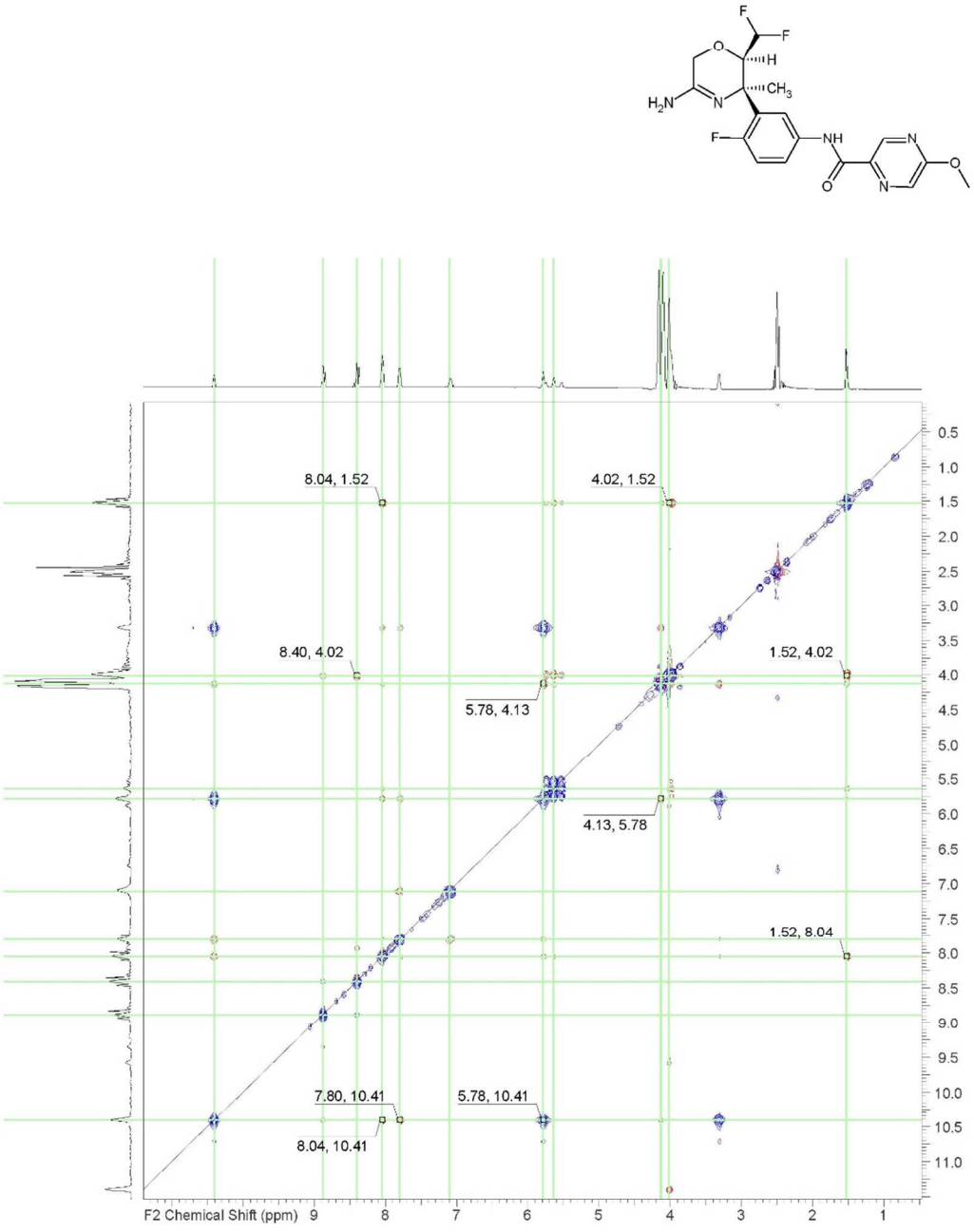


Figure S1. 1H NOESY of compound **25**

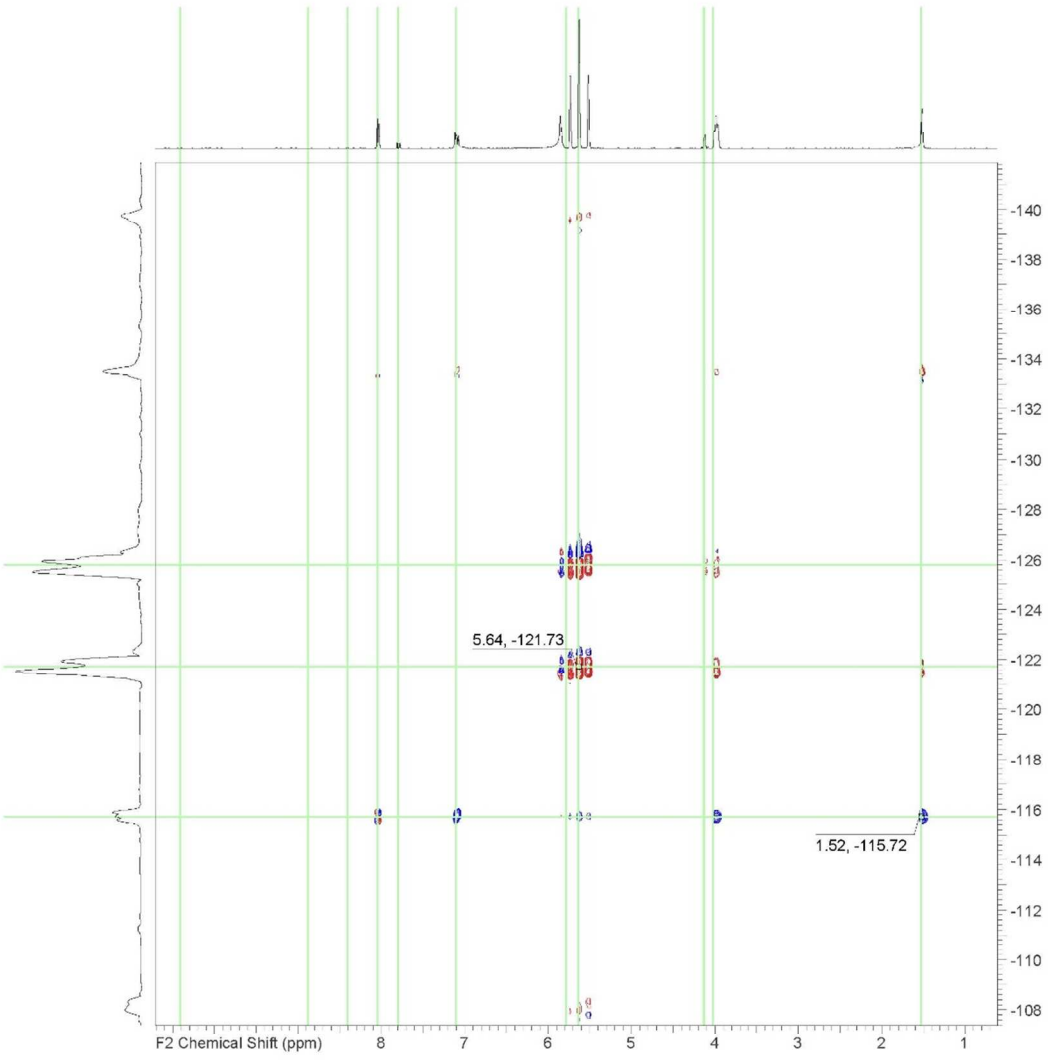


Figure S2. $^{19}F$-$^{1}H$ NOESY for compound **25**, with $^{1}H$ signals on horizontal axis and $^{19}F$ signals on vertical axis

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