Supporting information

BAY-069, A Novel (Trifluoromethyl)pyrimidinedione-based BCAT1/2 Inhibitor And Chemical Probe

Judith Günther,*† Roman C. Hillig,†# Katja Zimmermann,† Stefan Kaulfuss,†# Clara Lemos,†# Duy Nguyen,†# Hartmut Rehwinkel,† Matthew Habgood,§ǁ Christian Lechner,†# Roland Neuhaus,†# Ursula Ganzer,†# Mark Drewes,Ξ Jijie Chai,† and Léa Bouché*†,╙

1Research & Development, Pharmaceuticals, Bayer Pharma AG, Müllerstrasse 178, 13353 Berlin, Germany
2Research & Development, Pharmaceuticals, Bayer Pharma AG, Apather Weg 18a, 42113 Wuppertal, Germany
3Evotec (UK) Ltd., 114 Innovation Drive, Milton Park, Abingdon, Oxfordshire, OX14 4RZ, UK
4Research & Development BCS, Bayer Pharma AG, Alfred-Nobel-Strasse 50, 40789 Monheim, Germany
5 School of Life Sciences, Tsinghua University, 100084 Beijing, China
For correspondence: lea.bouche@roche.com, judith.guenther@bayer.com

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1. BCAT1 Protein Production, Crystallization, and Co-Complex Structure Determination

An expression construct comprising human full length BCAT1 (Uniprot entry P5467) with an N-terminal hexa-His tag and a HRV3C cleavage site was transformed into BL21(DE3) cells (BL) for protein expression. Cells were lysed using a microfluidizer and the supernatant was purified via Nickel-NTA affinity chromatography. The tag was cleaved using HRV3C Protease (Novagen #71493-3) and the protein further purified via ion exchange (Source Q) and size exclusion chromatography (Superdex200). The final buffer was 10 mM Tris-HCl pH 8.0, 100 mM NaCl, 3 mM DTT. The protein was concentrated to 17 mg/mL, flash-frozen in liquid nitrogen and stored at -80 °C.

For crystallization, BCAT1 was thawed and supplemented with 3 mM fresh DTT (from a 100 mM stock in water), 10 mM phenylpropionic acid (3PP (Sigma-Aldrich), 500 mM stock in DMSO) and 1.5 mM co-factor PLP (100 mM stock in DMSO) and incubated over night at 20 °C. Crystals were grown by vapor diffusion using the hanging drop method. Drops made from 1 µL of protein were mixed with 1 µL of reservoir buffer (16-20% PEG 3350 (w/v), 225 mM MgCl₂) incubated for 5 min, streak seeded (with crystals obtained previously under identical conditions) and stored at 20 °C. Thick rod-shaped crystals (Figure S1 A) grew within 1 to 4 days.

Inhibitor co-complexes were generated by soaking. Inhibitor solutions were prepared as 100 mM stock solution in DMSO or as 10 mM stock solution in reservoir buffer. For the DMSO approach, the 100 mM stock was diluted 1:5 with reservoir solution to form a 20 mM stock solution, which was then added in 0.5 µL-steps to a drop with crystals (10 mM final ligand concentration). For the reservoir buffer approach, a 10 mM inhibitor solution in reservoir buffer (20% PEG3350 (w/v), 225 mM MgCl₂) was prepared and crystals were transferred directly from their original drop into this ligand solution. Crystals were soaked for 1-4 days, briefly immersed in cryo buffer (reservoir buffer supplemented with 10 mM inhibitor and 20% glycerol) and flash-frozen in liquid nitrogen.

Data sets were collected at 100 K either at beamline 14.1 at the Helmholtz-Zentrum Berlin (wavelength λ=0.9184 Å) using a PILATUS detector or at beamline P11 at PETRA III at the Deutches Elektronen-Synchrotron (wavelength 1.000 Å) (see Table S1). The crystals diffracted to a resolution of 1.6 – 2.6 Å. Data were processed using the programs XDS¹ and XDSAPP.² They belonged either to space group P2₁2₁2₁ with one BCAT1 dimer in the asymmetric unit or to space group P2₁2₁2₁ with two BCAT1 dimers in the asymmetric unit (see Table S1). The structures were solved using molecular replacement (program PHASER³ from the CCP4 program suite⁴ and PDB entry 2ABJ as a search model, refined using REFMAC5⁵ and rebuilt using the program COOT.⁶ For inhibitor parameterization, 3D models were generated using Discovery Studio (Dassault Systèmes BIOVIA) and parameter files were generated using software PRODRG.⁷ The final data collection and refinement statistics are summarized in Table S1.
### Crystallographic Data Collection and Refinement Statistics

**Supplementary Table S1.** BCAT1 Crystallographic Data Collection and Refinement Statistics (Values in Brackets Refer to the Highest Resolution Shell)

| Compound       | 3PP  | 1   | 2   | 10  | 12  | 21 (variant 5'-F) |
|----------------|------|-----|-----|-----|-----|-------------------|
| PDB ID         | 7NTR | 7NWB| 7NWB| 7NWE| 7NWM| 7NXN              |
| **Data Collection & processing** |      |     |     |     |     |                   |
| Beamline       | ESRF ID29 | BESSY BL14.1 | BESSY BL14.1 | BESSY BL14.1 | PETRA P11 | PETRA P11 |
| Wavelength     | 0.8726 | 0.9184 | 0.9184 | 0.9184 | 1.0332 | 1.0332 |
| Space group    | P212121 | P21 | P21 | P212121 | P212121 | P212121 |
| Unit cell parameters, a (Å), b (Å), c (Å), β (°) | 66.8 | 103.8 | 110.4 | 90.0 | 66.5 | 66.3 |
|                | 82.0 | 110.4 | 107.0 | 103.2 | 66.7 | 106.2 |
|                | 103.8 | 110.4 | 107.7 | 107.8 | 109.3 | 109.3 |
|                | 110.2 | 107.8 | 103.4 | 90.0  | 109.3 | 109.3 |
|                | 90.0  | 103.2 | 90.0  | 90.0  | 109.3 | 109.3 |
| Resolution limit [Å] | 75.5-2.2 | 47.38-2.64 | 45.24-2.38 | 47.78-2.54 | 47.84-2.15 | 47.76-1.70 |
| (2.34 – 2.21) | 47.38-2.64 | 45.24-2.38 | 47.78-2.54 | 47.84-2.15 | 47.76-1.70 | 47.76-1.70 |
| No. of unique reflections | 39094 | 50175 | 70341 | 24853 | 40857 | 85902 |
| Multiplicity   | 5.1 (5.1) | 2.7 (2.5) | 3.4 (3.5) | 7.2 (7.2) | 4.5 (4.6) | 6.6 (6.5) |
| Completeness [%] | 99.3 (96.1) | 90.9 (90.5) | 98.5 | 99.2 (95.2) | 99.0 (99.5) | 99.7 (99.2) |
| I/σ(I)         | 9.9 (1.9) | 9.6 (1.9) | 6.5 (1.7) | 9.6 (1.3) | 13.0 (1.5) | 20.6 (1.6) |
| CC1/2          | 0.996 (0.719) | 0.993 (0.733) | 0.983 (0.639) | 0.997 (0.598) | 0.999 (0.722) | 1.000 (0.709) |
| Rmeas [%]      | 14.4 (91.0) | 12.1 (69.5) | 21.9 (91.1) | 17.7 (149.0) | 8.1 (120.2) | 6.3 (128.6) |
| Wilson B factor [Å²] | 39.0 | 44.4 | 33.4 | 57.6 | 51.7 | 34.0 |
| **Refinement** |     |     |     |     |     |                   |
| Resolution limit [Å] | 42.5-2.2 | 47.4-2.6 | 45.24-2.38 | 47.78-2.54 | 47.84-2.15 | 41.55-1.70 |
| Rwork/Rfree [%] | 16.99/24.11 | 18.56/22.75 | 22.06/26.26 | 21.06/24.54 | 21.82/27.30 | 18.96/22.37 |
| No. of unique reflections | 36147 | 48075 | 70342 | 23610 | 38813 | 83801 |
| RMSD bond length [Å] | 0.014 | 0.005 | 0.004 | 0.003 | 0.007 | 0.007 |
| RMSD bond angles [°] | 1.683 | 1.361 | 1.243 | 1.237 | 1.376 | 1.381 |
| Average B factor [Å²] | 34.7 | 42.6 | 35.9 | 55.5 | 51.4 | 33.5 |
### Supplementary Table S1 (Continued). (Values in Brackets Refer to the Highest Resolution Shell)

| Compound       | 24 (variant 5'-F) | 35       | 38       | 36/BAY-069 | Compound A |
|----------------|-------------------|----------|----------|------------|------------|
| PDB ID         | 7NXO              | 7NY2     | 7NY9     | 7NYA       | 7NWA       |

#### Data Collection & processing

|                           | PETRA P11 | PETRA P11 | PETRA P11 | BESSY BL14.1 | BESSY BL14.1 |
|---------------------------|-----------|-----------|-----------|--------------|--------------|
| Beamline                  | PETRA P11 | PETRA P11 | PETRA P11 | BESSY BL14.1 | BESSY BL14.1 |
| Wavelength                | 1.0332    | 1.0332    | 1.0332    | 0.9184       | 0.9184       |
| Space group               | P2;2;2;1  | P2;2;2;1  | P2;2;2;1  | P2;2;2;1     | P2;2;2;1     |
| Unit cell parameters, a (Å), b (Å), c (Å), β (°) | 66.9  | 66.8      | 66.4      | 66.6         | 67.0         |
|                           | 102.9     | 103.8     | 103.0     | 103.3        | 106.3        |
|                           | 110.0     | 110.2     | 109.4     | 108.1        | 109.0        |
|                           | 90.0      | 90.0      | 90.0      | 90.0         | 90.0         |
| Resolution limit [Å]      | 46.61-1.71| 47.42-2.31| 48.32-1.60| 47.90-1.85   | 48.49-1.59   |
|                           | (1.81-1.71)| (2.45-2.31)| (1.70-1.60)| (1.96-1.85)  | (1.68-1.59)  |
| No. of unique reflections | 82785     | 32595     | 99549     | 64223        | 103620       |
| Multiplicity              | 6.6 (6.5) | 5.9 (4.5) | 6.6 (6.3) | 6.7 (6.7)    | 4.3 (3.2)    |
| Completeness [%]          | 99.5 (97.9)| 98.0 (89.8)| 99.8 (99.6)| 99.8 (99.4)  | 98.3 (92.3)  |
| I/σ(I)                    | 15.8 (1.5) | 19.6 (4.1) | 17.3 (1.7) | 10.8 (1.4)   | 15.9 (2.1)   |
| CC1/2                     | 0.999 (0.738) | 0.999 (0.910) | 0.999 (0.726) | 0.998 (0.633) | 0.999 (0.751) |
| Rmeas [%]                 | 7.6 (116.8)| 7.5 (37.3) | 7.5 (106.0) | 15.1 (146.0) | 5.7 (57.2)   |
| Wilson B factor [Å²]      | 33.5      | 39.2      | 28.0      | 31.1         | 28.3         |

#### Refinement

|                           | 46.61-1.71| 47.42-2.31| 48.32-1.60| 47.90-1.85   | 48.49-1.59   |
|                           | (1.81-1.71)| (2.45-2.31)| (1.70-1.60)| (1.96-1.85)  | (1.68-1.59)  |
| Rwork/Rfree [%]           | 18.97/21.02| 20.64/27.22| 17.42/20.52| 18.27/22.13  | 16.80/19.89  |
| No. of unique reflections | 80683     | 32595     | 97447     | 62095        | 98439        |
| RMSD bond length [Å]      | 0.006     | 0.010     | 0.005     | 0.007        | 0.009        |
| RMSD bond angles [°]      | 1.347     | 1.730     | 1.257     | 1.360        | 1.543        |
| Average B factor [Å²]     | 30.6      | 32.3      | 23.6      | 28.1         | 23.7         |
Structural Biology: Supplementary Results

We determined the co-crystal structure of BCAT1 in complex with Compound A from a BCAT2 inhibitor series from GSK\(^8\) (Figure 1). The structure confirms the binding mode which was published for a very closely related inhibitor bound to BCAT2 (described as compound 66 by Bertrand et al.,\(^8\) PDB accession code 5BWX). A superimposition of the BCAT1 cocrystal structures with BAY-069 and Compound A (Figure S5) shows that the two inhibitors both insert a hydrophobic group into the substrate binding pocket near the PLP co-factor. Otherwise, however, they occupy different parts of the BCAT1 active site. While the naphthalene bicycle of BAY-069 occupies a deep groove between Phe\(^{49}\) and Tyr\(^{193}\), this groove is closed by an induced fit in the X-ray with compound A. Compound A instead kinks off and places its central scaffold between Thr\(^{260}\) and Tyr\(^{193}\) while it inserts its outer dichloro-fluoro-phenyl ring in a pocket between Gln\(^{244}\) and the Cys\(^{335}\)-Cys\(^{338}\) disulfide bond. This subpocket is, in turn, closed in the co-complex structure with BAY-069.
**Supplementary Figure S1.** (A) Crystals of BCAT1 grown in the presence of substrate mimic 3-phenyl propionic acid (3PP). The largest crystal is approx. 60 x 60 x 400 µm³. (B) Overall fold of the BCAT1 dimer exemplified by the cocrystal structure with 3PP (PDB accession code 7NTR). Protein in ribbon representation, bound co-factor PLP in stick representation with carbon atoms depicted in magenta, 3PP with carbon atoms in yellow. The two symmetric active sites are mostly formed by residues from one chain, with the other chain contributing only one loop. (C) Zoom into the binding site of 3PP, in front of the co-factor PLP (magenta carbon atoms) which is covalently attached to the side chain of Lys$_{222}$.
Supplementary Figure S2. Co-crystal structures of BCAT1, (A) in complex with compound 12 (PDB accession code 7NWM, carbon atoms of inhibitor in cyan), (B) in complex with 10 (carbon atoms of the inhibitor in orange, PDB accession code 7NWE). Shown are the active sites in the A chains. Together with 35 (Figure 5), the three closely related compounds feature very similar binding modes, with the pyrimidinedione core forming H bonds with the side chain of Tyr\textsuperscript{193} and Gln\textsuperscript{244} as well as the backbone oxygen of Val\textsuperscript{175}. 
Supplementary Figure S3. Superimposition of the BCAT1 in complex with 36/BAY-069 (PDB accession code 7NYA, protein carbon atoms in gray, inhibitor carbon atoms in green) with the BCAT1:compound A complex (PDB accession code 7NWA, protein carbon atoms in blue, inhibitor carbon atoms in lilac). Depicted is the active site in the A chains in two different orientations (A) and (B). The two inhibitors trigger two different induced fits, with the movement of Tyr^{193} enabling either BAY-069 to bind into a groove between Tyr^{193} and Phe^{49}, or compound A to insert its difluoro-chloro-phenyl moiety into a sub pocket between Tyr^{193} and Gln^{244}.
2. Computational Studies

All quantum mechanical simulation were carried out using the General Atomic and Molecular Electronic Structure System (GAMESS-US).

All molecular sketching, 3D structure building, manipulation, and visualisation in preparation of the FMO calculations was carried out using the Molecular Operating Environment (MOE).\textsuperscript{10}

The calculation of interaction energies between each ligand and the protein target was carried out as described in;\textsuperscript{11} the process can be summarised as follows. The protein is represented by the set of residues having close contacts with the ligand, as defined by MOE’s ‘near ligand’ option. Non-hydrogen geometries are taken directly from available crystal structures; hydrogen atoms are added in using MOE. A quantum mechanical simulation is carried out of each residue in this set, and of the ligand, in isolation. These simulations produce energies and atomic charges (derived via the CHelpG scheme\textsuperscript{12}). A second set of quantum mechanical simulations is performed, of each pairwise system formed by the ligand and one of the residues set. The pairwise system in each of these simulations is embedded in the set of charges derived from all the neglected residues. For example, in a system consisting of a ligand molecule plus five close-contacted protein residues, five quantum mechanical calculations would be carried out: the ligand plus residue one, embedded in the atomic charges calculated for residues 2-5; then the ligand plus residue two, embedded in the atomic charges calculated for residues 1 and 3-5; and so on. The individual energies calculated for the isolated ligand and residues are then subtracted from the energies calculated for the pairwise system, to give an interaction energy for each pair. These are summed to give an overall interaction energy for the ligand with the protein. All quantum mechanical simulations for FMO were carried out at MP2 level of theory with a 6-31G* basis set.

Using this method, the binding energy of 34 was calculated to be 0.9 kcal mol\textsuperscript{-1} stronger than that of 12 (ΔE = -0.9 kcal mol\textsuperscript{-1}).

For purposes of torsion angle scanning, an initial geometry was obtained by sketching and 3D building in MOE. QM simulations were then carried out at the HF level of theory using a 3-21G* basis set. The initial molecule was subjected to unconstrained quantum mechanical local energy minimisation / geometry optimisation in GAMESS-US. The indicated dihedral angle θ was then altered in steps of 10° up to a full rotation of 360°. At each step, constrained energy minimisation was carried out, with the dihedral θ fixed at the chosen value and the rest of the molecular geometry subject to minimisation. The energy barrier to rotation was calculated as the maximum molecular energy calculated for any step in this dihedral scan, minus the minimum molecular energy.
Supplementary Figure S4. Example biphenyl molecule with scanned dihedral angle indicated.

Supplementary Figure S5. Calculated rotational energy barriers of selected (trifluoromethyl)pyrimidinediones.

All docking studies were performed using Glide\textsuperscript{13, 14} as part of Schrodinger’s maestro suite\textsuperscript{15} version 2016-3 to 2017-3. Protein structures were prepared using the protein preparation wizard with default parameters, with ligand protonation states being manually adapted as required. The preparation of 3D ligand structures for docking employed an inhouse Pipeline Pilot\textsuperscript{16} script that utilizes Corina\textsuperscript{17} for 3D structure generation and SimPlus ADMET predictor\textsuperscript{18} for assignment of protonation states. The Cavbase search\textsuperscript{19} for pockets showing a similar 3D-arrangement of recognition properties to the active site of hBCAT1 was carried out using the database Relibase+ version 3.3.0 augmented by inhouse X-ray structures. The query pocket was defined as a 4Å envelope around the cocrystallized ligand 35 in the X-ray structure [PDB code 7NY2], all pseudocentres (donor/acceptor/pi/aromatic/aliphatic) were included. The query was run across all available X-ray data using default parameters. Other BCAT structures were removed from the top of the hit list and subsequent hits were visually inspected upon superimposition with the query pocket. No relevant similarities were detected that could point to off-target activities of the (trifluoromethyl)-pyrimidinedione series.
Supplementary Figure S6
Query setup for the Cavbase query using X-ray structure of BCAT1 in complex with compound 35. Within an envelope of 4Å radius around the cocrystallized ligand all pseudocentres (donor/acceptor/pi/aromatic/aliphatic) were picked.

3. Chemistry
Synthesis of intermediates 44 and 45

Supplementary Scheme S1: Synthetic route for the negative control BAY-771 (43)

Ethyl (3-Bromonaphthalen-1-yl)carbamate (44)
To an ice-cooled solution of commercially available 3-bromonaphthalen-1-amine (CAS: 90766-34-0; 1.00 g, 4.50 mmol) in pyridine (6.8 mL), ethyl chloroformate (520 µL, 5.40 mmol) was added dropwise at 0 °C. After complete addition, the corresponding suspension was stirred at rt for 2 h. Upon reaction completion, 1 M HCl (20 mL) was added, and the resulting precipitate was collected by filtration, washed with water, and dried under reduced pressure to give 44 as a gray solid (1.10 g, crude material, 90% purity). $^1$H NMR (400 MHz, [D$_6$]DMSO): $\delta = 1.28$ (t, 3H), 4.19 (d, 2H), 7.54–7.59 (m, 2H), 7.85, 7.92, 7.99, 8.14 (4 m, 1H each), 9.77 (bs, 1H) ppm. LC-MS (Method 3): $^1$R = 1.28 min; MS (ESI+): $m/z$ = 294 [M + H]$^+$.  

3-(3-Bromonaphthalen-1-yl)-6-(trifluoromethyl)pyridine-2,4(1H,3H)-dione (45)

To a suspension of sodium hydride (60% in mineral oil; 102 mg, 2.55 mmol) in DMF (4.4 mL) was added a solution of ethyl 3-amino-4,4,4-trifluorobut-2-enoate (350 µL, 2.4 mmol) in DMF (0.3 mL) at 0 °C and the yellow mixture was stirred for 2 h at rt. Then, a solution of ethyl (3-bromonaphthalen-1-yl)carbamate (44; 500 mg, 1.70 mmol) in DMF (10 mL) was slowly added. The resulting orange mixture was heated at 90 °C for 18 h under nitrogen atmosphere. Upon completion of the reaction, the mixture was cooled to 0 °C and then diluted with water and ethyl acetate. The layers were separated, and the water phase was extracted three times with ethyl acetate. The combined organic layers were dried over anhydrous sodium sulfate, filtered, and the solvent was removed under reduced pressure. The residue was purified by HT-HPLC (acid) giving 45 in two fractions (230 mg, 85% purity, contains ethyl 3-amino-4,4,4-trifluorobut-2-enoate, 34% yield) as an ochre solid. $^1$H NMR (400 MHz, [D$_6$]DMSO): $\delta = 6.45$ (s, 1H), 7.55–7.65, 7.80–7.83 (2 m, 2H each), 8.03 (d, 1H), 8.36 (m, 1H), 12.68 (bs, 1H) ppm. LC-MS (Method 3): $^1$R = 1.06 min; MS (ESI+): $m/z$ = 384 [M + H]$^+$. 

S 13
Analytics of chemical probe (36a) and negative control (43)

Supplementary Figure S7: UPLC-MS for racemate 36
Supplementary Figure S8: determination of enantiomeric ratio for 36
**Supplementary Figure S9**: determination of enantiomeric excess for separated atropisomer 36a

**Supplementary Figure S10**: determination of enantiomeric excess for separated atropisomer 36b
Supplementary Figure S11: UPLC-MS for chemical probe (36a)
Supplementary Figure S12: $^1$H-NMR of chemical probe (36a)

Supplementary Figure S13: $^{19}$F-NMR of chemical probe (36a)
Supplementary Figure S14: UPLC-MS for negative control (43)
Supplementary Figure S15: $^1$H-NMR of negative control (43)

Supplementary Figure S16: $^{19}$F-NMR of negative control (43)
Preparation of the starting materials

Ethyl 4-chloro-4,4-difluoro-3-oxobutanoate (46)

To a solution of ethyl acetate (2.2 g, 25.2 mmol) in tetrahydrofuran (50 mL) was added lithium diisopropylamide (9.5 mL, 2 mol/L in THF) at -78 °C and the resulting mixture was stirred at this temperature for 30 min. Then ethyl 2-chloro-2,2-difluoroacetate (2.0 g, 12.6 mmol) was added to the above mixture at -78 °C and the resulting solution was stirred at RT for overnight under nitrogen atmosphere. Upon completion of the reaction, aq. ammonium chloride solution was added at 0 °C and the resulting mixture was extracted with ethyl acetate. The combined organic layer was dried over anhydrous sodium sulfate and the solvent was removed in vacuo to give the title compound (3.0 g, 59% yield, 50% purity) as light red oil. LCMS (ESIneg): m/z = 199 [M-H].

Ethyl 3-amino-4-chloro-4,4-difluorobut-2-enoate (47)

To a solution of aforementioned ethyl 4-chloro-4,4-difluoro-3-oxobutanoate (46, 3.0 g, 7.5 mmol, 50% purity) in methanol (50 mL) was added ammonium acetate (2.3 g, 29.9 mmol). The resulting mixture was stirred at RT for overnight. Upon completion of the reaction, the solvent was removed in vacuo and the residue was purified by silica gel column chromatography (petroleum ether: ethyl acetate = 10: 1) to give the title compound (800 mg, 48% yield) as light-yellow oil. LCMS (ESIpos): m/z =200 [M+H]+.

Ethyl 3-amino-4,4-difluoro-4-phenylbut-2-enoate (48)

To a solution of diisopropylamine (4.0 mL, 29.41 mmol) in THF (95 mL) was added n-butyllithium (2.5 M in hexane) (11.7 mL, 29.41 mmol) at -40 °C, and the mixture was allowed to stir at -10 °C for 30 minutes. Again, the mixture was cooled to -50 °C; then ethyl acetate (2.3 mL, 39.2 mmol) was added at -50 °C and the resulting mixture was stirred at this temperature for 30 min. Then a solution of commercially available 2,2-difluoro-2-phenylacetonitrile (CAS: 2002-72-4, 3.0 g, 19.6 mmol) in THF (5 mL) was added to the above mixture at -78 °C and the resulting solution was stirred for the same temperature for 2 h. After completion of starting material, the reaction was stopped by adding aq. saturated aqueous ammonium chloride solution at 0 °C, and the mixture was extracted with ethyl acetate. The combined organic layer was dried over anhydrous sodium sulfate, filtered and the solvent was removed in vacuo. The residue was purified with silica gel column chromatography (petroleum ether: ethyl acetate = 8:1) to give the title compound (3.16 g, 65%) as a yellow
oil. ¹H NMR (300 MHz, [D]₆ DMSO): δ = 1.18 (t, 3H), 4.05 (q, 2 H), 4.70 (s, 1H), 7.50-7.57 (m, 5H), 7.61-7.63 (m, 2H) ppm; LCMS (ESIpos): m/z = 242 [M+H]^+.

4-Amino-5-fluoro-2-(2-methylphenoxy)benzonitrile (49)

To a slurry of sodium hydride (60% in mineral oil, 1.50 g, 36.9 mmol) in 1-methyl-2-pyrrolidinone (100 mL) were added o-cresol (5.30 g, 49.3 mmol) and commercially available 4-amino-2,5-difluorobenzonitrile (CAS: 112279-61-5, 4.00 g, 24.6 mmol) at 0 °C. The resulting mixture was stirred at 100 °C for overnight under nitrogen atmosphere. After cooling to RT, aq. ammonium chloride solution was added at 0 °C and the organic solvent was removed in vacuo. The residue was diluted with water and the resulting mixture was extracted with ethyl acetate. The combined organic layer was dried over anhydrous sodium sulfate and concentrated in vacuo. The residue was purified by silica gel column chromatography (petroleum ether: ethyl acetate = 4:1) to give the title compound (3.0 g, 45% yield) as a yellow solid. LCMS (ESIpos): m/z = 243 [M+H]^+.

4-Amino-5-fluorobenzonitrile (50)

This intermediate can be synthesized in analogy to the previously described procedure (example 49) from commercially available 4-amino-2,5-difluorobenzonitrile (CAS: 112279-61-5) and 2-chlorophenol (CAS: 95-57-8).

4-Amino-5-methoxy-2-(2-methylphenoxy)benzonitrile (51)

To a stirred solution of 4-amino-2-bromo-5-methoxybenzonitrile (CAS: 2384436-37-5, 25.0 g, 0.058 mol) in DMF (250 mL) was added o-cresol (31.6 g, 0.292 mol), caesium carbonate (95.3 g, 0.292 mol) and copper powder (18.6 g, 0.292 mol). The reaction mixture was heated under reflux for 16 h. After completion of the reaction, the reaction mixture was cooled to room temperature, and filtered over a Celite® pad. The filtrate was added to ice water (1 L) and was extracted with ethyl acetate (2x500 mL). The combined organic layers
were evaporated completely. The crude reaction product was purified by RP column chromatography using C18 column using 70% ACN in water as an eluent to give the title compound (9.2 g, 62%) as yellow oil. ¹H-NMR (300 MHz, [D]₆ DMSO): δ = 7.35 (d, 1H), 7.28-7.10 (m, 2H), 7.07 (s, 1H), 6.96 (d, 1H), 6.00 (s, 1H), 5.92 (br s, 2H), 3.78 (s, 3H), 2.19 (s, 3H) ppm.

4-Amino-5-bromo-2-(2-methyloxy)benzonitrile (52)

To a solution of 4-amino-2-(2-methyloxy)benzonitrile (55, 5.0 g, 19.6 mmol, 88% purity) in AcOH (100 mL) was added N-bromosuccinimide (3.50 g, 19.6 mmol) and the resulting mixture was stirred at room temperature for overnight. Upon completion of the reaction, the solvent was removed in vacuo and the residue was re-dissolved with water. The resulting mixture was extracted with ethyl acetate and the combined organic layer was dried over anhydrous sodium sulfate. The solvent was removed in vacuo and the residue was purified by silica gel column chromatography (petroleum ether: ethyl acetate = 3: 1) to give 5.5 g (80% yield) of the product as light-yellow oil.

4-Amino-5-hydroxy-2-(o-tolyl oxy)benzonitrile (53)

To a solution of 4-amino-5-bromo-2-(2-methyloxy)benzonitrile (52, 1.70 g, 4.80 mmol) in 1,4-dioxane (50 mL) were added bis(pinacolato)diboron (2.4 g, 9.50 mmol), potassium acetate (1.40 g, 14.3 mmol) and Pd(dppe)Cl₂ (349 mg, 0.5 mmol). The resulting mixture was stirred at 90 °C overnight under nitrogen atmosphere. After cooling to RT, hydrogen peroxide (486 mg, 14.3 mmol, 30% aq. solution) was added and the resulting mixture was stirred at RT for another 1 h. Upon completion of the reaction, the solvent was removed in vacuo and the residue was diluted with water. The resulting mixture was extracted with ethyl acetate and the combined organic layer was dried over anhydrous sodium sulfate. The solvent was removed in vacuo and the residue was purified by silica gel column chromatography (petroleum ether: ethyl acetate = 2: 1) to give the title compound (800 mg, 63%) as brown oil. LCMS (ESIpos): m/z = 241 [M+H]+.

4-Amino-5-proxy-2-(o-tolyl oxy)benzonitrile (54)
To a solution of aforementioned 4-amino-5-hydroxy-2-(o-tolyloxy)benzonitrile (53, 400 mg, 1.5 mmol, in 10 mL DMF) were added cesium carbonate (976 mg, 3.0 mmol) and 1-iodopropane (382 mg, 2.2 mmol). The resulting mixture was stirred at RT for 2 h. Upon completion of the reaction, the solvent was removed in vacuo and the residue was diluted with water. The resulting mixture was extracted with ethyl acetate and the combined organic layer was dried over anhydrous sodium sulfate. The solvent was removed in vacuo and the residue was purified by silica gel column chromatography (petroleum ether: ethyl acetate = 2:1) to give the title compound (310 mg, 78%) as a light-yellow solid. LCMS (ESIpos): m/z = 283 [M+H]^+.

4-Amino-2-(2-methylphenoxy)benzonitrile (55)

To a solution of commercially available 4-amino-2-bromobenzonitrile (CAS: 53312-82-6, 15 g, 73.8 mmol), in 1-methyl-2-pyrrolidinone (120 mL) were added o-cresol (15.9 g, 147.7 mmol), cesium carbonate (72.2 g, 221.5 mmol), copper(I) iodide (3.7 g, 36.9 mmol), and commercially available 2,2,6,6-tetramethylheptane-3,5-dione (CAS: 1118-71-4, 13.6 g, 73.8 mmol). The resulting mixture was stirred at 120 °C overnight under nitrogen atmosphere. After cooled to RT, the solvent was removed in vacuo and the residue was diluted with water. The resulting mixture was extracted with ethyl acetate and the combined organic layer was dried over anhydrous sodium sulfate. The solvent was removed in vacuo and the residue was purified by silica gel column chromatography (petroleum ether: ethyl acetate = 3:1) to give the title compound (14.0 g, 76%) as a yellow solid. LCMS (ESIpos): m/z = 225 [M+H]^+.

4-Amino-5-chloro-2-(2-methylphenoxy)benzonitrile (56)

To a solution of aforementioned 4-amino-2-(2-methylphenoxy)benzonitrile (55, 3.0 g, 12.0 mmol) in acetic acid (100 mL) was added N-chlorosuccinimide (1.6 g, 12.0 mmol), and the resulting mixture was stirred at
RT overnight. Upon completion of the reaction, the solvent was removed in vacuo and the residue was redissolved with water. The resulting mixture was extracted with ethyl acetate and the combined organic layer was dried over anhydrous sodium sulfate. The solvent was removed in vacuo and the residue was purified by silica gel column chromatography (petroleum ether: ethyl acetate = 8:1) to give 2.1 g (57% yield) of the title compound as a yellow solid. LCMS (ESIpos): m/z = 259 [M+H]+.

**4-Amino-5-iodo-2-(2-methylphenoxy)benzonitrile (57)**

![Chemical structure of 4-Amino-5-iodo-2-(2-methylphenoxy)benzonitrile](attachment:image.png)

To a solution of aforementioned 4-amino-2-(2-methylphenoxy)benzonitrile (55, 1.0 g, 4.0 mmol), in acetic acid (50 mL) was added N-iodosuccinimide (903 mg, 4.0 mmol), and the resulting mixture was stirred at RT overnight. Upon completion of the reaction, water was added, and the resulting mixture was extracted with ethyl acetate. The combined organic layer was dried over anhydrous sodium sulfate and concentrated in vacuo. The residue was purified by silica gel column chromatography (petroleum ether: ethyl acetate = 5:1) to give the title compound (900 mg, 64%) as a yellow solid. LCMS (ESIpos): m/z = 351 [M+H]+.

Compound 62 was synthesized in a 5 steps synthetic route starting from commercially available 4-nitronaphthalen-1-amine (CAS: 776-34-1):

![Synthetic route](attachment:image.png)
Supplementary Scheme S2: Synthetic route for (62)

2-Bromo-4-nitronaphthalen-1-amine (58)

To a stirred solution of commercially available 4-nitronaphthalen-1-amine (CAS: 776-34-1, 250 g, 1.33 mol) in acetonitrile (4 L) were added ammonium acetate (10.2 g, 0.132 mol) and N-bromosuccinimide (260 g, 1.46 mol, portion wise) at 10 °C. The resulting mixture was allowed to stir at RT for 1.5 h. A solid precipitated during the reaction, which was filtered, washed with water, and dried over vacuum giving the title compound (300 g, 86% yield) as a pale-yellow solid. LCMS (ESIpos): m/z = 267 [M+H]+.

2-(2-Methylphenoxy)-4-nitronaphthalen-1-amine (59)

To a solution of aforementioned 2-bromo-4-nitronaphthalen-1-amine (58, 21.0 g, 74.7 mmol) in 1-methyl-2-pyrrolidinone (150 mL) were added o-cresol (80.8 g, 746 mmol), copper(i) chloride (7.40 g, 74.7 mmol), cesium carbonate (48.6 g, 149.3 mmol) and 2,2,6,6-tetramethylheptane-3,5-dione (27.4 g, 149 mmol). The resulting mixture was stirred at 120 °C overnight under nitrogen atmosphere. After cooling to RT, the solvent was removed in vacuo and the residue was diluted with water. The resulting mixture was extracted with ethyl acetate and the combined organic layer was dried over anhydrous sodium sulfate. The solvent was removed in vacuo and the residue was purified by silica gel column chromatography (petroleum ether: ethyl acetate = 2:1) to give 5.5 g (90% purity, 23% yield) of the title compound as a brown solid. LCMS (ESIpos): m/z = 295 [M+H]+.

1-Bromo-2-(2-methylphenoxy)-4-nitronaphthalene (60)

To a solution of aforementioned 2-(2-methylphenoxy)-4-nitronaphthalen-1-amine (59, 5.50 g, 16.8 mmol) in acetonitrile (80 mL) were added copper(II) bromide (4.9 g, 21.9 mmol) and tert-butyl nitrite (2.3 g, 21.9 mmol). The resulting mixture was stirred at 70 °C for 2 h under nitrogen atmosphere. After cooled to RT, the solvent was removed in vacuo and the residue was purified by silica gel column chromatography (petroleum ether:...
ethyl acetate = 10:1) to give the title compound (3.0 g, 45% yield) as a brown solid. \(^1\)H-NMR (400 MHz, [D]_6 DMSO): δ [ppm] = 2.28 (s, 3H), 6.92 (d, 1H), 7.18 (t, 1H), 7.25 (t, 1H), 7.41 (d, 1H), 7.81-7.85 (m, 2H), 7.91 (t, 1H), 8.35 (d, 1H), 8.41 (d, 1H).

2-(2-Methylphenoxy)-4-nitronaphthalene-1-carbonitrile (61)

To a solution of aforementioned 1-bromo-2-(2-methylphenoxy)-4-nitronaphthalene (60, 3.0 g, 7.5 mmol) in DMF (20 mL) were added zinc cyanide (2.7 g, 22.6 mmol), 1,2-bis(dimethylamino)ethane (876 mg, 7.5 mmol), Pd(dba)_2 (690 mg, 0.8 mmol) and Xantphos (873 mg, 1.5 mmol). The resulting mixture was irradiated with microwaves for 10 min at 180 °C. After cooling to RT, water was added and the resulting mixture was extracted with ethyl acetate. The combined organic layer was dried over anhydrous sodium sulfate and concentrated in vacuo. The residue was purified by silica gel column chromatography (petroleum ether: ethyl acetate = 10:1) to give the title compound (720 mg, 25% yield) as a brown solid.

4-Amino-2-(2-methylphenoxy)naphthalene-1-carbonitrile (62)

To a solution of aforementioned 2-(2-methylphenoxy)-4-nitronaphthalene-1-carbonitrile (61, 720 mg, 80% purity, 1.9 mmol) in acetic acid (40 mL) was added iron powder (1.6 g, 28.4 mmol) and the resulting mixture was stirred at RT for 2 h. Upon completion of the reaction, water was added, and the resulting mixture was extracted with ethyl acetate. The combined organic layer was washed with water, brine, dried over anhydrous sodium sulfate and concentrated in vacuo. The residue was purified by silica gel column chromatography (petroleum ether: ethyl acetate = 2:1) to give the title compound (550 mg, 80% purity, 84%) as a yellow solid. LCMS (ESIpos): m/z = 275 [M+H]^+.
Compound 64 was synthesized in a two-step synthetic route starting from 4-amino-5-methoxy-2-(2-methylphenoxy)benzonitrile (51):

Supplementary Scheme S3: Synthetic route for (64)

1-(4-Cyano-2-methoxy-5-(o-tolyloxy)phenyl)urea (63)

To a stirred solution of 4-amino-5-methoxy-2-(2-methylphenoxy)benzonitrile (51, 10 g, 0.040 mol) in acetic acid (700 mL) and water (30 mL) was added sodium cyanate (6.40 g, 0.098 mol). The reaction mixture was stirred at room temperature for 16 h. The reaction was monitored by thin layer chromatography. After completion of the reaction, the solid formed during reaction was filtered off, washed with petrol ether (200 mL) and dried under vacuum to give the title compound (10 g, 86% yield) as off-white solid. $^1$H NMR (300 MHz, [D]$_6$ DMSO): $\delta$ = 8.45 (s, 1H), 7.88 (s, 1H), 7.40 (s, 1H), 7.33 (br d, 1H), 7.25-7.17 (m, 1H), 7.15-7.07 (m, 1H), 6.85 (d, 1H), 6.47 (br s, H), 3.90 (s, 3H), 2.21 (s, 3H) ppm.

5-Methoxy-2-(o-tolyloxy)-4-(2,4,6-trioxotetrahydropyrimidin-1(2H)-yl)benzonitrile (64)

To stirred ethanol (200 mL) at 0 °C was added metallic sodium (6.20 g, 0.269 mol) and the mixture was stirred at 0 °C until the sodium metal was dissolved completely. Then was added the aforementioned 1-(4-cyano-2-methoxy-5-(o-tolyloxy)phenyl)urea (63, 10 g, 0.033 mol) and diethyl malonate (27.0 g, 0.168 mol). The reaction mixture was stirred for 16 h at reflux temperature. After completion of the reaction, the solvent...
was evaporated under vacuum, and the thus obtained solid was acidified with 20% aq. HCl (up to 6.5-7 pH). The solid was filtered off and washed with water (50 mL) and dried under vacuum to give the title compound (6.8 g) as an off-white solid.

\[ \text{H NMR (300 MHz, [D\textsubscript{6}]DMSO): } \delta = 11.56 \text{ (br s, 1 H), 7.72 (s, 1H), 7.36 (br d, 1H), 7.32-7.23 (m, H), 7.23-7.14 (m, 1H), 7.01 (d, 1H), 6.81 (s, 1H), 3.80 (s, 3H), 3.36 (s, H), 2.16 (s, 3H).} \]

All carbamates were synthesized following to the general procedure GP1 as in the example 65 below.

**General Procedure 1: Formation of the carbamate**

To an ice-cooled solution of the aniline (1.0 eq.) in pyridine (1.5 mL/mmol), was added dropwise the commercially available either ethyl chloroformiate (CAS: 541-41-3, 1.2 eq.) or methyl carbonochloridoate (CAS: 79-22-1). After complete addition, the corresponding suspension was stirred at 0 °C for 2 h, or until the reaction was complete. Upon reaction completion, the reaction mixture was diluted with an aq. solution of 1 M HCl (10 mL/mmol). The resulting precipitate (carbamate) was collected by filtration and washed with water and dried under vacuum overnight and used without any further purification step in the next reaction step.

**Ethyl [5-(2-chlorophenoxy)-4-cyano-2-fluorophenyl]carbamate (65)**

To an ice-cooled solution of 4-amino-2-(2-chlorophenoxy)-5-fluorobenzonitrile (50) (200 mg, 0.761 mmol) in pyridine (1.2 mL), ethyl chloroformate (93 µL, 0.975 mmol) was added dropwise to the yellow solution. After complete addition, the corresponding suspension was stirred at 0 °C for 4 h. UPLCMS control indicated remaining starting material so that additional ethyl chloroformiate (0.5 eq.) was added dropwise. Upon reaction completion (further 16 h), the reaction mixture was diluted with an aq. solution of 1 M HCl (15 mL). The resulting precipitate was collected by filtration and washed with water and dried under vacuum overnight and used without any further purification in the next reaction step to give the title compound (233 mg, 85% pure, 73% yield) as a light orange solid; \( \text{H NMR (400 MHz, [D\textsubscript{6}]DMSO): } \delta = 1.17 \text{ (t, } J = 7.1 \text{ Hz, 3H), 4.08 (q, } J = 7.1 \text{ Hz, 2H), 7.29-7.40, 7.45-7.50 (2 m, 3H, 1H), 7.56 (d, } J = 7.6 \text{ Hz, 1H), 7.94 (d, } J = 7.9 \text{ Hz, 1H), 10.02 (bs, 1 H) ppm; LCMS (ESIpos): } m/z = 319 [M+H]^{+}. \)

**Supplementary Table S2.** Analytics of carbamates 66-69

| name | structure | analytics |
|------|-----------|-----------|
| Ethyl (4-cyano-3-fluorophenyl)carbamate (66) | ![Structure](image) | \( \text{H NMR (400 MHz, [D\textsubscript{6}]DMSO): } \delta = 1.25 \text{ (t, } J = 7.1 \text{ Hz, 3H), 4.17 (d, } J = 7.1 \text{ Hz, 2H), 7.36 (dd, } J = 8.6, 2.0 \text{ Hz, 1H), 7.62 (dd, } J = 12.6, 1.9 \text{ Hz, 1H), 7.81 (dd, } J = \) |
### Ethyl (4-cyano-5-fluoro-2-methylphenyl)carbamate (67)

| δ (ppm) | J (Hz) | Assignment |
|---------|--------|------------|
| 1.27    | 7.1    | 3H         |
| 2.22    | 3H     | 3H         |
| 4.18    | 7.1    | 2H         |
| 7.69    | 7.6    | 1H         |
| 7.79    | 12.4   | 1H         |

1H NMR (400 MHz, [D₆]DMSO): δ = 1.27 (t, J = 7.1 Hz, 3H), 2.22 (s, 3H), 4.18 (q, J = 7.1 Hz, 2H), 7.69 (d, J = 7.6 Hz, 1H), 7.79 (d, J = 12.4 Hz, 1H), and 9.34 (bs, NH) ppm; LCMS (ESIpos): m/z: [M+H]⁺ = 222.

### Ethyl (4-cyano-5-fluoro-2-methoxyphenyl)carbamate (68)

| δ (ppm) | J (Hz) | Assignment |
|---------|--------|------------|
| 1.25    | 7.1    | 3H         |
| 4.17    | 7.1    | 2H         |
| 7.50    | 6.1    | 1H         |
| 7.95    | 7.3    | 1H         |

1H NMR (400 MHz, [D₆]DMSO): δ = 1.25 (t, J = 7.1 Hz, 3H), 4.17 (q, J = 7.1 Hz, 2H), 7.50 (d, J = 6.1 Hz, 1H), 7.95 (d, J = 7.3 Hz, 1H), 9.14 (s, 1H) ppm; LCMS (ESIpos): m/z: [M+H]⁺ = 239.

### Ethyl (5-bromo-4-cyano-2-methoxyphenyl)carbamate (69)

| δ (ppm) | J (Hz) | Assignment |
|---------|--------|------------|
| 1.24    | 7.1    | 3H         |
| 3.85    | 3H     | 3H         |
| 4.16    | 7.1    | 2H         |
| 7.58    | 8.25   | 1H each    |
| 9.11    |        | bs, NH     |

1H NMR (400 MHz, [D₆]DMSO): δ = 1.24 (t, J = 7.1 Hz, 3H), 3.85 (s, 3H), 4.16 (q, J = 7.1 Hz, 2H), 7.58, 8.25 (2 s, 1H each), and 9.11 (bs, NH) ppm; LCMS (ESIpos): m/z: [M+H]⁺ = 298.

### Methyl [2-bromo-4-cyano-5-(2-methylphenoxy)phenyl]carbamate (70)

To a solution of 4-amino-5-bromo-2-((o-tolyl)oxy)benzonitrile (48, 5.5 g, 16.3 mmol, 90% purity) in pyridine (80 mL) was added methyl carbonochloridate (7.7 g, 81.6 mmol) and the resulting mixture was stirred at room temperature for 4 hours. Upon completion of the reaction, the solvent was removed in vacuo and the residue was dissolved with water. The resulting mixture was extracted with ethyl acetate and the combined organic
layer was dried over anhydrous sodium sulfate. The solvent was removed in vacuo and the residue was purified by silica gel column chromatography (petroleum ether: ethyl acetate = 3:1) to give 2.1 g (28% yield) of the product as a light-yellow solid. LCMS (ESI neg): m/z = 359 [M-H].

Supplementary Table S3. Analytics of carbamates 71-78

| name | structure | analytics |
|------|-----------|-----------|
| Methyl 4-cyano-2-proxy-5-(o-tolyl)oxyphenylcarbamate (71) | ![Structure](image1.png) | LCMS (ESI pos): m/z = 341 [M+H]+. |
| Methyl [2-chloro-4-cyano-5-(2-methylphenoxy)phenyl]carbamate (72) | ![Structure](image2.png) | LCMS (ESI pos): m/z = 317 [M+H]+. |
| Methyl 4-cyano-2-iodo-5-(o-tolyl)oxyphenylcarbamate (73) | ![Structure](image3.png) | LCMS (ESI pos): m/z = 407 [M-H]. |
| Ethyl (5-bromo-2-methoxyphenyl)carbamate (74) | ![Structure](image4.png) | ¹H NMR (400 MHz, [D]₆ DMSO): δ = 1.23 (t, J = 7.1 Hz, 3H), 4.11 (q, J = 7.0 Hz, 2H), 6.91 (d, J = 8.9 Hz, 1H), 7.21 (dd, J = 8.7, 2.4 Hz, 1H), 7.88 (d, J = 2.2 Hz, 1H), 8.58 (s, 1H) ppm; LCMS (ESI pos): m/z = 273 [M+H]+. |
| Ethyl (5-bromo-2-fluorophenyl)carbamate (75) | ![Structure](image5.png) | ¹H NMR (400 MHz, [D]₆ DMSO): δ = 1.24 (t, J = 7.1 Hz, 3H), 4.14 (d, J = 7.1 Hz, 2H), 7.21, 7.27 (2 m, 1H each), 7.92 (dd, J = 1... |
| Chemical Structure | Chemical Information |
|--------------------|---------------------|
| ![Chemical Structure](image.png) | 7.1, 2.0 Hz, 1H), 9.55 (bs, NH) ppm; LCMS (ESIpos): m/z = 262 [M+H]^+. |
| ![Chemical Structure](image.png) | \( ^1 \text{H NMR (400 MHz, [D]_6 DMSO): } \delta = 1.23 \text{ (t, J = 7.1 Hz, 3H)}, 2.29 \text{ (s, 3H), 4.12 \text{ (q, J = 7.1 Hz, 2H), 7.30 \text{ (d, J = 7.3 Hz, 1H), 7.87 \text{ (bd, J = 7.7 Hz, 1H), 7.58 \text{ (m, 1H), 9.42 (bs, NH) ppm; LCMS (ESIneg): m/z = 274 [M-H].}}) } \). |
| ![Chemical Structure](image.png) | \( ^1 \text{H NMR (400 MHz, [D]_6 DMSO): } \delta = 1.22 \text{ (t, J = 7.0 Hz, 3H), 2.17, 3.80 \text{ (2s, 3H each), 4.08 \text{ (q, J = 7.0 Hz, 2H), 6.96 \text{ (s, 1H), 7.47, 8.80 \text{ (2 bs, 1H each), LCMS (ESIpos): m/z = 288 [M+H]^+.}}) } \). |
| ![Chemical Structure](image.png) | LCMS (ESIpos): m/z = 333 [M+H]^+. |

**General Procedure 2: Formation of the pyrimidine diones**

To a cooled suspension of commercially available NaH (CAS: 7440-23-5, 60% in mineral oil, 1.5 eq.) in DMF (2.6 mL/ mmol), the acrylate ester (1.1 eq) was added dropwise. The reaction mixture was stirred at RT for around 40 min (until no formation of gas was observed anymore). Then the carbamate (1.0 eq., dissolved in a sufficient amount of DMF) was added and the reaction solution was heated to 90 °C for 18 h- 20 h, or until the reaction was complete. Upon reaction completion, the mixture was poured into water, and the resulting precipitate was filtered off, and the aq. solution was acidified until pH = 3 with an aq. solution 2 M HCl (3.5 mL/mmol). The solution was then diluted with DCM. The organic phase was extracted twice. The combined organic layers were washed with brine and dried with sodium sulfate. After filtration, the solvent was removed under vacuum. The resulted residue was purified using preparative HPLC (basic) giving the desired cyclized product.
Reaction was divided in three vials: to a cooled suspension of NaH (60% in mineral oil, 745 mg, 18.6 mmol) in DMF (32.4 mL), ethyl-3-amino-4,4,4-trifluorobut-2-enoate (2.6 mL, 17 mmol, dissolved in 0.3 mL DMF) was added dropwise. The reaction mixture was stirred at RT for around 40 min (until no formation of gas was observed anymore). Then the ethyl carbamate (60, 2.72 g, 12.4 mmol, dissolved in 0.3 mL DMF) was added and the reaction solution was heated to 90 °C for 19 h. Upon reaction completion, the mixture (containing the reunited three vials) was poured into water (220 mL), and the resulting precipitate was filtered off, and the aq. solution was acidified until pH = 3 with an aq. solution 2 M HCl (18 mL). The solution was then diluted with DCM. The organic phase was extracted twice. The combined organic layers were washed with brine and dried with sodium sulfate. After filtration, the solvent was removed under vacuum. The resulted residue was purified using HPLC-HT (basic) giving the desired title product in two fractions (1.27 g with 80% purity; 27.3 mg with 95% purity; 30%) as both colorless solids. Analytics of the 95%pure fraction: \(^1\)H NMR (400 MHz, [D]$_6$ DMSO): \(\delta = 6.42\) (s, 1H), 7.46 (dd, \(J = 8.2, 1.7\) Hz, 1H), 7.68 (dd, \(J = 10.1, 1.5\) Hz, 1H), 8.09 (t, \(J = 7.4\) Hz, 1H), 12.71 (bs, NH) ppm; LCMS (ESIpos): \(m/z = 300\) [M+H]$^+$. 

**Supplementary Table S4. Analytics of pyrimidine diones 80-91**

| name | structure | analytics |
|------|-----------|-----------|
| 4-[2,6-Dioxo-4-(trifluoromethyl)-3,6-dihydropyrimidin-1(2H)-yl]-2-fluoro-5-methylbenzonitrile (80) | ![Structure](image1) | \(^1\)H NMR (400 MHz, [D]$_6$ DMSO): \(\delta = 2.09\) (s, 3H), 6.45 (s, 1H), 7.66 (d, \(J = 9.9\) Hz, 1H), 8.0 (d, \(J = 7.1\) Hz, 1H), 12.78 (bs, NH) ppm; LCMS (ESIpos): \(m/z = 313\) [M+H]$^+$. |

Using acrylester 47:

| 4-[4-(Chloro(difluoro)methyl)-2,6-dioxo-3,6-dihydropyrimidin-1(2H)-yl]^-2-fluoro-5-methoxybenzonitrile (81) | ![Structure](image2) | \(^1\)H NMR (400 MHz, [D]$_6$ DMSO): \(\delta = 3.81\) (s, 3H), 6.23 (bs, 1H), 7.71 (d, \(J = 9.1\) Hz, 1H), 7.79 (d, \(J = 5.6\) Hz, 1H), 12.76 (bs, NH) ppm; LCMS (ESIpos): \(m/z = 346\) [M+H]$^+$. |
| Chemical Structure | Spectroscopy Data |
|--------------------|------------------|
| 3-(5-Bromo-2-chlorophenyl)-6-(trifluoromethyl)pyrimidine-2,4(1H,3H)-dione (82) | **^1H NMR** (400 MHz, [D₆]DMSO): \( \delta = 6.40 \) (s, 1H), 7.07 (t, \( J = 0.13 \) Hz, 1H), 7.62 (d, \( J = 8.9 \) Hz, 1H), 7.71 (dd, \( J = 8.6, 2.3 \) Hz, 1H), 7.83 (m, 1H), 12.81 (bs, NH) ppm; *possible ammonium salt; LCMS (ESIpos): m/z = 369 [M+H]^+. |
| 4-[4-(Difluoromethyl)-2,6-dioxo-3,6-dihydropyrimidin-1(2H)-yl]-2-fluoro-5-methoxybenzonitrile (83) | **^1H NMR** (400 MHz, [D₆]DMSO): \( \delta = 3.80 \) (s, 3H), 6.09 (s, 1H), 6.83 (t, \( J = 6.8 \) Hz, 1H), 7.70 (d, \( J = 9.1 \) Hz, 1H), 7.77 (d, \( J = 5.6 \) Hz, 1H), 12.24 (bs, NH) ppm; LCMS (ESIneg): m/z = 310 [M-H]^-. |
| 2-Bromo-4-[2,6-dioxo-4-(pentafluoroethyl)-3,6-dihydropyrimidin-1(2H)-yl]-5-methoxybenzonitrile (84) | **^1H NMR** (400 MHz, [D₆]DMSO): \( \delta = 3.82 \) (s, 3H), 6.30 (bs, 1H), 7.07 (t, \( J = 51 \) Hz, 1H)^*, 7.83 (s, 1H), 7.91 (bs, 1H), 12.74 (bs, NH) ppm; *possibly ammonium salt; LCMS (ESIpos): m/z = 442 [M+H]^+. |
| 2-Bromo-4-{4-[difluoro(phenyl)methyl]-2,6-dioxo-3,6-dihydropyrimidin-1(2H)-yl}-5-methoxybenzonitrile (85) | **^1H NMR** (400 MHz, [D₆]DMSO): \( \delta = 3.79 \) (s, 3H), 6.11 (bs, 1H), 7.57-7.63 (m, 3H), 7.11 (d, \( J = 9.4 \) Hz, 1H), 7.75 (m, 3H), 12.25 (bs, NH) ppm; LCMS (ESIneg): m/z = 447 [M-H]^-. |
| 3-(5-Bromo-2-fluorophenyl)-6-(trifluoromethyl)pyrimidine-2,4(1H,3H)-dione (86) | **^1H NMR** (400 MHz, [D₆]DMSO): \( \delta = 6.42 \) (s, 1H), 7.42 (t, \( J = 9.3 \) Hz, 1H), 7.72 (m, 1H), 7.79 (dd, \( J = 6.6, 2.5 \) Hz, 1H), 12.8 (bs, NH) ppm; LCMS (ESIpos): m/z = 352 [M+H]^+. |
| Compound                                                                 | 1H NMR (400 MHz, [D₆]DMSO) | LCMS (ESIpos) |
|------------------------------------------------------------------------|-----------------------------|---------------|
| 3-(5-Bromo-2-methoxyphenyl)-6-(trifluoromethyl)pyrimidine-2,4(1H,3H)-dione (87) | δ = 3.74 (s, 3H), 6.36 (s, 1H), 7.15 (d, J = 8.9 Hz, 1H), 7.55 (d, J = 2.6 Hz, 1H), 7.61 (dd, J = 2.6, 8.9 Hz, 1H), 12.64 (bs, NH) ppm; m/z = 367 [M+H]^+. | m/z = 367 [M+H]^+. |
| 3-(5-Bromo-2-fluoro-4-methylphenyl)-6-(trifluoromethyl)pyrimidine-2,4(1H,3H)-dione (88) | δ = 2.39 (s, 3H), 6.24 (bs, 1H), 7.46 (d, J = 10.9 Hz, 1H), 7.69 (d, J = 7.1 Hz, 1H), 12.76 (bs, NH) ppm; m/z = 368 [M+H]^+. | m/z = 368 [M+H]^+. |
| 3-(5-Bromo-4-chloro-2-methoxyphenyl)-6-(trifluoromethyl)pyrimidine-2,4(1H,3H)-dione (89) | δ = 1.22 (t, J = 7.0 Hz, 3H), 4.12 (q, J = 7.0 Hz, 2H), 7.28, 8.04 (2s, 1H each), 8.74 (bs, NH) ppm; m/z = 307 [M+H]^+. | m/z = 307 [M+H]^+. |
| 3-(5-Bromo-4-methoxy-2-methylphenyl)-6-(trifluoromethyl)pyrimidine-2,4(1H,3H)-dione (90) | δ = 2.02, 3.88 (2s, 3H each), 6.36, 7.10, 7.52 (3s, 1H each), 12.56 (bs, NH) ppm; m/z: [M-H]^− = 378. | m/z: [M-H]^− = 378. |
| 2-Bromo-4-[2,6-dioxo-4-(trifluoromethyl)-3,6-dihydropyrimidin-1(2H)-yl]-5-methoxybenzonitrile (91) | δ = 3.83 (s, 3H), 6.42 (s, 1H), 7.85 (s, 1H), 7.93 (s, 1H), 12.71 – 12.88 (br.s, 1H); m/z = 389.9 [M+H]^+. | m/z = 389.9 [M+H]^+. |
Supplementary Scheme S4: Synthetic scheme for the synthesis of compounds 1-8

2-Amino-N-methylpyridine-4-carboxamide (92)

To a solution of 2-aminoisonicotinic acid (4.00 g, 28.4 mmol), in DMF (100 mL) were added methanamine hydrochloride (2.30 g, 34.1 mmol), N-ethyl-N-isopropylpropan-2-amine (7.30 g, 56.7 mmol), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (6.50 g, 34.1 mmol) and 1-hydroxybenzotriazole (5.20 g, 34.1 mmol). The resulting mixture was stirred at room temperature for overnight. Upon completion of the reaction, the solid was removed by filtration and the filtrate was purified by C\textsubscript{18} reversed phase column chromatography [Column: 180 g, Mobile phase A: water; Mobile phase B: acetonitrile; Gradient: 0% B to 12% B in 20 min] to give 3.0 g (64% yield) of the title compound as a white solid. LCMS (ESIpos): m/z = 152 [M+H]+.

N-Methyl-2-(pyridin-2-yl)-3-[[(2,4,4-trimethylpentan-2-yl)amino]imidazo[1,2-a]pyridine-7-carboxamide (93)

To a solution of 2-amino-N-methylisonicotinamide (3.0 g, 18.3 mmol) in methanol (100 mL) were added picolinaldehyde (1.90 g, 18.3 mmol), indium(III) trifluoromethanesulfonate (1.00 g, 1.80 mmol), and 2-isocyano-2,4,4-trimethylpentane (2.50 g, 18.3 mmol). The resulting mixture was stirred at 80 °C for 30 min under nitrogen atmosphere. After cooling to room temperature, the solvent was removed in vacuo and the residue was diluted with water. The resulting mixture was extracted with ethyl acetate and the combined organic layer was dried over anhydrous sodium sulfate. The solvent was removed in vacuo to give 6.00 g (84% yield) of the title compound as an orange solid. \textsuperscript{1}H-NMR (400 MHz, [D]\textsubscript{6} DMSO): δ = 1.01 (s, 6H), 1.10 (s, 9H), 1.68 (s, 2H), 2.82 (d, 3H), 5.55 (s, 1H), 7.28-7.36 (m, 2H), 7.91 (t, 1H), 8.01 (s, 1H), 8.11 (d, 1H), 8.42 (d, 1H), 8.62 (d, 2H) ppm; LCMS (ESIpos): m/z = 360 [M+H]+.
To a solution of N-methyl-2-(pyridin-2-yl)-3-(2,4,4-trimethylpentan-2-ylamino)imidazo[1,2-a]pyridine-7-carboxamide (3.00 g, 7.9 mmol), in dichloromethane (100 mL) was added trifluoroacetic acid (10 mL), and the resulting mixture was stirred at room temperature for overnight. Upon completion of the reaction, the solvent was removed in vacuo and the residue was diluted with n-hexane. The precipitated solid was collected by filtration and the filter cake was dried in vacuo to give 1.80 g (85% yield) of the title compound as an orange solid.

**1H-NMR (400 MHz, [D]6 DMSO):** δ = 2.85 (d, 3H), 7.37-7.40 (m, 1H), 7.63 (d, 1H), 8.01-8.06 (m, 2H), 8.11 (s, 1H), 8.63 (d, 1H), 8.70 (d, 1H), 8.89 (d, 1H), 11.32 (br, 2H) ppm; LCMS (ESIpos): m/z = 268 [M+H]+.

To a solution of 3-amino-N-methyl-2-(pyridin-2-yl)imidazo[1,2-a]pyridine-7-carboxamide (1.0 g, 3.6 mmol) in pyridine (30 mL) was added methyl carbonochloridate (330 mg, 3.6 mmol), and the resulting mixture was stirred at room temperature for overnight. Upon completion of the reaction, water was added and the resulting mixture was extracted with ethyl acetate. The combined organic layer was dried over anhydrous sodium sulfate and concentrated in vacuo. The residue was purified by silica gel column chromatography (dichloromethane: methanol = 10: 1) to give 350 mg (30% yield) of the title compound as a light-yellow solid.

**LCMS (ESIpos):** m/z = 326 [M+H]+.
4. Biology

Supplementary Figure S17. BCAT1 biochemical activity of compound 36a (BAY-069) in the presence of bovine serum albumin (BSA) or human serum albumin (HSA).

Supplementary Table S5. Antiproliferative activity of 36a (BAY-069) and A in a panel of cancer cell lines.

Cancer cell viability was determined by CellTiter-Glo® after treatment with the indicated compounds.

| Compound | MDA-MB-231 IC_{50} | U-87 MG IC_{50} | SEM IC_{50} | CAL-51 IC_{50} | HCC-33 IC_{50} | NCI-H2110 IC_{50} |
|----------|---------------------|-----------------|-------------|----------------|----------------|------------------|
| BAY-069  | >50 µM              | >50 µM          | >50 µM      | 47.7 µM        | >50 µM         | >50 µM           |
| A        | >50 µM              | >50 µM          | >50 µM      | >50 µM         | >50 µM         | >50 µM           |

Supplementary Figure S18. BCAT1 and BCAT2 protein expression in a panel of cancer cell lines, determined by Western Blot.
Supplementary Figure S19: Correlation between biochemical BCAT1 and cellular activity (BCAA in U-87 MG): Outliers (red) show low Caco permeability (Papp A-B), green reflects good and yellow medium Caco permeability.
Supplementary Table S6. Summary of experimental results obtained for 36a (BAY-069) in the Eurofins LeadProfilingScreen panel.21

| Cat #  | Assay Name                                      | Batch* | Spec. | Rep. | Conc. | % Inh. | IC50* | Ki | pN | R  |
|--------|------------------------------------------------|--------|-------|------|-------|--------|-------|----|----|----|
| 107000 | Aldose Reductase                               | 433410 | rat   | 2    | 10 µM | -1     |       |    |    |    |
| 107710 | ATPase, Na+/K+; Heart, Pig                     | 433438 | pig   | 2    | 10 µM | -13    |       |    |    |    |
| 112020 | Carbonic Anhydrase II                          | 433411 | hum   | 2    | 10 µM | 1      |       |    |    |    |
| 104010 | Cholinesterase, Acetyl, ACES                   | 433409 | hum   | 2    | 10 µM | 8      |       |    |    |    |
| 116020 | Cyclooxygenase COX-1                           | 433651 | hum   | 2    | 10 µM | -10    |       |    |    |    |
| 118010 | Cyclooxygenase COX-2                           | 433652 | hum   | 2    | 10 µM | 7      |       |    |    |    |
| 124010 | HMG-CoA Reductase                              | 433419 | hum   | 2    | 10 µM | 0      |       |    |    |    |
| 132000 | Leukotriene LTc4 Synthase                      | 433418 | gp    | 2    | 10 µM | 29     |       |    |    |    |
| 199017 | Lipooxygenase 15-LO                            | 433427 | hum   | 2    | 10 µM | 15     |       |    |    |    |
| 140010 | Monoamine Oxidase MAO-A                        | 433440 | hum   | 2    | 10 µM | 8      |       |    |    |    |
| 140120 | Monoamine Oxidase MAO-B                        | 433441 | hum   | 2    | 10 µM | 8      |       |    |    |    |
| 142000 | Nitric Oxide Synthase, Neuronal (nNOS)         | 433420 | rat   | 2    | 10 µM | -4     |       |    |    |    |
| 199010 | Nitric Oxide Synthetase, Inducible (iNOS)      | 433425 | mouse | 2    | 10 µM | 14     |       |    |    |    |
| 107300 | Peptidase, Angiotensin Converting Enzyme       | 433437 | rabbit| 2    | 10 µM | 1      |       |    |    |    |
| 152000 | Phosphodiesterase PDE3                         | 433452 | hum   | 2    | 10 µM | -8     |       |    |    |    |
| 154420 | Phosphodiesterase PDE4D2                       | 433454 | hum   | 2    | 10 µM | 2      |       |    |    |    |
| 156000 | Phosphodiesterase PDE5                         | 433453 | hum   | 2    | 10 µM | -19    |       |    |    |    |
| 194020 | Thromboxane Synthase                           | 433426 | hum   | 2    | 10 µM | 45     |       |    |    |    |
| 200510 | Adenosine A1                                   | 433503 | hum   | 2    | 10 µM | 27     |       |    |    |    |
| 200610 | Adenosine A2A                                   | 433505 | hum   | 2    | 10 µM | -6     |       |    |    |    |
| 200720 | Adenosine A2                                   | 433689 | hum   | 2    | 10 µM | -2     |       |    |    |    |
| 203100 | Adrenergic A1A                                 | 433443 | rat   | 2    | 10 µM | 5      |       |    |    |    |
| 203630 | Adrenergic A1A                                 | 433428 | hum   | 2    | 10 µM | 2      |       |    |    |    |
| 203710 | Adrenergic A2B                                 | 433429 | hum   | 2    | 10 µM | -2     |       |    |    |    |
| 203810 | Adrenergic A2C                                 | 433430 | hum   | 2    | 10 µM | 6      |       |    |    |    |
| 204010 | Adrenergic B1                                  | 433456 | hum   | 2    | 10 µM | 3      |       |    |    |    |
| 204110 | Adrenergic B2                                  | 433457 | hum   | 2    | 10 µM | -11    |       |    |    |    |
| 204200 | Adrenergic B3                                  | 433459 | hum   | 2    | 10 µM | 15     |       |    |    |    |
| 206000 | Androgen (Testosterone)                        | 433476 | hum   | 2    | 10 µM | 9      |       |    |    |    |
| 210030 | Angiotensin AT1                                | 433527 | hum   | 2    | 10 µM | 7      |       |    |    |    |
| 210120 | Angiotensin AT2                                | 433528 | hum   | 2    | 10 µM | 6      |       |    |    |    |
| 212520 | Bradykinin B1                                 | 433509 | hum   | 2    | 10 µM | -8     |       |    |    |    |
| 212620 | Bradykinin B2                                 | 433464 | hum   | 2    | 10 µM | 1      |       |    |    |    |
| 217030 | Cannabinoid CB1                                | 433671 | hum   | 2    | 10 µM | 16     |       |    |    |    |
| Cat #   | Assay Name                                                                 | Batch* | Spec. | Rep. | Conc.   | % Inh. | IC50* | Kᵢ | nh | R  |
|---------|----------------------------------------------------------------------------|--------|-------|------|---------|--------|-------|----|----|----|
| 217100  | Cannabinoid CB₂                                                            | 433530 | hum   | 2    | 10 µM   | -5     |       |    |    |    |
| 219500  | Dopamine D₁                                                               | 433525 | hum   | 2    | 10 µM   | 7      |       |    |    |    |
| 219600  | Dopamine D₂                                                               | 433523 | hum   | 2    | 10 µM   | -5     |       |    |    |    |
| 219700  | Dopamine D₃β                                                              | 433524 | hum   | 2    | 10 µM   | -5     |       |    |    |    |
| 219800  | Dopamine D₃                                                               | 433525 | hum   | 2    | 10 µM   | 14     |       |    |    |    |
| 224010  | Endothelin ETₐ                                                             | 433548 | hum   | 2    | 10 µM   | -2     |       |    |    |    |
| 224110  | Endothelin ETᵦ                                                             | 433549 | hum   | 2    | 10 µM   | -9     |       |    |    |    |
| 226010  | Estrogen ERα                                                                | 433601 | hum   | 2    | 10 µM   | 6      |       |    |    |    |
| 226810  | GABAₐ, Chloride Channel, TBOB                                              | 433540 | rat   | 2    | 10 µM   | 15     |       |    |    |    |
| 226600  | GABAₐ, Flunitrazepam, Central                                             | 433465 | rat   | 2    | 10 µM   | 4      |       |    |    |    |
| 228510  | GABAᵦ, Non-Selective                                                        | 433506 | rat   | 2    | 10 µM   | -15    |       |    |    |    |
| 232030  | Glucocorticoid                                                              | 433479 | hum   | 2    | 10 µM   | 13     |       |    |    |    |
| 232600  | Glutamate, AMPA                                                             | 433538 | rat   | 2    | 10 µM   | 11     |       |    |    |    |
| 232710  | Glutamate, Kainate                                                          | 433539 | rat   | 2    | 10 µM   | -4     |       |    |    |    |
| 232810  | Glutamate, NMDA, Agonism                                                   | 433532 | rat   | 2    | 10 µM   | 11     |       |    |    |    |
| 232910  | Glutamate, NMDA, Glycine                                                   | 433536 | rat   | 2    | 10 µM   | -2     |       |    |    |    |
| 239300  | Growth Hormone Secretagogue (GHS, Ghrelin)                                  | 433558 | hum   | 2    | 10 µM   | 1      |       |    |    |    |
| 239610  | Histamine H₁                                                                | 433467 | hum   | 2    | 10 µM   | -2     |       |    |    |    |
| 239710  | Histamine H₂                                                                | 433541 | hum   | 2    | 10 µM   | -2     |       |    |    |    |
| 239820  | Histamine H₃                                                                | 433654 | hum   | 2    | 10 µM   | 3      |       |    |    |    |
| 243000  | Insulin                                                                     | 433555 | rat   | 2    | 10 µM   | 15     |       |    |    |    |
| 252200  | Motilin                                                                     | 433504 | hum   | 2    | 10 µM   | 4      |       |    |    |    |
| 252610  | Muscarinic M₁                                                               | 433435 | hum   | 2    | 10 µM   | 8      |       |    |    |    |
| 252710  | Muscarinic M₂                                                               | 433435 | hum   | 2    | 10 µM   | 4      |       |    |    |    |
| 252810  | Muscarinic M₃                                                               | 433436 | hum   | 2    | 10 µM   | -7     |       |    |    |    |
| 252910  | Muscarinic M₄                                                               | 433442 | hum   | 2    | 10 µM   | -5     |       |    |    |    |
| 258730  | Nicotinic Acetylcholine α3β4                                               | 433470 | hum   | 2    | 10 µM   | -8     |       |    |    |    |
| 260130  | Opiate δ (OP1, DOP)                                                         | 433461 | hum   | 2    | 10 µM   | -3     |       |    |    |    |
| 260210  | Opiate κ (OP2, KOP)                                                         | 433462 | hum   | 2    | 10 µM   | -7     |       |    |    |    |
| 260410  | Opiate µ (OP3, MOP)                                                         | 433463 | hum   | 2    | 10 µM   | 0      |       |    |    |    |
| 299005  | Progesterone PR-B                                                           | 433474 | hum   | 2    | 10 µM   | -4     |       |    |    |    |
| 299036  | Purinergic P2X                                                               | 433451 | rat   | 2    | 10 µM   | 11     |       |    |    |    |
| 268810  | Purinergic P2Y                                                               | 433697 | rat   | 2    | 10 µM   | -4     |       |    |    |    |
| 271110  | Serotonin (5-Hydroxytryptamine) 5-HT₁α                                     | 433545 | hum   | 2    | 10 µM   | 9      |       |    |    |    |
| 271650  | Serotonin (5-Hydroxytryptamine) 5-HT₂α                                     | 433484 | hum   | 2    | 10 µM   | 1      |       |    |    |    |
| 271700  | Serotonin (5-Hydroxytryptamine) 5-HT₂β                                     | 433519 | hum   | 2    | 10 µM   | 1      |       |    |    |    |
| 271800  | Serotonin (5-Hydroxytryptamine) 5-HT₃C                                     | 433544 | hum   | 2    | 10 µM   | 3      |       |    |    |    |
| 202020  | Transporter, Adenosine                                                      | 433553 | hum   | 2    | 10 µM   | 28     |       |    |    |    |
| 220320  | Transporter, Dopamine (DAT)                                                | 433478 | hum   | 2    | 10 µM   | 33     |       |    |    |    |
| 226400  | Transporter, GABA                                                           | 433531 | rat   | 2    | 10 µM   | 46     |       |    |    |    |
| 204410  | Transporter, Norepinephrine (NET)                                          | 433477 | hum   | 2    | 10 µM   | 22     |       |    |    |    |
| 274030  | Transporter, Serotonin (5-Hydroxytryptamine)(SERT)                         | 433471 | hum   | 2    | 10 µM   | 3      |       |    |    |    |
| 287530  | Vasopressin V₁α                                                             | 433516 | hum   | 2    | 10 µM   | 7      |       |    |    |    |
5. Pharmacokinetics

| BAY Number | BAY-069 | BAY-069 | BAY-069 |
|------------|---------|---------|---------|
| **Dose**   | 25 mg/kg| 50 mg/kg| 100 mg/kg|
| **AUC_{0-tLast}** | 16 h·mg/L| 53 h·mg/L| 270 h·mg/L|
| **AUC_{0-tLast,norm}** | 0.63 h·kg/L| 1.1 h·kg/L| 2.7 h·kg/L|
| **AUC_{0-tLast,u}** | 0.022 h·mg/L| 0.074 h·mg/L| 0.38 h·mg/L|
| **C_{max,u}** | 17 nM| 46 nM| 130 nM|
| **IC_{50 Assay}** | U87MG| U87MG| U87MG|
| **IC_{50}** | 410 nM| 410 nM| 410 nM|
| **IC_{50,u}** | 410 nM| 410 nM| 410 nM|
| **C_{max,u} / IC_{50,u}** | 0.043| 0.11| 0.31|

**Supplementary Table S7.** High dose exposure study BAY-069 in mouse

**Supplementary Figure S20:** Total plasma levels of BAY-069 after po and iv administration to Wistar rat
Supplementary Figure S21: Unbound plasma levels of BAY-069 vs. IC50,u after p.o. dosing of 25 & 50 & 100 mg/kg to NMRI nu/nu mice
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