Supporting Information

Hydantoin analogs inhibit the fully assembled ClpXP protease without affecting the individual peptidase and chaperone domains

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Supporting Tables

Supporting Table 1 is available as an .xlsx file for download.
Biochemical Procedures

**In vitro Creatine Kinase Assay**
To account for inhibitors of the ATP regeneration system a counter-screen against creatine kinase was performed using Kinase-Glo assay (Promega). Assays were performed in PZ buffer (25 mM Hepes, pH 7.6, 200 mM KCl, 5 mM MgCl₂, 1 mM DTT, 10% glycerol) in a white flat-bottom 96-well plate (Brand) with 50 µL reaction volume at r.t. 0.5 µL of compound stock (100x, 50 µM final concentration) or DMSO were mixed with 47 µL of master mix (creatine kinase in PZ buffer, 52 µg/mL final concentration). After incubation for 10 min at r.t. 3 µL of substrate mix (20 µM ADP, 20 µM creatine phosphate; final concentrations) were added and incubated for another 10 min at r.t. 50 µL of Kinase-Glo reagent were added and luminescence was recorded after 10 min incubation at r.t. using an Infinite M200 Pro (Tecan). DMSO-treated samples were normalized to 100% activity and samples without creatine kinase were used as a negative control.

**In vitro ClpXP Protease Assay**
The SaClpXP protease activity was monitored using a fluorescent GFP substrate, which was tagged with a SsrA degradation tag. Assays were performed in PZ buffer (25 mM Hepes, pH 7.6, 200 mM KCl, 5 mM MgCl₂, 1 mM DTT, 10% glycerol) with 60 µL reaction volume at 30 °C. GFP fluorescence was monitored in white, flat-bottom well plates (Brand) using an Infinite M200 Pro (Tecan; λ_{ex} = 465 nm, λ_{em} = 535 nm). Degradation reactions contained 0.6 µM ClpX₆, 0.3 µM ClpP₁₄, 0.25 µM GFP-SsrA and an ATP regeneration system (4 mM ATP, 16 mM creatine phosphate, 20 U/mL creatine phosphokinase). 0.6 µL of inhibitor (in DMSO) were added to the wells followed by all other reaction partners (in 50 µL) except the substrate. After pre-incubation for 15 min at 30 °C, the substrate (10 µL) was added and fluorescence was monitored. Unless stated otherwise all data were collected in duplicates and in three independent experiments. The slope of the curves in the linear region was determined via linear regression using GraphPad Prism. DMSO-treated samples were normalized to 100% activity and samples without ClpX were used as a negative control.

**In vitro ClpP Peptidase Assay**
Activity of SaClpP was measured using a fluorogenic peptide substrate (Suc-Leu-Tyr-AMC, Bachem) in duplicates. 1 µL of DMSO or compound stock was pipetted to a black flat-bottom 96-well plate (Greiner) and 88 µL of assay-buffer (100 mM Hepes, pH 7.0, 100 mM NaCl) containing 1 µM ClpP (final concentration) were added. After incubation for 15 min at 32 °C, the reaction was started by addition of 10 µL substrate (0.2 mM final concentration) and fluorescence was recorded for 60 min in an infinite M200 pro plate reader (Tecan; λ_{ex} = 380 nm, λ_{em} = 440 nm). Data was analyzed by calculating the initial slope (GraphPad Prism) and normalizing DMSO-treated samples to 100% activity.
**In vitro ClpX ATPase Assay**

In a transparent flat-bottom 96-well plate 0.6 µL of DMSO or compound stock were mixed with 54 µL ATPase buffer (100 mM Hepes, pH 7.0, 200 mM KCl, 20 mM MgCl$_2$, 1 mM DTT, 1 mM NADH, 2 mM phosphoenolpyruvate, 50 U/mL lactate dehydrogenase, 50 U/mL pyruvate kinase, 5% (v/v) glycerol) containing SaClpX (4 µM final concentration). After incubation for 10 min at 37 °C, 6 µL 200 mM ATP (in H$_2$O) were added to start the assay. The amount of NADH/H$^+$ was monitored by absorbance measurement ($\lambda = 340$ nm) using a Tecan M200Pro.

**Analytical Size Exclusion Chromatography**

Analytical size exclusion chromatography experiments were performed using a Superdex 200 10/300 gL (GE) or a Superose 6 Increase 10/300 gL (GE) column at 4 °C. For most experiments PZ buffer (with 0.5 mM ATP) was used. Samples (200 µL) were mixed, incubated 10 min at 37 °C and loaded into a 500 µL loop. Elution was monitored at 280 nm. Runs were referenced against the salt peak of the conductivity trace and normalized to the highest peak for easier comparison.

**Thermal Shift Assay**

To each well of a white 96-well PCR plate, 50 µL of a 10 µM SaClpX (or SaClpP) solution in PZ-buffer (or PBS) containing Sypro Orange (1:2000, Sigma-Aldrich) were added. To this solution 0.5 µL of DMSO or 100× compound stock was added and fluorescence intensity was measured while heating from 20 °C to 89.6 °C (0.3 K steps) in a CFX96 Real-Time System (BioRad). Data was analyzed using Bio-Rad CFX Manager 3.0.

**Intact Protein Mass Spectrometry**

A solution of 3 µM SaClpX wt or SaClpX E183Q (or 1 µM ClpP) in PZ-Buffer (containing 0.5 mM ATP) was incubated (60 min at 30 °C) with up to 100 µM of inhibitor (1% DMSO final concentration). Measurements were performed on a Dionex Ultimate 3000 HPLC system coupled to a Thermo LTQ-FT Ultra mass spectrometer with an electrospray ionization source (spray voltage 4.2 kV, tube lens 110 V, capillary voltage 48 V, sheath gas 60 arb, aux gas 10 arb). 5 µL of reaction mixture were on-line desalted using a Massprep desalting cartridge (Waters). The mass spectrometer was operated in positive mode collecting full scans at high-resolution (R = 200,000) from m/z = 600 to m/z = 2000. Collected spectra were deconvoluted using the Thermo Xcalibur Xtract algorithm.

**Western Blot Analysis**

*S. aureus* NCTC 8325 cultures were grown for 20 h in presence of compound at 37 °C while shaking. For western blot analysis of Hla, 10 µL of bacterial supernatant were subjected to SDS-PAGE using Tris-Glycine gels. Blotting was performed according to manufacturer’s instructions at 100 V for 60 min on PVDF membrane using the Tetra Blotting Module (Bio-Rad). Membranes were blocked (TBS containing...
5% skimmed milk and 0.1% Tween-20) and incubated with primary anti-Hla antibody (1:4000; anti-
\textit{Staphylococcus} alpha hemolysin antibody, polyclonal rabbit; Abcam ab50536) overnight at 4 °C. After
extensive washing (3 × 15 min) with blocking buffer the membranes were incubated with secondary
antibody (1:10000; goat anti-rabbit ATTO 488 conjugate; Sigma Aldrich 18772) for 60 min at r.t.
followed by washing (3 × 15 min blocking buffer; 15 min TBST). Bands were detected by fluorescence
scan using a LAS4000 (GE).

\textbf{Secretome Analysis}

5 mL of B-Medium were inoculated 1:100 from an overnight culture of \textit{S. aureus} NCTC 8325 (NRS77)
and incubated (37 °C, 200 rpm) until OD_{600} = 0.3 - 0.4. The culture was then diluted to 3×10^4 CFU/mL
with fresh B-Medium and split into 1.5 mL aliquots in 14 mL PP plastic tubes (17×100 mM, VWR). 15 µL
of DMSO or compound stocks (3 mM 1-E1 or 1-E2, final concentration 30 µM) were added to the
aliquots following incubation for 19-20 h at 37 °C and 200 rpm. On the next day the cultures were
transferred to 2 mL tubes and centrifuged (5 min, 6000 g).

The supernatant was removed and sterile filtered (0.22 µm) into a 50 mL Falcon tube and 12 mL cold
acetone (-80 °C) were added. Proteins were allowed to precipitate overnight at -80 °C. The precipitate
was centrifuged (15 min, 18000 g) and washed twice with 1 mL cold methanol (-80 °C) with
resuspension (5 s ultrasonic bath) and centrifugation steps (10 min, 18000 g) in between. The washed
pellet was dissolved in 200 µL X-buffer (20 mM Hepes, pH 7.5, 7 M urea, 2 M thiourea) and transferred
in low-bind Eppendorf tubes for further analysis.

Proteins were reduced by addition of 0.2 µL dithiothreitol (DTT, 1 M) and incubation for 45 min at r.t.
and 450 rpm). Alkylation was performed by adding 2 µL iodoacetamide (IAA, 550 mM) and incubation
for 30 min at r.t. in the dark with subsequent quenching of the reaction with 0.5 µL DTT (1 M) for
30 min. The samples were pre-digested by addition of 1 µL LysC (0.5 mg/mL) and incubation at r.t. for
4 h. After diluting with 600 µL triethylammonium bicarbonate buffer (TEAB, 50 mM) 1.5 µL trypsin
(0.5 mg/mL in 50 mM acetic acid) was added followed by overnight incubation at 37 °C. Digestion was
stopped by addition of 8 µL formic acid (FA). Desalting of the samples was conducted on 50 mg SepPak
C18 columns (Waters). The columns were equilibrated with 1 mL acetonitrile (ACN), 1 mL elution
buffer (80% ACN, 0.5% FA) and 3 mL aqueous 0.5% FA solution. The acidified samples were loaded by
gravity flow, washed five times with 1 mL 0.5% FA and then labeled with five times 1 mL of the
respective dimethyl labeling agents (light (L): 30 mM NaBH$_3$CN, 0.2 % CH$_2$O, 45 mM sodium phosphate
buffer, pH 7.5; medium (M): 30 mM NaBH$_3$CN, 0.2 % CD$_2$O, 45 mM sodium phosphate buffer, pH 7.5;
heavy (H): 30 mM NaBD$_3$CN, 0.2 % $^{13}$CD$_2$O, 45 mM sodium phosphate buffer, pH 7.5). Labels were
switched throughout the replicates. Column bound peptides were washed two more times with 1 mL
0.5% FA and then eluted with two times 250 µL elution buffer. 900 µL of each sample were combined
in a 15 mL tube, frozen in liquid nitrogen and lyophilized. Prior to LC-MS/MS measurement the samples were dissolved in 40 µL 1% FA and filtered with 0.22 µm ultrafree centrifugal filters (Merck) equilibrated with 300 µL 1% FA. The filtrates were transferred into MS vials and queued for LC-MS/MS measurement.

Samples were analyzed with an UltiMate 3000 nano HPLC system (Dionex) using Acclaim C18 PepMap100 75 µm ID x 2 cm trap and Acclaim Pepmap RSLC C18 (75 µm ID x 50 cm) separation columns in an EASY-spray setting coupled to an Orbitrap Fusion (Thermo Fisher).

Samples were loaded on the trap and washed with 0.1% TFA, then transferred to the analytical column. Orbitrap Fusion was operated in a 3 s top speed data dependent mode. Full scan acquisition was performed in the orbitrap at a resolution of 120000 and an AGC target of 2e5 in a scan range of 300 – 1500 m/z. Monoisotopic precursor selection as well as dynamic exclusion (exclusion duration: 60 s, exclusion mass width relative to low mass: 10 ppm, exclusion mass width relative to high mass: 10 ppm) was enabled. Precursors with charge states of 2 – 7 and intensities greater than 5e3 were selected for fragmentation. Isolation was performed in the quadrupole using a window of 1.6 m/z. Precursors were collected to an AGC target of 3e3 for a maximum injection time of 250 ms with “inject ions for all available parallelizable time” set to true. Fragments were generated using higher-energy collisional dissociation (HCD, normalized collision energy: 27%) and detected in the ion trap at a rapid scan rate. Internal calibration was performed using the ion signal of fluoranthene cations (EASY-IC).
Organic Synthesis

General remarks
All reactions were carried out under argon in oven-dried glassware unless noted otherwise. All chemicals were of reagent grade or better and used without further purification. Chemicals and solvents were purchased from Sigma Aldrich. Solvents for chromatography and workup purposes were generally of reagent grade. In all reactions, temperatures were measured externally. $^1$H NMR and $^{13}$C spectra of small molecules were recorded on Bruker instruments (250MHz, 360 MHz or 500 MHz) and referenced to the residual proton signal of the deuterated solvent. Carbon samples were reference externally against the residual $^{13}$C signal of CDCl$_3$. HR-MS-ESI spectra were recorded with a Thermo Scientific LTQ FT.

1-(3,4-Dimethylphenyl)-2-phenylethane-1,2-dione was obtained from 1-(3,4-dimethylphenyl)-2-phenylethan-1-one according to the known procedure.$^5$

Isolation of single 1 enantiomers was achieved by using chiral high-performance liquid chromatography using a Daicel Chiralpak AD-H column (250×4.6mm) and heptane/2-propanol as eluents. Compounds 5 and 6 were purchased from Enamine.

**General Procedure 1 (GP1) for the synthesis of Substituted Imines**

A solution of the aldehyde (15 mmol) in MeOH (22.5 mL) was cooled to 0° C and a solution of the amine (90 mmol) in THF (45 mL) followed by acetic acid (1.0 mL) were added to it. The reaction mixture was stirred for 4 h at 0° C and subsequently 18 h at r. t. The volatiles were removed under reduced pressure and the residue was taken up in EtOAc and washed with H$_2$O. The organic phase was dried over Na$_2$SO$_4$, filtered, and the solvent was evaporated under reduced pressure. The residue was directly used for the next step without further purification.

$N$-((5-Chlorothien-2-yl)methylene)-methylamine

$N$-((5-Chlorothien-2-yl)methylene)-methylamine was obtained according to GP1 from 5-chlorothien-2-carbaldehyde (2.2 g, 15 mmol), methylamine (45 mL, 90 mmol, 2 M solution in THF) and acetic acid (1.0 mL) in MeOH (22.5 mL). Yield: 2.2 g (92%). – $^1$H NMR (300 MHz, CDCl$_3$): 8.21 (q, $J$ = 1.6 Hz, 1 H), 7.03 (d, $J$ = 3.8 Hz, 1 H), 6.87 (d, $J$ = 3.8 Hz, 1 H), 3.44 (d, $J$ = 1.6 Hz, 3 H).
**N-((5-Chlorothien-2-yl)methylene)-propylamine**

\[
\text{Cl} \quad \text{N} \sim \text{Pr}
\]

\(N-((5\text{-Chlorothien-2-yl})\text{methylene})\)-propylamine was obtained according to GP1 from 5-chlorothien-2-carbaldehyde (1.0 g, 6.8 mmol), propylamine (2.4 g, 41 mmol) and acetic acid (0.5 mL) in MeOH (11 mL) and THF (22 mL). Yield: 1.2 g (94%). – \(^1\text{H} \text{NMR (300 MHz, CDCl}_3\text{)}: 8.21 (\text{td, } J = 1.3, 0.4 \text{ Hz, 1 H}), 7.03 (d, J = 3.9 \text{ Hz, 1 H}), 6.87 (d, J = 3.9 \text{ Hz, 1 H}), 3.50 (\text{td, } J = 6.9, 1.3 \text{ Hz, 2 H}), 1.75 – 1.61 (m, 2 H), 0.92 (t, J = 7.4 \text{ Hz, 3 H}).

**General Procedure 2 (GP2) for the Synthesis of Substituted Amines by Reduction of the Corresponding Imines**

\[
\text{R}_1 \text{N} \sim \text{R}_2 \xrightarrow{\text{NaBH}_4, \text{EtOH}} \text{R}_1 \text{HN} \sim \text{R}_2
\]

\(\text{NaBH}_4\) (0.78 g, 20.6 mmol) was slowly added to the solution of the imine (13.8 mmol, obtained from the previous step) in EtOH (20 mL) at r. t. The reaction mixture was stirred for 2 h at r. t. and the solvent was removed under reduced pressure. The residue was taken up in Et\(_2\)O and washed with saturated aq. K\(_2\)CO\(_3\). The organic phase was dried over Na\(_2\)SO\(_4\), filtered and concentrated under reduced pressure to yield a desired amine which was used for the next step without further purification.

**\(N\)-Methyl-(5-chlorothien-2-yl)methylamine**

\[
\text{Cl} \quad \text{NMe}
\]

\(N\)-Methyl-(5-chlorothien-2-yl)methylamine was obtained according to GP2 from \(N-((5\text{-chlorothien-2-yl})\text{methylene})\)-methylamine (2.2 g, 13.8 mmol) and \(\text{NaBH}_4\) (0.8 g, 20.6 mmol) in EtOH (20 mL). Yield: 1.9 g (86%). – \(^1\text{H} \text{NMR (300 MHz, CDCl}_3\text{)}: 6.76 (d, J = 3.7 \text{ Hz, 1 H}), 6.71 (dt, J = 3.8, 1.0 \text{ Hz, 1 H}), 3.87 (d, J = 1.0 \text{ Hz, 2 H}), 2.48 (s, 3 H). –\text{HRMS (ESI) calcd. for C}_6\text{H}_9\text{ClNS [M+H]}^+ 162.0144, found 162.0137.}
**N-Propyl-(5-chlorothien-2-yl)methylamine**

*N-Propyl-(5-chlorothien-2-yl)methylamine* was obtained according to GP2 from *N*-(5-chlorothien-2-yl)methylene-propylamine (1.2 g, 6.4 mmol) and NaBH₄ (0.36 g, 9.6 mmol) in EtOH (10 mL). Yield: 1.1 g (91%). – ¹H NMR (300 MHz, CDCl₃): 6.73 (d, J = 3.7 Hz, 1 H), 6.67 (dt, J = 3.8, 1.0 Hz, 1 H), 3.88 (d, J = 1.0 Hz, 2 H), 2.60 (t, J = 7.1 Hz, 2 H), 1.56 – 1.46 (m, 2 H), 0.92 (t, J = 7.4 Hz, 3 H).

**General Procedure 3 (GP3) for the Synthesis Various 2-Bromoacetamides**

To a solution of an amine (3.0 mmol) in CH₂Cl₂ (24 mL) cooled to 0 °C was added a solution of 2-bromoacetyl bromide (0.3 mL, 689 mg, 3.43 mmol) in CH₂Cl₂ (12 mL). The mixture was stirred for 1 h at r. t. then cooled once more to 0 °C and quenched with saturated aq. NaHCO₃. The organic phase was separated, washed with saturated aq. NaHCO₃, dried over Na₂SO₄, filtered and concentrated under reduced pressure to yield a desired amide. The crude product was purified by column chromatography (hexane: ethyl acetate).

**N-Benzyl-N-methyl 2-Bromoacetamide**

*N-Benzyl-N-methyl 2-bromoacetamide* was obtained according to GP3 from the *N*-methylbenzylamine (726 mg, 6.0 mmol) and 2-bromoacetyl bromide (0.6 mL, 1378 mg, 6.86 mmol) in CH₂Cl₂ (72 mL). Yield: 1.1 g (76%) as a ca. 3 : 2 mixture of 2 rotamers. – ¹H NMR (300 MHz, CDCl₃): 7.45–7.20 (m, 5 H), 4.62 (s, 0.8 H), 4.61 (s, 1.2 H), 3.95 (s, 1.2 H), 3.90 (s, 0.8 H), 3.02 (s, 1.8 H), 2.98 (s, 1.2 H). Spectral data are identical to those published in the literature.⁶

**N-(5-Chlorothien-2-yl)methyl-N-methyl 2-Bromoacetamide**

*N-(5-Chlorothien-2-yl)methyl-N-methyl 2-bromoacetamide* was obtained according to GP3 from the *N*-methyl-(5-chlorothien-2-yl)methylamine (680 mg, 4.2 mmol) and 2-bromoacetyl bromide (0.43 mL, 1 g, 5.0 mmol) in CH₂Cl₂ (48 mL). The crude product was purified by column chromatography (hexane...
EtOAc = 2 : 1). Yield: 450 mg (38%) as a ca. 4 : 1 mixture of 2 rotamers. – \(^1\)H NMR (400 MHz, CDCl\(_3\)): 6.81–6.72 (m, 2 H), 4.64 (s, 0.4 H), 4.59 (s, 1.6 H), 3.91 (s, 0.4 H), 3.88 (s, 1.6 H), 3.07 (s, 2.4 H), 2.97 (s, 0.6 H). – \(^1\)C NMR (100 MHz, CDCl\(_3\)): 169.17, 166.83, 137.44, 130.22, 126.31, 126.08, 125.55, 125.48, 49.80, 46.86, 35.55, 33.94, 26.00, 25.61. – HRMS (ESI) calcd. for C\(_8\)H\(_{10}\)BrClNOS [M+H]\(^+\) 281.9355, found 281.9347.

\(\text{N-(5-Chlorothien-2-yl)methyl-N-propyl 2-Bromoacetamide}\)

\(\begin{align*}
\text{N-(5-Chlorothien-2-yl)methyl-N-propyl 2-bromoacetamide was obtained according to GP3 from the N-} \\
\text{propyl-(5-chlorothien-2-yl)methylamine (1.1 g, 5.8 mmol) and 2-bromoacetetyl bromide (0.56 mL, 1.3 g,} \\
\text{6.4 mmol) in CH}_2\text{Cl}_2 \text{ (45 mL). The crude product was purified by column chromatography (hexane :} \\
\text{EtOAc = 2 : 1}). \text{ Yield: 1.3 g (72%) as a ca. 3 : 1 mixture of 2 rotamers. –} \(^1\)\text{H NMR (400 MHz, CDCl}_3\text{): 6.80–} \\
\text{6.70 (m, 2 H), 4.63 (s, 0.5 H), 4.58 (s, 1.5 H), 3.88 (s, 1.5 H), 3.86 (s, 0.5 H), 3.37–3.32 (m, 0.5 H), 3.31–} \\
\text{3.25 (m, 1.5 H), 1.71–1.61 (m, 1.5 H), 1.61–1.52 (m, 0.5 H), 0.93 (t, } J = 7.4 \text{ Hz, 2.25 H), 0.90 (t, } J = 7.4 \\
\text{Hz, 0.75 H). – HRMS (ESI) calcd. for C}_{10}\text{H}_{14}\text{BrClNOS [M+H]}^+ \text{ 309.9668, found 309.9663.}\end{align*}\)

**General Procedure 4 (GP4) for the Synthesis of Substituted Hydantoins**

\[
\begin{array}{c}
\text{Ar}_1\text{O} \quad \text{Ar}_2\text{O} + \text{H}_2\text{NCONH}_2 \\
\text{1. NaOH, EtOH/H}_2\text{O} \quad \text{2. HCl}
\end{array}
\]

A mixture of the corresponding 1,2-diarylethane-1,2-dione (25 mmol), urea (3 g, 50 mmol) and NaOH (15 mL of a 40% aqueous solution, 150 mmol) in EtOH (70 mL) was heated at reflux for 3 h with stirring. The reaction mixture was cooled down to r. t. and poured into H\(_2\)O (120 mL). The solution obtained was acidified by conc. HCl, the product precipitated was filtered off and recrystallized from EtOH.
5,5-Diphenylimidazolidine-2,4-dione

5,5-Diphenylimidazolidine-2,4-dione was obtained according to GP4 from 1,2-diphenylethane-1,2-dione (benzil) (5.3 g, 25 mmol), urea (3g, 50 mmol) and NaOH (15 mL of a 40% aqueous solution, 150 mmol) in EtOH (70 mL). Yield: 2.6 g (41%). – $^1$H NMR (300 MHz, DMSO-d$_6$): 11.09 (s, 1 H), 9.30 (s, 1 H), 7.45 – 7.30 (m, 10 H). Spectral data are identical to those published in the literature.  

5,5-Di(4-methylphenyl)imidazolidine-2,4-dione

5,5-Di(4-methylphenyl)imidazolidine-2,4-dione was obtained according to GP4 from 1,2-di(4-methylphenyl)ethane-1,2-dione (2.5 g, 10.5 mmol), urea (1.26 g, 21.0 mmol) and NaOH (7 mL of a 40% aqueous solution, 70 mmol) in EtOH (40 mL). Yield: 1.9 g (66%). – $^1$H NMR (400 MHz, DMSO-d$_6$): 11.00 (s, 1 H), 9.19 (s, 1 H), 7.26–7.12 (m, 8 H), 2.28 (s, 6 H). – $^{13}$C NMR (101 MHz, DMSO-d$_6$): 175.43, 156.53, 137.73, 137.63, 129.52, 127.03, 70.33, 21.10.

5-(3,4-Dimethylphenyl)-5-phenylimidazolidine-2,4-dione

5-(3,4-Dimethylphenyl)-5-phenylimidazolidine-2,4-dione was obtained according to GP4 from 1-(3,4-dimethylphenyl)-2-phenylethane-1,2-dione (0.87 g, 3.65 mmol), urea (0.44 g, 7.30 mmol) and NaOH (4
mL of a 40% aqueous solution, 40 mmol) in EtOH (30 mL). Yield: 0.56 g (55%). – ¹H NMR (400 MHz, DMSO-\textit{d}_6): 11.02 (s, 1 H), 9.21 (s, 1 H), 7.41–7.29 (m, 5 H), 7.16–7.09 (m, 2 H), 7.07–7.02 (m, 1 H), 2.20 (s, 3 H), 2.19 (s, 3 H). – ¹³C NMR (101 MHz, DMSO-\textit{d}_6): 175.39, 156.44, 140.57, 137.86, 136.71, 136.56, 129.93, 128.89, 128.38, 127.99, 127.04, 124.47, 70.49, 20.09, 19.44.

**General Procedure 5 (GP5) for the Alkylation of Substituted Hydantoins**

![General Procedure 5 (GP5) for the Alkylation of Substituted Hydantoins](image)

To a solution of the corresponding substituted hydantoin (1.6 mmol) and \textit{N},\textit{N}-disubstituted 2-bromacetamide (1.6 mmol) in DMF (10 mL) was added Cs$_2$CO$_3$ (587 mg, 1.8 mmol). This mixture was stirred for 3 h at r. t. H$_2$O (50 mL) was added to the reaction and the mixture obtained was extracted with EtOAc (3 x 30 mL). The combined organic phases were dried over Na$_2$SO$_4$, filtered, concentrated and separated by column chromatography (hexane : EtOAc = 10 : 1 to 1 : 1) yielding the desired product.

3-(2-(\textit{N}-(5-Chlorothien-2-yl)methyl-\textit{N}-methyl-amino)-2-oxo-ethyl)-5-(3,4-dimethylphenyl)-5-phenylimidazolidine-2,4-dione (1)

3-(2-(\textit{N}-(5-Chlorothien-2-yl)methyl-\textit{N}-methyl-amino)-2-oxo-ethyl)-5-(3,4-dimethylphenyl)-5-phenylimidazolidine-2,4-dione was obtained according to GP4 from 5-(3,4-dimethylphenyl)-5-phenylimidazolidine-2,4-dione (448 mg, 1.6 mmol), \textit{N}-(5-chlorothien-2-yl)-\textit{N}-methyl 2-bromoacetamide (450 mg, 1.6 mmol) and Cs$_2$CO$_3$ (587 mg, 1.8 mmol) in DMF (10 mL). Purification by column chromatography (hexane : EtOAc = 4 : 1 to 1 : 1) yielded the desired product as a ca. 3 : 1 mixture of two rotamers. Yield: 440 mg (57%). – ¹H NMR (500 MHz, DMSO-\textit{d}_6): 9.57 (s, 0.25 H), 9.55 (s, 0.75 H), 7.43–7.32 (m, 5 H), 7.23–7.08 (m, 3 H), 7.06 (d, \textit{J} = 3.7 Hz, 0.25 H), 6.98 (d, \textit{J} = 3.7 Hz, 1 H),
6.93 (d, $J = 3.7$ Hz, 0.75 H), 4.75 (s, 0.5 H), 4.58, 4.54 (ABq, $J = 5.0$ Hz, 1.5 H), 4.40 (s, 0.5 H), 4.34 (s, 1.5 H), 3.02 (s, 2.25 H), 2.85 (s, 0.75 H), 2.23–2.17 (m, 6 H). $^{13}$C NMR (126 MHz, DMSO-$d_6$): 174.12, 174.09, 166.25, 165.78, 155.45, 155.44, 140.36, 140.32, 139.51, 139.41, 137.52, 137.40, 136.75, 136.65, 129.88, 128.85, 128.55, 128.43, 128.38, 128.21, 127.51, 127.34, 127.28, 126.70, 126.60, 124.88, 124.85, 69.79, 46.51, 34.11, 33.92, 20.15, 19.49. –HRMS (ESI) calcd. for C$_{25}$H$_{25}$ClN$_3$O$_3$S [M+H]$^+$ 482.1305, found 482.1296.

3-(2-(N-Benzyl-N-methyl-amino)-2-oxo-ethyl)-5,5-diphenylimidazolidine-2,4-dione (2)

3-(2-(N-Benzyl-N-methyl-amino)-2-oxo-ethyl)-5,5-diphenylimidazolidine-2,4-dione was obtained according to GP4 from 5,5-diphenylimidazolidine-2,4-dione (252 mg, 1.0 mmol), N-benzyl-N-methyl 2-bromoacetamide (242 mg, 1.0 mmol) and Cs$_2$CO$_3$ (652 mg, 2.0 mmol) in DMF (6 mL). Purification by column chromatography (hexane : EtOAc = 10 : 1 to 3 : 1 to 1 : 1) yielded the desired product as a mixture 3 : 2 of two rotamers. Yield: 120 mg (29%). –$^1$H NMR (500 MHz, DMSO-$d_6$): 9.64 (s, 1 H), 7.45–7.40 (m, 10 H), 7.40–7.19 (m, 5 H), 4.64 (s, 0.8 H), 4.53 (s, 1.2 H), 4.43 (s, 1.2 H), 4.38 (s, 0.8 H), 2.98 (s, 1.8 H), 2.83 (s, 1.2H). –$^{13}$C NMR (126 MHz, DMSO-$d_6$): 174.08, 174.02, 166.22, 166.07, 155.53, 155.49, 140.15, 140.09, 137.63, 137.17, 129.29, 129.00, 128.93, 128.92, 127.91, 127.64, 127.50, 127.49, 127.18, 69.99, 69.97, 51.94, 50.90, 34.27, 34.24. –HRMS (ESI) calcd. for C$_{25}$H$_{24}$N$_3$O$_3$ [M+H]$^+$ 414.1818, found 414.1815.

3-(2-(N-(5-Chlorothien-2-yl)methyl-N-propyl-amino)-2-oxo-ethyl)-5,5-diphenylimidazolidine-2,4-dione (3)
3-(2-((N-(5-Chlorothien-2-yl)methyl)-N-methyl-amino)-2-oxo-ethyl)-5,5-diphenylimidazolidine-2,4-dione was obtained according to GP4 from 5,5-diphenylimidazolidine-2,4-dione (479 mg, 1.9 mmol), N-(5-chlorothien-2-yl)-N-propyl 2-bromoacetamide (600 mg, 1.9 mmol) and Cs$_2$CO$_3$ (685 mg, 2.1 mmol) in DMF (10 mL). Purification by column chromatography (hexane : EtOAc = 10 : 1 to 1 : 1) yielded the desired product as a mixture ca. 3 : 1 of two rotamers. Yield: 315 mg (34%). – $^1$H NMR (500 MHz, DMSO-$d_6$): 9.65 (s, 1 H), 7.48–7.33 (m, 10 H), 7.04 (d, $J = 3.8$ Hz, 0.25 H), 7.00–6.93 (m, 1.75 H), 4.75 (s, 0.5 H), 4.56 (s, 1.5 H), 4.39 (s, 0.5 H), 4.36 (s, 1.5 H), 3.30 (t, $J = 7.4$ Hz, 1.5 H), 3.23 (t, $J = 7.4$ Hz, 0.5 H) 1.64–1.53 (m, 1.5 H), 1.51–1.40 (m, 0.5 H), 0.86 (t, $J = 7.4$ Hz, 2.25 H), 0.80 (t, $J = 7.4$ Hz, 0.75 H). – $^{13}$C NMR (126 MHz, DMSO-$d_6$): 173.96, 166.22, 165.74, 155.45, 140.22, 140.14, 140.10, 128.91, 128.65, 128.25, 127.50, 127.47, 127.26, 127.19, 126.50, 126.31, 70.01, 55.34, 48.35, 47.94, 44.75, 21.83, 20.74, 11.56, 11.29. –HRMS (ESI) calcd. for C$_{25}$H$_{25}$ClN$_3$O$_3$S [M+H]$^+$ 482.1305, found 482.1296.

3-(2-((N-(5-Chlorothien-2-yl)methyl)-N-methyl-amino)-2-oxo-ethyl)-5,5-diphenylimidazolidine-2,4-dione (4)

3-(2-((N-(5-Chlorothien-2-yl)methyl)-N-methyl-amino)-2-oxo-ethyl)-5,5-diphenylimidazolidine-2,4-dione was obtained according to GP4 from 5,5-diphenylimidazolidine-2,4-dione (402 mg, 1.6 mmol), N-(5-chlorothien-2-yl)methyl-N-methyl 2-bromoacetamide (450 mg, 1.6 mmol) and Cs$_2$CO$_3$ (587 mg, 1.8 mmol) in DMF (10 mL). Purification by column chromatography (hexane : EtOAc = 10 : 1 to 3 : 1 to 1 : 1) yielded the desired product as a mixture ca. 3 : 1 of two rotamers. Yield: 180 mg (25%). – $^1$H NMR (500 MHz, DMSO-$d_6$): 9.66 (s, 0.25 H), 9.64 (s, 0.75 H), 7.43–7.34 (m, 10 H), 7.06 (d, $J = 3.7$ Hz, 0.25 H), 6.98 (d, $J = 3.7$ Hz, 1 H), 6.93 (d, $J = 3.7$ Hz, 0.75 H), 4.76 (s, 0.5 H), 4.57 (s, 1.5 H), 4.42 (s, 0.5 H), 4.36 (s, 1.5 H), 3.02 (s, 2.25 H), 2.86 (s, 0.75 H). – $^{13}$C NMR (126 MHz, DMSO-$d_6$): 174.02, 173.96, 166.22, 165.77, 155.43, 140.14, 140.06, 128.94, 128.67, 128.18, 127.95, 127.49, 127.27, 126.70, 126.63, 70.02, 69.97, 55.41, 46.48, 34.09, 33.94. –HRMS (ESI) calcd. for C$_{23}$H$_{23}$ClN$_3$O$_3$S [M+H]$^+$ 454.0992, found 454.0991.
3-(2-(N-(5-Chlorothien-2-yl)-N-methyl-amino)-2-oxo-ethyl)-5,5-di(4-methylphenyl)imidazolidine-2,4-dione (7)

3-(2-(N-(5-Chlorothien-2-yl)-N-methyl-amino)-2-oxo-ethyl)-5,5-di(4-methylphenyl)imidazolidine-2,4-dione was obtained according to GP4 from 5,5-di(4-methylphenyl)imidazolidine-2,4-dione (448 mg, 1.6 mmol), N-(5-chlorothien-2-yl)-N-methyl 2-bromoacetamide (450 mg, 1.6 mmol) and Cs₂CO₃ (587 mg, 1.8 mmol) in DMF (10 mL). Purification by column chromatography (hexane : EtOAc = 10 : 1 to 1 : 1) yielded the desired product as a ca. 4 : 1 mixture of two rotamers. Yield: 440 mg (57%). – ¹H NMR (500 MHz, DMSO-d₆): 9.55 (s, 0.2 H), 9.53 (s, 0.8 H), 7.30–7.25 (m, 4 H), 7.23–7.18 (m, 4 H), 7.06 (d, \(J = 3.8 \) Hz, 0.2 H), 6.98 (d, \(J = 3.8 \) Hz, 1 H), 6.93 (d, \(J = 3.8 \) Hz, 0.8 H), 4.75 (s, 0.4 H), 4.56 (s, 1.6 H), 4.40 (s, 0.4 H), 4.34 (s, 1.6 H), 3.02 (s, 2.4 H), 2.85 (s, 0.6 H), 2.30 (s, 6 H). – ¹³C NMR (126 MHz, DMSO-d₆): 174.23, 174.18, 166.23, 165.78, 155.46, 139.51, 139.40, 137.92, 137.38, 137.30, 129.39, 128.19, 128.18, 127.39, 127.33, 127.25, 126.67, 126.62, 69.68, 69.64, 55.40, 46.47, 34.08, 33.91, 21.09. –HRMS (ESI) calcd. for C₂₅H₂₅ClN₃O₃S [M+H]⁺ 482.1305, found 482.1300.
$N$-((5-Chlorothien-2-yl)methylene)-methylamine

SpinWorks 4:

![Spectrum Image]

file: D:\Spektren\vk89110\fid  expt: <zg30>
transmitter freq.: 300.131853 MHz
time domain size: 65536 points
width: 6009.62 Hz = 20.0233 ppm = 0.091699 Hz/pt
number of scans: 16

freq. of 0 ppm: 300.130007 MHz
processed size: 65536 complex points
LB: 0.000  GF: 0.0000
N-Methyl-(5-chlorothien-2-yl)methylamine

SpinWorks 4:

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freq. of 0 ppm: 300.130007 MHz
processed size: 65536 complex points
LB: 0.000  GF: 0.0000

expt: <zg30>
time domain size: 65536 points
width: 6009.62 Hz = 20.0233 ppm = 0.091699 Hz/pt
number of scans: 16
N-(5-Chlorothien-2-yl)methyl-N-methyl 2-Bromoacetamide

SpinWorks 4:

file: D:\Spektren\vk889\vk889\10\fid  extpt: <zg30>
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time domain size: 65536 points
width: 8012.82 Hz = 20.0254 ppm = 0.122266 Hz/pt
number of scans: 16

freq. of 0 ppm: 400.130010 MHz
processed size: 65536 complex points
LB: 0.000  GF: 0.0000
SpinWorks 4:

file: D:\Spektren\vk889\vk889\11\fid
expt: <zgpg30>
transmitter freq.: 100.622829 MHz
time domain size: 65536 points
width: 28409.09 Hz = 282.3325 ppm = 0.433488 Hz/pt
number of scans: 1024

freq. of 0 ppm: 100.612769 MHz
processed size: 32768 complex points
LB: 0.000   GF: 0.0000
$N$-(5-Chlorothien-2-yl)methyl-$N$-propyl 2-Bromoacetamide

SpinWorks 4:

file: D:\Spektren\vk901\vk901\10\fid  expt: <zg30>
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time domain size: 65536 points
width: 8012.82 Hz = 20.0254 ppm = 0.122266 Hz/pt
number of scans: 16

freq. of 0 ppm: 400.130000 MHz
processed size: 65536 complex points
LB: 0.000  GF: 0.0000
N-Benzyl-N-methyl 2-Bromoacetamide

SpinWorks 4:

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transmitter freq.: 300.131853 MHz
time domain size: 65536 points
width: 6009.62 Hz = 20.0233 ppm = 0.091699 Hz/pt
number of scans: 16
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processed size: 65536 complex points
LB: 0.000  GF: 0.0000
5,5-Di(4-methylphenyl)imidazolidine-2,4-dione

file: D:\Spektren\TG_13\12\fid  expt: <zg30>
transmitter freq.: 300.131853 MHz
time domain size: 65536 points
width: 6009.62 Hz = 20.0233 ppm = 0.091699 Hz/pt
number of scans: 16
freq. of 0 ppm: 300.130002 MHz
processed size: 65536 complex points
LB: 0.000  GF: 0.0000
5-(3,4-Dimethylphenyl)-5-phenylimidazolidine-2,4-dione

SpinWorks 4: TG_12 post column

file: D:\Spektren\TG_12\Vid  expt: zg30
transmitter freq.: 400.132471 MHz
time domain size: 65536 points
width: 8012.82 Hz = 20.0254 ppm = 0.122266 Hz/pt
number of scans: 16
freq. of 0 ppm: 400.130003 MHz
processed size: 65536 complex points
LB: 0.000  GF: 0.0000
3-(2-((N-(5-Chlorothien-2-yl)methyl-N-methyl-amino)-2-oxo-ethyl)-5-(3,4-dimethylphenyl)-5-phenylimidazolidine-2,4-dione (1)}
SpinWorks 4: VK896

weC13CPD DMSO C:\aksieber 43

file: D:\Spektren\vk896\VK896\1\fid  expt: <zgpg30>
transmitter freq.: 125.829462 MHz
time domain size: 65536 points
width: 32894.74 Hz = 261.4232 ppm = 0.501934 Hz/pt
number of scans: 1024
3-(2-(N-Benzyl-N-methyl-amino)-2-oxo-ethyl)-5,5-diphenylimidazolidine-2,4-dione (2)

SpinWorks 4: VK882-11
weproton DMSO C:\ aksieber

file: D:\Spektren\VK882-11\10\fid  expt: <zg30>
transmitter freq.: 500.363002 MHz
time domain size: 65536 points
width: 9014.42 Hz = 18.0158 ppm = 0.137549 Hz/pt
number of scans: 64

freq. of 0 ppm: 500.360003 MHz
processed size: 65536 complex points
LB: 0.000  GF: 0.0000
file: D:\Spektren\VK882-11\11\fid  expt: <zgpg30>
transmitter freq.: 125.829462 MHz
freq. of 0 ppm: 125.815622 MHz
processed size: 131072 complex points
LB: 0.000  GF: 0.0000

freq. of 0 ppm: 125.815622 MHz
processed size: 131072 complex points
LB: 0.000  GF: 0.0000

number of scans: 1024

SpinWorks 4: VK882-11
weC13CPD DMSO C:\ aksieber

PPM

file: D:\Spektren\VK882-11\11\fid  expt: <zgpg30>
transmitter freq.: 125.829462 MHz
time domain size: 65536 points
width: 32894.74 Hz = 261.4232 ppm = 0.501934 Hz/pt
number of scans: 1024
3-(2-(N-(5-Chlorothien-2-yl)methyl-N-propyl-amino)-2-oxo-ethyl)-5,5-diphenylimidazolidine-2,4-dione (3)
file: D:\Spektren\vk902-12\20\fid expt: <zgpg30>  
transmitter freq.: 100.622829 MHz  
time domain size: 65536 points  
width: 28409.09 Hz = 282.3325 ppm = 0.433488 Hz/pt  
number of scans: 1024  
freq. of 0 ppm: 100.612769 MHz  
processed size: 32768 complex points  
LB: 0.000  GF: 0.0000
3-(2-(N-(5-Chlorothien-2-yl)methyl-N-methyl-amino)-2-oxo-ethyl)-5,5-diphenylimidazolidine-2,4-dione (4)
SpinWorks 4: VK890-14

weC13CPD DMSO C:\ aksieber

file: ...\Spektren\vk890-14\VK890-14\11\fid  expt: <zgpg30>
transmitter freq.: 125.829462 MHz
time domain size: 65536 points
width: 32894.74 Hz = 261.4232 ppm = 0.501934 Hz/pt
number of scans: 1024

freq. of 0 ppm: 125.815622 MHz
processed size: 131072 complex points
LB: 0.000  GF: 0.0000
3-(2-(N-(5-Chlorothien-2-yl)methyl-N-methyl-amino)-2-oxo-ethyl)-5,5-di(4-methylphenyl)imidazolidine-2,4-dione (7)

SpinWorks 4: VK894
weproton DMSO C:\ aksieber

file: D:\Spektren\vk894\VK894\10\fid  expt: <zg30>
transmitter freq.: 500.360003 MHz
frequ. of 0 ppm: 500.360003 MHz
processed size: 65536 complex points
number of scans: 64

PPM

freq. of 0 ppm: 500.360003 MHz
processed size: 65536 complex points
L8: 0.0000 GF: 0.0000
SpinWorks 4: VK894

weC13CPD DMSO C:\akteiber

file: D:\Spektren\vk894\VK894\11\fid  expt: <zgpg30>
transmitter freq.: 125.829462 MHz
time domain size: 65536 points
width: 32894.74 Hz = 261.4232 ppm = 0.501934 Hz/pt
number of scans: 1024

freq. of 0 ppm: 125.815622 MHz
processed size: 131072 complex points
LB: 0.000  GF: 0.0000
References