

# ChemMedChem

## Supporting Information

### **Development of a NanoBRET Assay Platform to Detect Intracellular Ligands for the Chemokine Receptors CCR6 and CXCR1**

Max E. Huber, Silas L. Wurnig, Aurélien F. A. Moumbock, Lara Toy, Evi Kostenis, Ana Alonso Bartolomé, Martyna Szpakowska, Andy Chevigné, Stefan Günther, Finn K. Hansen,\* and Matthias Schiedel\*

# Supporting Information

## Development of a NanoBRET Assay Platform to Detect Intracellular Ligands for the Chemokine Receptors CCR6 and CXCR1

Max E. Huber,<sup>[a]#</sup> Silas L. Wurnig,<sup>[b]#</sup> Aurélien F. A. Moumbock,<sup>[c]</sup> Lara Toy,<sup>[a]</sup> Evi Kostenis,<sup>[d]</sup> Ana Alonso Bartolomé,<sup>[e,f]</sup> Martyna Szpakowska,<sup>[e]</sup> Andy Chevigné,<sup>[e]</sup> Stefan Günther,<sup>[c]</sup> Finn K. Hansen,<sup>\*[b]</sup> Matthias Schiedel<sup>\*[a,g]</sup>

---

[a] Department of Chemistry and Pharmacy, Medicinal Chemistry, Friedrich-Alexander-University Erlangen-Nürnberg, Nikolaus-Fiebiger-Straße 10, 91058 Erlangen, Germany

[b] Department of Pharmaceutical & Cell Biological Chemistry, Pharmaceutical Institute, University of Bonn, An der Immenburg 4, 53121 Bonn, Germany

[c] Institute of Pharmaceutical Sciences, Albert-Ludwigs-Universität Freiburg, Hermann-Herder-Straße 9, 79104 Freiburg, Germany

[d] Molecular, Cellular and Pharmacobiology Section, Institute for Pharmaceutical Biology, University of Bonn, Nussallee 6, 53115 Bonn, Germany

[e] Immuno-Pharmacology and Interactomics, Department of Infection and Immunity, Luxembourg Institute of Health, rue Henri Koch 29, 4354 Esch-sur-Alzette, Luxembourg

[f] Faculty of Science, Technology and Medicine, University of Luxembourg, 2 Avenue de l'Université, L-4365 Esch-sur-Alzette, Luxembourg

[g] Institute of Medicinal and Pharmaceutical Chemistry, Technische Universität Braunschweig, Beethovenstraße 55, 38106 Braunschweig, Germany

Correspondence to: finn.hansen@uni-bonn.de and matthias.schiedel@tu-braunschweig.de

## Table of Contents

Page	Contents
<b>S3</b>	<b>Experimental Procedures</b>
<b>S3</b>	<b>Synthesis</b>
S3	General remarks
S4	Synthesis and compound characterization
<b>S14</b>	<b>Biological tests</b>
S14	Cell culture
S14	Transient transfection using polyethylenimine
S14	Membrane preparation
S14	cDNA constructs
S14	ELISA
S15	Emission and excitation spectra of the fluorescent ligands
S15	Emission spectra of Nluc-labeled CCR1 (CCR1_Nluc) protein
S15	NanoBRET binding assays
S16	Cellular NanoBiT $\beta$ -arrestin recruitment assays
<b>S17</b>	<b>Computational Methods</b>
S17	Molecular docking
<b>S18</b>	<b>Supplementary Figures</b>
<b>S25</b>	<b>Supplementary NMR Spectra</b>
<b>S45</b>	<b>Supplementary HPLC Chromatograms</b>
<b>S55</b>	<b>Supplementary References</b>

## Experimental Procedures

### Synthesis

#### General remarks:

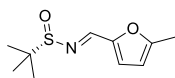
Chemicals were obtained from abcr GmbH (Karlsruhe, Germany), Acros Organics (Geel, Belgium), Carbolution Chemicals (Sankt Ingberg, Germany), Sigma-Aldrich (Steinheim, Germany), TCI Chemicals (Eschborn, Germany) or VWR (Langenfeld, Germany) and used without further purification. Technical-grade solvents were distilled prior to use. For all HPLC purposes, acetonitrile in HPLC-grade quality (HiPerSolv CHROMANORM, VWR, Langenfeld, Germany) was used. Water was purified with a PURELAB flex® (ELGA VEOLIA, Celle, Germany). The following literature known compounds were either purchased from commercial suppliers (MedChemExpress: navarixin (**1**), PF-07054894 (**2**), danirixin (**8**), SB225002 (**9**), reparixin (**11**), CCR6 antagonist **1** (**12**), CCR6 inhibitor **1** (**13**)) or synthesized according to previously published procedures (Mz438 (**3**),<sup>[1]</sup> LT220 (**4**),<sup>[1]</sup> LT221 (**5**),<sup>[1]</sup> linker-ligand conjugate Xi (**6**),<sup>[1]</sup> CCR2-RA (**14**),<sup>[2]</sup> SD-24 (**15**),<sup>[2]</sup> vercirnon (**16**),<sup>[3]</sup> AAA30 (**17**),<sup>[3]</sup> MHS-37 (**28**)).<sup>[3]</sup> Thin-layer chromatography (TLC) was carried out on prefabricated plates (silica gel 60, F254, Merck). Components were visualized either by irradiation with ultraviolet light (254 nm or 366 nm) or by appropriate staining. Column chromatography was carried out on silica gel (60 Å, 40–60 µm, Acros Organics, Geel, Belgium). If no solvent is stated, an aqueous solution was prepared with demineralized water. Mixtures of two or more solvents are specified as “solvent A”/“solvent B”, 3/1, v/v; meaning that 100 mL of the respective mixture consists of 75 mL of “solvent A” and 25 mL of “solvent B”. The uncorrected melting points were determined using a Büchi (Essen, Germany) Melting Point M-560 apparatus. Diastereomeric ratios were determined by <sup>1</sup>H NMR spectroscopy. Proton (<sup>1</sup>H) and carbon (<sup>13</sup>C) NMR spectra were recorded either on a Bruker AVANCE 500 MHz at a frequency of 500 MHz (<sup>1</sup>H) and 126 MHz (<sup>13</sup>C) or a Bruker AVANCE III HD 600 MHz at a frequency of 600 MHz (<sup>1</sup>H) and 151 MHz (<sup>13</sup>C). The chemical shifts are given in parts per million (ppm). As solvents, deuterated chloroform (CDCl<sub>3</sub>), deuterated methanol (methanol-*d*<sub>4</sub>) and deuterated dimethyl sulfoxide (DMSO-*d*<sub>6</sub>) were used. The residual solvent signal (CDCl<sub>3</sub>: <sup>1</sup>H NMR: 7.26 ppm, <sup>13</sup>C NMR: 77.1 ppm; DMSO-*d*<sub>6</sub>: <sup>1</sup>H NMR: 2.50 ppm, <sup>13</sup>C NMR: 39.52 ppm; methanol-*d*<sub>4</sub>: <sup>1</sup>H NMR: 3.31 ppm, 4.87 ppm, <sup>13</sup>C NMR: 49.0 ppm) was used for calibration. The multiplicity of each signal is reported as singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m) or combinations thereof. Multiplicities and coupling constants are reported as measured and might disagree with the expected values.

High-resolution electrospray-ionization mass spectra (HRMS-ESI) were acquired with a Bruker Daltonik GmbH micrOTOF coupled to a LC Packings Ultimate HPLC system and controlled by micrOTOFControl3.4 and HyStar 3.2-LC/MS, with a Bruker Daltonik GmbH ESI-qTOF Impact II coupled to a Dionex UltiMate™ 3000 UHPLC system and controlled by micrOTOFControl 4.0 and HyStar 3.2-LC/MS or with a micrOTOF-Q mass spectrometer (Bruker, Bremen, Germany) with ESI-source coupled with an HPLC Dionex UltiMate 3000 (Thermo Scientific, Heysham, United Kingdom). Low-resolution electrospray-ionization mass spectra (LRMS-ESI) were acquired with an Advion expression® compact mass spectrometer (CMS) coupled with an automated TLC plate reader Plate Express® (Advion, Ithaca, NY, USA).

For high performance liquid chromatography (HPLC) we used a Thermo Fisher Scientific (Heysham, United Kingdom) UltiMate™ 3000 UHPLC system with a Nucleodur 100-5 C18 (250 × 4.6 mm, Macherey Nagel, Düren, Germany) with a flow rate of 1 mL/min and a temperature of 25 °C, or a 100-5 C18 (100 × 3 mm, Macherey Nagel, Düren, Germany) with a flow rate of 0.5 mL/min and a temperature of 25 °C, and an appropriate gradient. For preparative purposes, an AZURA Prep. 500/1000 gradient system with a Nucleodur 110-5 C18 HTec (150 × 32 mm, Macherey Nagel, Düren, Germany) column with 20 mL/min was used. Detection was implemented with UV absorption measurement at wavelengths of λ = 220 nm and λ = 250 nm. Bidest. H<sub>2</sub>O (A) and MeCN (B) were used as eluents with an addition of 0.1% TFA in case of eluent A. Purity: The purity of all final compounds was 95% or higher. Purity was determined via HPLC with the Nucleodur 100-5 C18 (250 × 4.6 mm, Macherey Nagel, Düren, Germany) at 250 nm. After column equilibration for 5 min, a linear gradient from 5% A to 95% B in 7 min followed by an isocratic regime of 95% B for 10 min was used.

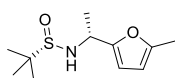
## Synthesis and compound characterization:

### (*S,E*)-2-Methyl-*N*-[(5-methylfuran-2-yl)methylene]propane-2-sulfonamide (**20**)<sup>[1]</sup>



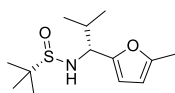
(*S*)-2-Methylpropane-2-sulfonamide (**19**, 2.00 g, 16.2 mmol, 1.00 eq) was dissolved in dichloromethane (10 mL). 5-Methylfuran-2-carboxaldehyde (**18**, 1.69 mL, 16.2 mmol, 1.00 eq), titanium(IV) ethoxide (7.50  $\mu$ L, 35.6 mmol, 2.20 eq) and sodium sulfate (2 g) were added under stirring. The reaction mixture was stirred at room temperature overnight, filtered through celite, and rinsed with dichloromethane. Evaporation of the solvent gave the crude product in quantitative yield, which was used in the next step without further purification.

### (*S*)-2-Methyl-*N*-[(*R*)-1-(5-methylfuran-2-yl)ethyl]propane-2-sulfonamide (**21b**)



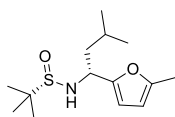
A 250 mL flask was sealed with a septum, flushed with N<sub>2</sub>, and filled with THF (20 mL). A 2 M methylmagnesium chloride solution in THF (5.15 mL, 10.3 mmol, 2.20 eq.) was added and the solution was cooled to 0 °C. (*S,E*)-2-Methyl-*N*-[(5-methylfuran-2-yl)methylene]propane-2-sulfonamide (**20**, 1.0 g, 4.7 mmol, 1.0 eq.) was dissolved in THF (20 mL) and added dropwise to the vigorously stirred solution. Afterwards, the reaction mixture was stirred for 72 h. The reaction mixture was then quenched by the addition of saturated ammonium chloride solution (50 mL) and extracted using ethyl acetate (3 x 100 mL). Drying over sodium sulfate, filtration, and evaporation of the solvent resulted in the crude product which was purified by column chromatography using a gradient of dichloromethane and ethyl acetate (dichloromethane to dichloromethane/ethyl acetate (8/2) to dichloromethane/ethyl acetate (5/5) (v/v)). The title compound was obtained as a dark orange to red oil (207 mg, 19%). <sup>1</sup>H NMR (600 MHz, DMSO-*d*<sub>6</sub>  $\delta$  [ppm]) 6.16 & 6.09 (d, *J* = 3.1 Hz, 1H), 5.99 – 5.93 (m, 1H), 5.49 & 5.32 (d, *J* = 7.1 Hz, 1H), 4.39 – 4.29 (m, 1H), 2.25 – 2.19 (m, 3H), 1.45 & 1.41 (d, *J* = 6.8 Hz, 3H), 1.13 – 1.05 (m, 9H); <sup>13</sup>C NMR (151 MHz, DMSO-*d*<sub>6</sub>  $\delta$  [ppm]) 154.9, 150.3, 106.4, 106.1, 55.0, 49.3, 22.5, 21.3, 13.2; LRMS *m/z* (ESI<sup>+</sup>) [found: 230.2, C<sub>11</sub>H<sub>20</sub>NO<sub>2</sub>S<sup>+</sup> requires [M+H]<sup>+</sup> 230.3]; R<sub>f</sub>: 0.24 (dichloromethane/ethyl acetate (1/1) (v/v)); diastereomeric ratio: *dr* = 7:3.

### (*S*)-2-Methyl-*N*-[(*R*)-2-methyl-1-(5-methylfuran-2-yl)propyl]propane-2-sulfonamide (**21c**)



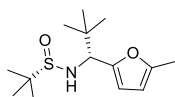
A 250 mL flask was sealed with a septum flushed with N<sub>2</sub> and filled with THF (20 mL). A 2 M isopropylmagnesium chloride solution in THF (5.15 mL, 10.3 mmol, 2.20 eq.) was added and the solution was cooled to 0 °C. (*S,E*)-2-Methyl-*N*-[(5-methylfuran-2-yl)methylene]propane-2-sulfonamide (**20**, 1.0 g, 4.7 mmol, 1.0 eq.) was dissolved in 20 mL THF and added dropwise to the vigorously stirred solution. Afterwards, the reaction mixture was stirred for 72 h. The reaction mixture was then quenched by the addition of saturated ammonium chloride solution (50 mL) and extracted using ethyl acetate (3 x 100 mL). Drying over sodium sulfate, filtration, and evaporation of the solvent resulted in the crude product which was purified by column chromatography using a gradient of dichloromethane and ethyl acetate (dichloromethane to dichloromethane/ethyl acetate (8/2) to dichloromethane/ethyl acetate (5/5) (v/v)). The title compound was obtained as a dark orange to red oil (220 mg, 18%). <sup>1</sup>H NMR (600 MHz, DMSO-*d*<sub>6</sub>  $\delta$  [ppm]) 6.08 (d, *J* = 3.1 Hz, 1H), 5.98 – 5.94 (m, 1H), 5.10 (d, *J* = 6.1 Hz, 1H), 3.97 (t, *J* = 6.3 Hz, 1H), 2.20 (s, 3H), 2.12 – 2.03 (m, *J* = 6.8 Hz, 1H), 1.07 (s, 9H), 0.91 (d, *J* = 6.8 Hz, 3H), 0.82 (d, *J* = 6.8 Hz, 3H); <sup>13</sup>C NMR (151 MHz, DMSO-*d*<sub>6</sub>  $\delta$  [ppm]) 152.9, 150.2, 107.9, 106.0, 59.7, 55.2, 32.0, 22.5, 19.4, 18.6, 13.3; LRMS *m/z* (ESI<sup>+</sup>) [found: 258.1, C<sub>13</sub>H<sub>24</sub>NO<sub>2</sub>S<sup>+</sup> requires [M+H]<sup>+</sup> 258.4]; R<sub>f</sub>: 0.14 (dichloromethane/ethyl acetate (7/3) (v/v)); diastereomeric ratio: *dr* = 1:0.

**(S)-2-Methyl-N-[(R)-3-methyl-1-(5-methylfuran-2-yl)butyl]propane-2-sulfonamide (21d)**



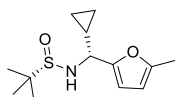
A 250 mL flask was sealed with a septum flushed with N<sub>2</sub> and filled with THF (20 mL). A 2 M isobutylmagnesium chloride solution in THF (5.15 mL, 10.3 mmol, 2.20 eq.) was added and the solution was cooled to 0 °C. (S,E)-2-Methyl-N-[(5-methylfuran-2-yl)methylene]propane-2-sulfonamide (**20**, 1.0 g, 4.7 mmol, 1.0 eq.) was dissolved in 20 mL THF and added dropwise to the vigorously stirred solution. Afterwards, the reaction mixture was stirred for 72 h. The reaction mixture was then quenched by the addition of saturated ammonium chloride solution (50 mL) and extracted using ethyl acetate (3 x 100 mL). Drying over sodium sulfate, filtration, and evaporation of the solvent resulted in the crude product which was purified by column chromatography using a gradient of dichloromethane and ethyl acetate (dichloromethane to dichloromethane/ethyl acetate (8/2) to dichloromethane/ethyl acetate (5/5) (v/v)). The title compound was obtained as a dark orange to red oil (233 mg, 18%). <sup>1</sup>H NMR (600 MHz, DMSO-*d*<sub>6</sub> δ [ppm]) 6.17 & 6.11 (d, *J* = 3.1 Hz, 1H), 5.97 – 5.92 (m, 1H), 5.43 & 5.26 (d, *J* = 6.1 Hz, 1H), 4.25 – 4.08 (m, 1H), 2.26 – 2.17 (m, 3H), 1.75 – 1.52 (m, 3H), 1.15 – 1.01 (m, 9H), 0.97 – 0.83 (m, 6H); <sup>13</sup>C NMR (151 MHz, DMSO-*d*<sub>6</sub> δ [ppm]) 154.9, 154.1, 150.3, 150.1, 107.0, 106.9, 106.1, 106.0, 55.5, 55.0, 52.1, 51.9, 43.6, 43.5, 24.0, 23.9, 22.7, 22.5, 22.4, 22.2, 21.5, 13.3, 13.2; LRMS *m/z* (ESI<sup>+</sup>) [found: 272.4, C<sub>14</sub>H<sub>26</sub>NO<sub>2</sub>S<sup>+</sup> requires [M+H]<sup>+</sup> 272.4]; R<sub>f</sub>: 0.36 (cyclohexane/dichloromethane/ethyl acetate (4/4/3) (v/v)); diastereomeric ratio: *dr* = 6:4.

**(S)-N-[(R)-2,2-Dimethyl-1-(5-methylfuran-2-yl)propyl]-2-methylpropane-2-sulfonamide (21e)<sup>[1]</sup>**



A 250 mL flask was sealed with a septum flushed with N<sub>2</sub> and filled with THF (20 mL). A 2M *tert*-butylmagnesium chloride solution in THF (5.15 mL, 10.3 mmol, 2.20 eq.) was added and the solution was cooled to 0 °C. (S,E)-2-Methyl-N-[(5-methylfuran-2-yl)methylene]propane-2-sulfonamide (**20**, 1.0 g, 4.7 mmol, 1.0 eq.) was dissolved in 20 mL THF and added dropwise to the vigorously stirred solution. Afterwards, the reaction mixture was stirred for 72 h. The reaction mixture was then quenched by the addition of saturated ammonium chloride solution (50 mL) and extracted using ethyl acetate (3 x 100 mL). Drying over sodium sulfate, filtration, and evaporation of the solvent resulted in the crude product which was purified by column chromatography using a gradient of dichloromethane and ethyl acetate (dichloromethane to dichloromethane/ethyl acetate (8/2) to dichloromethane/ethyl acetate (5/5) (v/v)). The title compound was obtained as a dark orange to red oil (690 mg, 54%). <sup>1</sup>H NMR (600 MHz, DMSO-*d*<sub>6</sub> δ [ppm]) 6.11 (d, *J* = 3.1 Hz, 1H), 5.98 – 5.95 (m, 1H), 4.64 (d, *J* = 6.3 Hz, 1H), 3.94 (d, *J* = 6.3 Hz, 1H), 2.21 (s, 3H), 1.05 (s, 9H), 0.92 (s, 9H); <sup>13</sup>C NMR (151 MHz, DMSO-*d*<sub>6</sub> δ [ppm]) 152.5, 149.9, 108.5, 106.0, 62.9, 55.3, 35.3, 26.5, 22.2, 13.2; LRMS *m/z* (ESI<sup>+</sup>) [found: 272.5, C<sub>14</sub>H<sub>26</sub>NO<sub>2</sub>S<sup>+</sup> requires [M+H]<sup>+</sup> 272.4]; R<sub>f</sub>: 0.28 (cyclohexane/ethyl acetate (7/3) (v/v)); diastereomeric ratio: *dr* = 1:0. The obtained analytical data are in good agreement with literature values.<sup>[1]</sup>

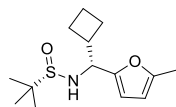
**(S)-N-[(R)-Cyclopropyl(5-methylfuran-2-yl)methyl]-2-methylpropane-2-sulfonamide (21f)<sup>[4]</sup>**



An oven dried 100 mL flask was flushed with N<sub>2</sub> and charged with freshly crushed magnesium turnings (253 mg, 10.3 mmol, 2.20 eq.) and a few iodine crystals were added. This mixture was dissolved in dry THF (15 mL) and stirring was started. Bromocyclopropane (870 μL, 1.31 g, 10.3 mmol, 2.20 eq.) was added dropwise to the solution and was stirred at 50 °C under nitrogen pressure until the magnesium disappeared and the solution turned to a greyish color. (S,E)-2-Methyl-N-[(5-methylfuran-2-yl)methylene]propane-2-sulfonamide (**20**, 1.0 g, 4.7 mmol, 1.0 eq.) was dissolved with THF (20 mL) and was added dropwise to the vigorously stirred grignard solution. Afterwards, the reaction mixture was stirred for 72 h. The reaction mixture was then quenched by the addition of saturated ammonium chloride solution (50 mL) and extracted using ethyl acetate (3 x 100 mL). Drying over sodium sulfate, filtration, and evaporation of the solvent resulted in the crude product which was purified by column chromatography using a gradient of dichloromethane and ethyl acetate (dichloromethane to dichloromethane/ethyl acetate (8/2) to dichloromethane/ethyl acetate (5/5) (v/v)). The title compound was obtained as a dark orange to red oil (200 mg, 17%). <sup>1</sup>H NMR (600 MHz, DMSO-*d*<sub>6</sub> δ [ppm]) 6.19 & 6.15 (d, *J* = 3.1 Hz, 1H), 5.98 – 5.95 (m, 1H), 5.49 & 5.33 (d, *J* = 6.2 Hz, 1H), 3.57 – 3.51 (m, 1H), 2.22 & 2.21 (s, 3H), 1.29 – 1.20 (m, 1H),

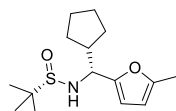
1.11 & 1.10 (s, 9H), 0.58 – 0.48 (m, 2H), 0.41 – 0.32 (m, 2H);  $^{13}\text{C}$  NMR (151 MHz,  $\text{DMSO}-d_6$   $\delta$  [ppm]) 154.9, 154.1, 150.3, 150.1, 107.0, 106.9, 106.1, 106.0, 55.5, 55.0, 52.1, 51.9, 43.6, 43.5, 24.0, 23.9, 22.7, 22.5, 22.5, 22.4, 22.2, 21.5, 13.3, 13.2; LRMS  $m/z$  (ESI $^+$ ) [found: 256.3,  $\text{C}_{13}\text{H}_{22}\text{NO}_2\text{S}^+$  requires  $[\text{M}+\text{H}]^+$  256.1];  $R_f$ : 0.10 (cyclohexane/dichloromethane/ethyl acetate (4/4/3) (v/v)); diastereomeric ratio:  $dr = 7:3$ . The obtained analytical data are in good agreement with literature values.<sup>[4]</sup>

**(S)-N-[(R)-Cyclobutyl(5-methylfuran-2-yl)methyl]-2-methylpropane-2-sulfonamide (21g)**



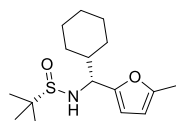
An oven dried 100 mL flask was flushed with  $\text{N}_2$  and charged with freshly crushed magnesium turnings (253 mg, 10.3 mmol, 2.20 eq.) and a few iodine crystals were added. This mixture was dissolved in dry THF (15 mL) and stirring was started. Bromocyclobutane (1.02 mL, 1.47 g, 10.3 mmol, 2.20 eq.) was added dropwise to the solution and was stirred at 50 °C under nitrogen pressure until the magnesium disappeared and the solution turned to a greyish color. (S,E)-2-Methyl-N-[(5-methylfuran-2-yl)methylene]propane-2-sulfonamide (**20**, 1.0 g, 4.7 mmol, 1.0 eq.) was dissolved with THF (20 mL) and was added dropwise to the vigorously stirred grignard solution. Afterwards, the reaction mixture was stirred for 72 h. The reaction mixture was then quenched by the addition of saturated ammonium chloride solution (50 mL) and extracted using ethyl acetate (3 x 100 mL). Drying over sodium sulfate, filtration, and evaporation of the solvent resulted in the crude product which was purified by column chromatography using a gradient of dichloromethane and ethyl acetate (dichloromethane to dichloromethane/ethyl acetate (8/2) to dichloromethane/ethyl acetate (5/5) (v/v)). The title compound was obtained as a dark orange to red oil (181 mg, 14%).  $^1\text{H}$  NMR (600 MHz,  $\text{DMSO}-d_6$   $\delta$  [ppm]) 6.10 & 6.05 (d,  $J = 3.1$  Hz, 1H), 5.97 – 5.92 (m, 1H), 5.34 & 5.15 (d,  $J = 7.4$  Hz, 1H), 4.15 – 3.98 (m, 1H), 2.77 – 2.65 (m, 1H), 2.20 & 2.19 (s, 3H), 2.10 – 1.95 (m, 1H), 1.94 – 1.63 (m, 4H), 1.17 – 0.99 (m, 10H);  $^{13}\text{C}$  NMR (151 MHz,  $\text{DMSO}-d_6$   $\delta$  [ppm]) 153.4, 153.1, 150.3, 150.3, 107.2, 107.0, 106.0, 106.0, 59.0, 58.8, 55.6, 55.2, 38.4, 25.6, 25.1, 22.6, 22.5, 16.9, 13.3, 13.2; LRMS  $m/z$  (ESI $^+$ ) [found: 270.2,  $\text{C}_{14}\text{H}_{24}\text{NO}_2\text{S}^+$  requires  $[\text{M}+\text{H}]^+$  270.1];  $R_f$ : 0.25 (cyclohexane/dichloromethane/ethyl acetate (4/4/3) (v/v)); diastereomeric ratio:  $dr = 6:4$ .

**(S)-N-[(R)-Cyclopentyl(5-methylfuran-2-yl)methyl]-2-methylpropane-2-sulfonamide (21h)**



A 250 mL flask was sealed with a septum flushed with  $\text{N}_2$  and filled with THF (20 mL). A 2 M cyclopentylmagnesium chloride solution in THF (5.15 mL, 10.3 mmol, 2.20 eq.) was added and the solution was cooled to 0 °C. (S,E)-2-Methyl-N-[(5-methylfuran-2-yl)methylene]propane-2-sulfonamide (**20**, 1.0 g, 4.7 mmol, 1.0 eq.) was dissolved in 20 mL THF and added dropwise to the vigorously stirred solution. Afterwards, the reaction mixture was stirred for 72 h. The reaction mixture was then quenched by the addition of saturated ammonium chloride solution (50 mL) and extracted using ethyl acetate (3 x 100 mL). Drying over sodium sulfate, filtration, and evaporation of the solvent resulted in the crude product which was purified by column chromatography using a gradient of dichloromethane and ethyl acetate (dichloromethane to dichloromethane/ethyl acetate (8/2) to dichloromethane/ethyl acetate (5/5) (v/v)). The title compound was obtained as a dark orange to red oil (171 mg, 13%).  $^1\text{H}$  NMR (600 MHz,  $\text{DMSO}-d_6$   $\delta$  [ppm]) 6.12 & 6.08 (d,  $J = 3.1$  Hz, 1H), 6.00 – 5.90 (m, 1H), 5.35 & 5.13 (d,  $J = 6.0$  Hz, 1H), 4.03 – 3.81 (m, 1H), 2.41 – 2.28 (m, 1H), 2.21 & 2.20 (s, 3H), 1.88 – 0.79 (m, 17H);  $^{13}\text{C}$  NMR (151 MHz,  $\text{DMSO}-d_6$   $\delta$  [ppm]) 154.7, 154.0, 150.1, 149.9, 107.3, 107.1, 106.0, 106.0, 71.7, 58.4, 58.2, 55.6, 55.3, 55.1, 44.0, 43.6, 35.0, 29.9, 29.8, 29.5, 29.2, 24.9, 24.9, 24.7, 22.9, 22.6, 22.4, 13.3, 13.2; LRMS  $m/z$  (ESI $^+$ ) [found: 284.4,  $\text{C}_{15}\text{H}_{26}\text{NO}_2\text{S}^+$  requires  $[\text{M}+\text{H}]^+$  284.4];  $R_f$ : 0.13 (cyclohexane/dichloromethane/ethyl acetate (4/4/3) (v/v)); diastereomeric ratio:  $dr = 6:4$ .

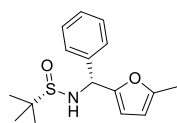
**(S)-N-[(R)-Cyclohexyl(5-methylfuran-2-yl)methyl]-2-methylpropane-2-sulfonamide (21i)**



A 250 mL flask was sealed with a septum flushed with  $\text{N}_2$  and filled with THF (20 mL). A 2 M cyclohexylmagnesium chloride solution in THF (5.15 mL, 10.3 mmol, 2.20 eq.) was added and the solution was cooled to 0 °C. (S,E)-2-methyl-N-[(5-

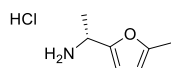
methylfuran-2-yl)methylene]propane-2-sulfinamide (**20**, 1.0 g, 4.7 mmol, 1.0 eq.) was dissolved in 20 mL THF and added dropwise to the vigorously stirred solution. Afterwards, the reaction mixture was stirred for 72 h. The reaction mixture was then quenched by the addition of saturated ammonium chloride solution (50 mL) and extracted using ethyl acetate (3 x 100 mL). Drying over sodium sulfate, filtration, and evaporation of the solvent resulted in the crude product which was purified by column chromatography using a gradient of dichloromethane and ethyl acetate (dichloromethane to dichloromethane/ethyl acetate (8/2) to dichloromethane/ethyl acetate (5/5) (v/v)). The title compound was obtained as a dark orange to red oil (696 mg, 50%). <sup>1</sup>H NMR (600 MHz, DMSO-*d*<sub>6</sub> δ [ppm]) 6.12 & 6.05 (d, *J* = 3.1 Hz, 1H), 5.97 -5.92 (m, 1H), 5.20 & 5.11 (d, *J* = 6.1 Hz 1H), 4.09 – 3.78 (m, 1H), 2.22 & 2.20 (s, 3H), 1.98 – 1.82 (m, 1H), 1.82 – 1.52 (m, 3H), 1.51 – 1.35 (m, 2H), 1.21 – 1.03 (m, 12H), 0.98 – 0.84 (m, 2H); <sup>13</sup>C NMR (151 MHz, DMSO-*d*<sub>6</sub> δ [ppm]) 154.1, 153.3, 150.3, 150.2, 107.8, 107.7, 106.2, 106.2, 68.3, 59.3, 59.2, 55.9, 55.3, 41.7, 35.5, 29.7, 29.6, 29.3, 29.1, 26.1, 25.8, 25.7, 25.7, 25.6, 25.5, 23.9, 22.8, 22.6, 22.1, 13.5, 13.4; LRMS *m/z* (ESI<sup>+</sup>) [found: 298.3, C<sub>16</sub>H<sub>28</sub>NO<sub>2</sub>S<sup>+</sup> requires [M+H]<sup>+</sup> 298.5]; R<sub>f</sub>: 0.22 (cyclohexane/dichloromethane/ethyl acetate (4/4/3) (v/v)); diastereomeric ratio: *dr* = 6:4.

**(S)-2-Methyl-N-[(R)-(5-methylfuran-2-yl)(phenyl)methyl]propane-2-sulfinamide (21j)**<sup>[5]</sup>



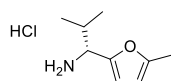
A 250 mL flask was sealed with a septum flushed with N<sub>2</sub> and filled with THF (20 mL). A 2 M phenylmagnesium chloride solution in THF (5.15 mL, 10.3 mmol, 2.20 eq.) was added and the solution was cooled to 0 °C. (S,E)-2-Methyl-N-[(5-methylfuran-2-yl)methylene]propane-2-sulfinamide (**20**, 1.0 g, 4.7 mmol, 1.0 eq.) was dissolved in 20 mL THF and added dropwise to the vigorously stirred solution. Afterwards, the reaction mixture was stirred for 72 h. The reaction mixture was then quenched by the addition of saturated ammonium chloride solution (50 mL) and extracted using ethyl acetate (3 x 100 mL). Drying over sodium sulfate, filtration, and evaporation of the solvent resulted in the crude product which was purified by column chromatography using a gradient of dichloromethane and ethyl acetate (dichloromethane to dichloromethane/ethyl acetate (8/2) to dichloromethane/ethyl acetate (5/5) (v/v)). The title compound was obtained as a dark orange to red oil (353 mg, 26%). <sup>1</sup>H NMR (600 MHz, DMSO-*d*<sub>6</sub> δ [ppm]) 7.44 – 7.39 (m, 2H), 7.37 – 7.31 (m, 2H), 7.31 – 7.24 (m, 1H), 6.10 (d, *J* = 3.10 Hz, 1H), 5.97 (d, *J* = 3.1 Hz, 1H), 5.94 (d, *J* = 6.5 Hz, 1H), 5.46 (d, *J* = 6.5 Hz, 1H), 2.18 (s, 3H), 1.13 (s, 9H); <sup>13</sup>C NMR (151 MHz, DMSO-*d*<sub>6</sub> δ [ppm]) 153.0, 151.0, 141.1, 128.1, 127.6, 127.3, 108.5, 106.3, 56.9, 55.5, 22.6, 13.3; LRMS *m/z* (ESI<sup>+</sup>) [found: 292.3, C<sub>16</sub>H<sub>22</sub>NO<sub>2</sub>S<sup>+</sup> requires [M+H]<sup>+</sup> 292.4]; R<sub>f</sub>: 0.26 (cyclohexane/dichloromethane/ethyl acetate (4/4/3) (v/v)); diastereomeric ratio: *dr* = 1:0. The obtained analytical data are in good agreement with literature values.<sup>[5]</sup>

**(R)-1-(5-Methylfuran-2-yl)ethan-1-amine hydrochloride (22b)**



(S)-2-Methyl-N-[(R)-1-(5-methylfuran-2-yl)ethyl]propane-2-sulfinamide (**21b**, 85 mg, 0.36 mmol, 1.0 eq.) was dissolved in diethyl ether (10 mL) and cooled to 0 °C. After addition of 2 M HCl in diethyl ether (0.45 mL, 0.90 mmol, 2.5 eq.) the mixture was stirred for 1 h. After evaporation of the solvent the title compound was obtained as a reddish oil that was used in the next step without further purification. LRMS *m/z* (ESI<sup>+</sup>) [found: 126.3, C<sub>7</sub>H<sub>12</sub>NO<sup>+</sup> requires [M+H]<sup>+</sup> 126.2].

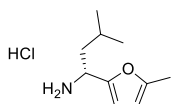
**(R)-2-Methyl-1-(5-methylfuran-2-yl)propan-1-amine hydrochloride (SLW165, 22c)**



(S)-2-Methyl-N-[(R)-2-methyl-1-(5-methylfuran-2-yl)propyl]propane-2-sulfinamide (**21c**, 300 mg, 1.20 mmol, 1.00 eq.) was dissolved in diethyl ether (10 mL) and cooled to 0 °C. After addition of 2 M HCl in diethyl ether (1.5 mL, 3.0 mmol, 2.5 eq.) the mixture was stirred for 1 h. After evaporation of the solvent the title compound was obtained as a reddish oil that was used in the next step without further purification. LRMS *m/z* (ESI<sup>+</sup>) [found: 154.4, C<sub>9</sub>H<sub>16</sub>NO<sup>+</sup> requires [M+H]<sup>+</sup> 154.2].

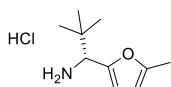


**(R)-3-Methyl-1-(5-methylfuran-2-yl)butan-1-amine hydrochloride (22d)**



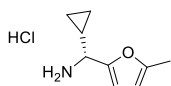
(S)-2-Methyl-N-[(R)-3-methyl-1-(5-methylfuran-2-yl)butyl]propane-2-sulfonamide (**21d**, 0.17 g, 0.60 mmol, 1.0 eq.) was dissolved in diethyl ether (10 mL) and cooled to 0 °C. After addition of 2 M HCl in diethyl ether (0.80 mL, 1.6 mmol, 2.5 eq.) the mixture was stirred for 1 h. After evaporation of the solvent the title compound was obtained as a reddish oil that was used in the next step without further purification. LRMS  $m/z$  (ESI<sup>+</sup>) [found: 168.3, C<sub>10</sub>H<sub>18</sub>NO<sup>+</sup> requires [M+H]<sup>+</sup> 168.2].

**(R)-2,2-Dimethyl-1-(5-methylfuran-2-yl)propan-1-amine hydrochloride (22e)<sup>[1]</sup>**



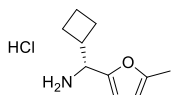
(S)-N-[(R)-2,2-Dimethyl-1-(5-methylfuran-2-yl)propyl]-2-methylpropane-2-sulfonamide (**21e**, 845 mg, 3.10 mmol, 1.00 eq.) was dissolved in diethyl ether (10 mL) and cooled to 0 °C. After addition of 2 M HCl in diethyl ether (3.9 mL, 7.8 mmol, 2.5 eq.) the mixture was stirred for 1 h. After evaporation of the solvent the title compound was obtained as a reddish oil that was used in the next step without further purification. LRMS  $m/z$  (ESI<sup>+</sup>) [found: 168.3, C<sub>10</sub>H<sub>18</sub>NO<sup>+</sup> requires [M+H]<sup>+</sup> 168.2]. The obtained analytical data are in good agreement with literature values.<sup>[1]</sup>

**(R)-Cyclopropyl(5-methylfuran-2-yl)methanamine hydrochloride (22f)**



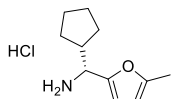
(S)-N-[(R)-Cyclopropyl(5-methylfuran-2-yl)methyl]-2-methylpropane-2-sulfonamide (**21f**, 0.17 g, 0.70 mmol, 1.0 eq.) was dissolved in diethyl ether (10 mL) and cooled to 0 °C. After addition of 2 M HCl in diethyl ether (850 µL, 1.70 mmol, 2.50 eq.) the mixture was stirred for 1 h. After evaporation of the solvent the title compound was obtained as a reddish oil that was used in the next step without further purification. LRMS  $m/z$  (ESI<sup>+</sup>) [found: 153.2, C<sub>9</sub>H<sub>14</sub>NO<sup>+</sup> requires [M+H]<sup>+</sup> 153.1].

**(R)-Cyclobutyl(5-methylfuran-2-yl)methanamine hydrochloride (22g)**



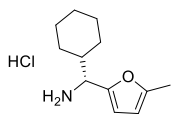
(S)-N-[(R)-Cyclobutyl(5-methylfuran-2-yl)methyl]-2-methylpropane-2-sulfonamide (**21g**, 0.16 g, 0.60 mmol, 1.0 eq.) was dissolved in diethyl ether (10 mL) and cooled to 0 °C. After addition of 2 M HCl in diethyl ether (0.75 mL, 1.5 mmol, 2.5 eq.) the mixture was stirred for 1 h. After evaporation of the solvent the title compound was obtained as a reddish oil that was used in the next step without further purification. LRMS  $m/z$  (ESI<sup>+</sup>) [found: 166.3, C<sub>10</sub>H<sub>16</sub>NO<sup>+</sup> requires [M+H]<sup>+</sup> 166.1].

**(R)-Cyclopentyl(5-methylfuran-2-yl)methanamine hydrochloride (22h)**



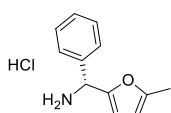
(S)-N-[(R)-Cyclopentyl(5-methylfuran-2-yl)methyl]-2-methylpropane-2-sulfonamide (**21h**, 0.15 g, 0.50 mmol, 1.0 eq.) was dissolved in diethyl ether (10 mL) and cooled to 0 °C. After addition of 2 M HCl in diethyl ether (650 µL, 1.25 mmol, 2.50 eq.) the mixture was stirred for 1 h. After evaporation of the solvent the title compound was obtained as a reddish oil that was used in the next step without further purification. LRMS  $m/z$  (ESI<sup>+</sup>) [found: 180.3, C<sub>11</sub>H<sub>18</sub>NO<sup>+</sup> requires [M+H]<sup>+</sup> 180.3].

**(R)-Cyclohexyl(5-methylfuran-2-yl)methanamine hydrochloride (22i)**



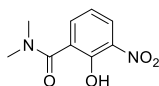
(S)-*N*-[(*R*)-Cyclohexyl(5-methylfuran-2-yl)methyl]-2-methylpropane-2-sulfonamide (**21i**, 0.60 g, 2.0 mmol, 1.0 eq.) was dissolved in diethyl ether (10 mL) and cooled to 0 °C. After addition of 2 M HCl in diethyl ether (2.5 mL, 5.0 mmol, 2.5 eq.) the mixture was stirred for 1 h. After evaporation of the solvent the title compound was obtained as a reddish oil that was used in the next step without further purification. LRMS *m/z* (ESI<sup>+</sup>) [found: 194.4, C<sub>12</sub>H<sub>19</sub>NO<sup>+</sup> requires [M+H]<sup>+</sup> 194.3].

**(R)-(5-Methylfuran-2-yl)(phenyl)methanamine hydrochloride (22j)**



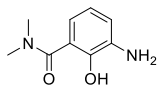
(S)-2-Methyl-*N*-[(*R*)-(5-methylfuran-2-yl)(phenyl)methyl]propane-2-sulfonamide (**21j**, 0.30 g, 1.0 mmol, 1.0 eq.) was dissolved in diethyl ether (10 mL) and cooled to 0 °C. After addition of 2 M HCl in diethyl ether (1.3 mL, 2.5 mmol, 2.5 eq.) the mixture was stirred for 1 h. After evaporation of the solvent the title compound was obtained as a reddish oil that was used in the next step without further purification. LRMS *m/z* (ESI<sup>+</sup>) [found: 188.3, C<sub>12</sub>H<sub>14</sub>NO<sup>+</sup> requires [M+H]<sup>+</sup> 188.2].

**2-Hydroxy-*N,N*-dimethyl-3-nitrobenzamide (24)<sup>[6]</sup>**



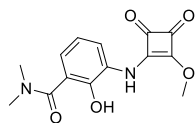
3-Nitrosalicylic acid (**23**, 1.5 g, 8.0 mmol, 1.0 eq.) was dissolved in dichloromethane (60 mL) at room temperature. 2 M Oxalyl chloride solution (5.20 mL, 1.35 g, 10.4 mmol, 1.30 eq.) and two drops of *N,N*-dimethylformamide were added to the vigorously stirred solution. The mixture was stirred overnight, and the solvent was evaporated under reduced pressure to give a white solid. The solid was dissolved in dichloromethane (30 mL) and cooled to 0 °C. To this solution a 2 M dimethyl amine solution (12.0 mL, 2.00 g, 24.1 mmol, 3.00 eq.) and DIPEA (5.70 mL, 4.24 g, 32.1 mmol, 4.00 eq.) were added. This reaction mixture was stirred overnight at room temperature and the solution was concentrated in vacuo, diluted with 1 M sodium hydroxide (50 mL) and washed with dichloromethane (100 mL). The aqueous phase was acidified with 6 M HCl (20 mL) and extracted with dichloromethane (3 x 100 mL). After drying over sodium sulfate, filtration, and evaporation of the solvent, the crude product was purified by column chromatography using ethyl acetate to obtain the title compound as an orange gum (434 mg, 26%). <sup>1</sup>H NMR (600 MHz, DMSO-*d*<sub>6</sub> δ [ppm]): 10.67 (s, 1H), 8.02 (dd, *J* = 8.3, 1.7 Hz, 1H), 7.55 (dd, *J* = 7.4, 1.7 Hz, 1H), 7.09 (dd, *J* = 8.3, 7.4 Hz, 1H), 3.23 – 2.63 (m, 6H); <sup>13</sup>C NMR (151 MHz, DMSO-*d*<sub>6</sub> δ [ppm]): 166.2, 148.5, 136.3, 134.2, 129.2, 125.5, 120.0, 40.1; LRMS *m/z* (ESI<sup>+</sup>) [found: 211.0, C<sub>9</sub>H<sub>11</sub>N<sub>2</sub>O<sub>4</sub><sup>+</sup> requires [M+H]<sup>+</sup> 211.1]; R<sub>f</sub>: 0.56 (ethyl acetate). The obtained analytical data are in good agreement with literature values.<sup>[6]</sup>

**3-Amino-2-hydroxy-*N,N*-dimethylbenzamide (25)<sup>[6]</sup>**



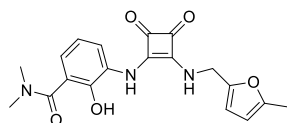
2-Hydroxy-*N,N*-dimethyl-3-nitrobenzamide (**24**, 400 mg, 1.90 mmol, 1.00 eq.) was dissolved in ethanol (10 mL) in a 25 mL flask sealed with a septum. 5% Pd/C (80 mg, 0.19 mmol, 0.10 eq.) was added under an inert atmosphere and the reaction mixture was stirred overnight under a hydrogen atmosphere at room temperature. The reaction mixture was then filtered through a short pad of celite and washed with ethyl acetate (30 mL) and concentrated to dryness to obtain the title compound as a green oil in quantitative yield that was used in the next step without further purification. LRMS *m/z* (ESI<sup>+</sup>) [found: 181.0, C<sub>9</sub>H<sub>13</sub>N<sub>2</sub>O<sub>2</sub><sup>+</sup> requires [M+H]<sup>+</sup> 181.1]. The obtained analytical data are in good agreement with literature values.<sup>[6]</sup>

## 2-Hydroxy-3-[(2-methoxy-3,4-dioxocyclobut-1-en-1-yl)amino]-*N,N*-dimethylbenzamide (26)<sup>[7]</sup>



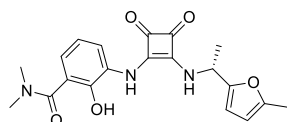
3-Amino-2-hydroxy-*N,N*-dimethylbenzamide (**25**, 320 mg, 1.80 mmol, 1.00 eq) and 3,4-dimethoxycyclobut-3-ene-1,2-dione (253 mg, 1.80 mmol, 1.00 eq.) were dissolved in methanol (10 mL) and stirred overnight. Evaporation of the solvent under reduced pressure afforded the title compound as a green gum (480 mg, 93% yield) that was used in the next step without further purification. LRMS *m/z* (ESI<sup>+</sup>) [found: 291.1, C<sub>14</sub>H<sub>15</sub>N<sub>2</sub>O<sub>5</sub><sup>+</sup> requires [M+H]<sup>+</sup> 291.3]; R<sub>f</sub>: 0.36 (ethyl acetate).

## 2-Hydroxy-*N,N*-dimethyl-3-[(2-[(5-methylfuran-2-yl)methyl]amino)-3,4-dioxocyclobut-1-en-1-yl]amino]benzamide (27a)



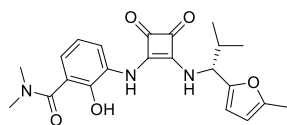
2-Hydroxy-3-[(2-methoxy-3,4-dioxocyclobut-1-en-1-yl)amino]-*N,N*-dimethylbenzamide (**26**, 90 mg, 0.30 mmol, 1.0 eq.) and commercially available (5-methylfuran-2-yl)methanamine (**22a**, 69  $\mu$ L, 0.60 mmol, 2.0 eq.) were dissolved in methanol (10 mL). After the addition of *N,N*-diisopropylethylamine (0.11 mL, 0.60 mmol, 2.0 eq.), the reaction was stirred for 3 days. Extraction between water and ethyl acetate (3  $\times$  50 mL), drying over sodium sulfate, filtration, and evaporation of the solvent gave the crude product which was purified by preparative HPLC (acetonitrile/water (0.1% TFA): gradient 5–95%) to obtain the title compound as a white-brown amorphous solid (24 mg, 21%). <sup>1</sup>H NMR (500 MHz, DMSO-*d*<sub>6</sub>,  $\delta$  [ppm]): 9.90 (bs, 1H), 9.25 (s, 1H), 8.62 (t, *J* = 6.0 Hz, 1H), 7.80 – 7.75 (m, 1H), 6.92 – 6.83 (m, 2H), 6.28 (d, *J* = 3.1 Hz, 1H), 6.05 (d, *J* = 3.1 Hz, 1H), 4.77 (d, *J* = 5.8 Hz, 2H), 2.94 (s, 6H), 2.26 (s, 3H); <sup>13</sup>C NMR (126 MHz, DMSO-*d*<sub>6</sub>,  $\delta$  [ppm]): 184.2, 180.4, 168.8, 168.3, 163.6, 151.7, 149.5, 143.4, 128.6, 124.3, 122.2, 120.9, 119.7, 108.9, 106.6, 40.1, 13.3; HRMS *m/z* (ESI<sup>+</sup>) [found: 370.1395, C<sub>19</sub>H<sub>20</sub>N<sub>3</sub>O<sub>5</sub><sup>+</sup> requires [M+H]<sup>+</sup> 370.1325]; HPLC retention time 11.59 min, 98.0%.

## (*R*)-2-Hydroxy-*N,N*-dimethyl-3-[(2-[(1-(5-methylfuran-2-yl)ethyl]amino)-3,4-dioxocyclobut-1-en-1-yl]amino]benzamide (27b)<sup>[6]</sup>



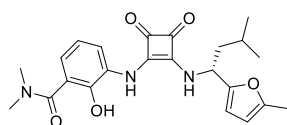
2-Hydroxy-3-[(2-methoxy-3,4-dioxocyclobut-1-en-1-yl)amino]-*N,N*-dimethylbenzamide (**26**, 55 mg, 0.20 mmol, 1.0 eq.) and (*R*)-1-(5-methylfuran-2-yl)ethan-1-amine hydrochloride (**22b**, 61 mg, 0.40 mmol, 2.0 eq.) were dissolved in methanol (10 mL). After the addition of *N,N*-diisopropyl-diethylamine (67  $\mu$ L, 0.40 mmol, 2.0 eq.), the reaction was stirred for 3 days. Extraction between water and ethyl acetate (3  $\times$  50 mL), drying over sodium sulfate, filtration, and evaporation of the solvent gave the crude product which was purified by preparative HPLC (acetonitrile/water (0.1% TFA): gradient 5–95%) to obtain the title compound as a white-brown amorphous solid (31 mg, 43%). <sup>1</sup>H NMR (500 MHz, DMSO-*d*<sub>6</sub>,  $\delta$  [ppm]): The two CH<sub>3</sub> groups of the dimethylamide moiety were not detectable due to the water peak, 11.99 (bs, 1H), 9.33 (s, 1H), 8.69 (d, *J* = 8.6 Hz, 1H), 8.02 (d, *J* = 8.5 Hz, 1H), 7.48 (dd, *J* = 8.0, 1.5 Hz, 1H), 6.92 (t, *J* = 8.0 Hz, 1H), 6.27 (d, *J* = 3.1 Hz, 1H), 6.08 – 6.04 (m, 1H), 5.38 – 5.28 (m, 1H), 2.26 (s, 3H), 1.56 (d, *J* = 6.9 Hz, 3H); <sup>13</sup>C NMR (126 MHz, DMSO-*d*<sub>6</sub>,  $\delta$  [ppm]): 184.1, 180.2, 172.2, 168.3, 163.2, 153.0, 151.4, 151.0, 127.5, 124.9, 123.8, 118.8, 112.8, 107.1, 106.5, 47.0, 40.1, 20.3, 13.3; HRMS *m/z* (ESI<sup>+</sup>) [found: 384.1494, C<sub>20</sub>H<sub>22</sub>N<sub>3</sub>O<sub>5</sub><sup>+</sup> requires [M+H]<sup>+</sup> 383.1481]; HPLC retention time 12.14 min, 98.0%. The obtained analytical data are in good agreement with literature values.<sup>[6]</sup>

**(*R*)-2-Hydroxy-*N,N*-dimethyl-3-[(2-[(2-methyl-1-(5-methylfuran-2-yl)propyl]amino)-3,4-dioxocyclobut-1-en-1-yl)amino]benzamide (27c)<sup>[6]</sup>**



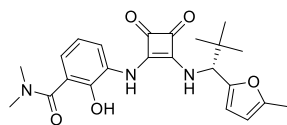
2-Hydroxy-3-[(2-methoxy-3,4-dioxocyclobut-1-en-1-yl)amino]-*N,N*-dimethylbenzamide (**26**, 60 mg, 0.20 mmol, 1.0 eq.) and (*R*)-2-methyl-1-(5-methylfuran-2-yl)propan-1-amine hydrochloride (**22c**, 78 mg, 0.40 mmol, 1.3 eq.) were dissolved in methanol (10 mL). After the addition of *N,N*-diisopropylethylamine (73  $\mu$ L, 0.40 mmol, 2.0 eq.), the reaction was stirred for 3 days. Extraction between water and ethyl acetate (3  $\times$  50 mL), drying over sodium sulfate, filtration, and evaporation of the solvent gave the crude product which was purified by preparative HPLC (acetonitrile/water (0.1% TFA): gradient 5–95%) to obtain the title compound as a white-brown amorphous solid (40 mg, 47%) <sup>1</sup>H NMR (600 MHz, DMSO-*d*<sub>6</sub>,  $\delta$  [ppm]): 9.95 (bs, 1H), 9.36 (s, 1H), 8.68 (d, *J* = 9.61 Hz, 1H), 7.78 (dd, *J* = 7.3, 2.3 Hz, 1H), 6.91 – 6.84 (m, 2H), 6.22 (d, *J* = 3.1 Hz, 1H), 6.06 – 6.03 (m, 1H), 5.08 – 5.02 (m, 1H), 2.94 (s, 6H), 2.27 (s, 3H), 2.23 – 2.14 (m, *J* = 6.8 Hz, 1H), 0.97 (d, *J* = 6.8 Hz, 3H), 0.89 (d, *J* = 6.8 Hz, 3H); <sup>13</sup>C NMR (126 MHz, DMSO-*d*<sub>6</sub>,  $\delta$  [ppm]): 184.0, 180.2, 168.7, 168.3, 163.4, 151.5, 151.2, 143.3, 128.6, 124.4, 122.2, 120.9, 119.8, 107.9, 106.4, 57.2, 40.1, 32.1, 19.1, 18.2, 13.3; HRMS *m/z* (ESI<sup>+</sup>) [found: 412.1792, C<sub>22</sub>H<sub>26</sub>N<sub>3</sub>O<sub>5</sub><sup>+</sup> requires [M+H]<sup>+</sup> 412.1794]; HPLC retention time 12.35 min, 98.0%. The obtained analytical data are in good agreement with literature values.<sup>[6]</sup>

**(*R*)-2-Hydroxy-*N,N*-dimethyl-3-[(2-[(3-methyl-1-(5-methylfuran-2-yl)butyl]amino)-3,4-dioxocyclobut-1-en-1-yl)amino]benzamide (27d)<sup>[8]</sup>**



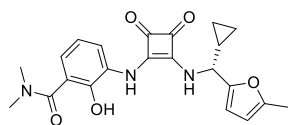
2-Hydroxy-3-[(2-methoxy-3,4-dioxocyclobut-1-en-1-yl)amino]-*N,N*-dimethylbenzamide (**26**, 75 mg, 0.30 mmol, 1.0 eq.) and (*R*)-3-methyl-1-(5-methylfuran-2-yl)butan-1-amine hydrochloride (**22d**, 0.12 g, 0.60 mmol, 2.0 eq.) were dissolved in methanol (10 mL). After the addition of *N,N*-diisopropylethylamine (0.11 mL, 0.60 mmol, 2.0 eq.), the reaction was stirred for 3 days. Extraction between water and ethyl acetate (3  $\times$  50 mL), drying over sodium sulfate, filtration, and evaporation of the solvent gave the crude product which was purified by preparative HPLC (acetonitrile/water (0.1% TFA): gradient 5–95%) to obtain the title compound as a white-brown amorphous solid (20 mg, 18%) <sup>1</sup>H NMR (600 MHz, DMSO-*d*<sub>6</sub>,  $\delta$  [ppm]): 9.92 (bs, 1H), 9.23 (s, 1H), 8.64 (d, *J* = 9.3 Hz, 1H), 7.82 – 7.77 (m, 1H), 6.91 – 6.84 (m, 2H), 6.24 (d, *J* = 3.1 Hz, 1H), 6.04 (dd, *J* = 3.1, 1.2 Hz, 1H), 5.35 – 5.28 (m, 1H), 2.94 (s, 6H), 2.26 (s, 3H), 1.81 – 1.71 (m, 2H), 1.65 – 1.57 (m, 1H), 0.94 (d, *J* = 6.6 Hz, 3H), 0.92 (d, *J* = 6.6 Hz, 3H); <sup>13</sup>C NMR (151 MHz, DMSO-*d*<sub>6</sub>,  $\delta$  [ppm]): 183.9, 180.1, 168.3, 168.3, 163.5, 152.6, 151.3, 143.2, 128.6, 124.4, 122.2, 120.7, 119.8, 107.2, 106.4, 49.6, 42.6, 40.1, 24.4, 22.5, 21.5, 13.3; HRMS *m/z* (ESI<sup>+</sup>) [found: 426.2031, C<sub>23</sub>H<sub>28</sub>N<sub>3</sub>O<sub>5</sub><sup>+</sup> requires [M+H]<sup>+</sup> 426.1951]; HPLC retention time 12.68 min, 98.0%.

**(*R*)-3-[(2-[(2,2-Dimethyl-1-(5-methylfuran-2-yl)propyl]amino)-3,4-dioxocyclobut-1-en-1-yl)amino]-2-hydroxy-*N,N*-dimethylbenzamide (10 / 27e)<sup>[6]</sup>**



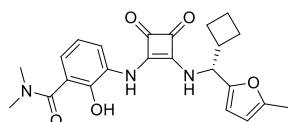
2-Hydroxy-3-[(2-methoxy-3,4-dioxocyclobut-1-en-1-yl)amino]-*N,N*-dimethylbenzamide (**26**, 60 mg, 0.20 mmol, 1.0 eq.) and (*R*)-2,2-dimethyl-1-(5-methylfuran-2-yl)propan-1-amine hydrochloride (**22e**, 84 mg, 0.40 mmol, 2.0 eq.) were dissolved in methanol (10 mL). After the addition of *N,N*-diisopropylethylamine (73  $\mu$ L, 0.40 mmol, 2.0 eq.), the reaction was stirred for 3 days. Extraction between water and ethyl acetate (3  $\times$  50 mL), drying over sodium sulfate, filtration, and evaporation of the solvent gave the crude product which was purified by preparative HPLC (acetonitrile/water (0.1% TFA): gradient 5–95%) to obtain the title compound as a white-brown amorphous solid (43 mg, 49%) <sup>1</sup>H NMR (600 MHz, DMSO-*d*<sub>6</sub>,  $\delta$  [ppm]): 9.97 (bs, 1H), 9.48 (s, 1H), 8.75 (d, *J* = 10.1 Hz, 1H), 7.76 – 7.72 (m, 1H), 6.91 – 6.85 (m, 2H), 6.19 (d, *J* = 3.1 Hz, 1H), 6.06 – 6.03 (m, 1H), 5.12 (d, *J* = 10.1 Hz, 1H), 2.95 (s, 6H), 2.28 (s, 3H), 0.98 (s, 9H); <sup>13</sup>C NMR (151 MHz, DMSO-*d*<sub>6</sub>,  $\delta$  [ppm]): 184.1, 180.3, 168.6, 168.3, 163.4, 151.0, 150.8, 143.5, 128.5, 124.4, 122.3, 121.2, 119.8, 108.5, 106.3, 60.2, 40.1, 35.7, 26.2, 13.4; HRMS *m/z* (ESI<sup>+</sup>) [found: 426.2028, C<sub>23</sub>H<sub>28</sub>N<sub>3</sub>O<sub>5</sub><sup>+</sup> requires [M+H]<sup>+</sup> 426.1951]; HPLC retention time 12.64 min, 98.0%. The obtained analytical data are in good agreement with literature values.<sup>[6]</sup>

**(R)-3-[(2-[(Cyclopropyl(5-methylfuran-2-yl)methyl]amino)-3,4-dioxocyclobut-1-en-1-yl)amino]-2-hydroxy-*N,N*-dimethylbenzamide (27f)<sup>[6]</sup>**



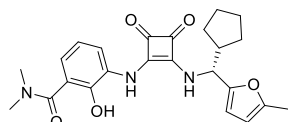
2-Hydroxy-3-[(2-methoxy-3,4-dioxocyclobut-1-en-1-yl)amino]-*N,N*-dimethylbenzamide (**26**, 55 mg, 0.20 mmol, 1.0 eq.) and (*R*)-cyclopropyl(5-methylfuran-2-yl)methanamine hydrochloride (**22f**, 75 mg, 0.4 mmol, 2.0 eq.) were dissolved in methanol (10 mL). After the addition of *N,N*-diisopropylethylamine (67  $\mu$ L, 0.40 mmol, 2.0 eq.), the reaction was stirred for 3 days. Extraction between water and ethyl acetate (3  $\times$  50 mL), drying over sodium sulfate, filtration, and evaporation of the solvent gave the crude product which was purified by preparative HPLC (acetonitrile/water (0.1% TFA): gradient 5–95%) to obtain the title compound as a white-brown amorphous solid (24 mg, 31%) <sup>1</sup>H NMR (500 MHz, DMSO-*d*<sub>6</sub>,  $\delta$  [ppm]): The two CH<sub>3</sub> groups of the dimethylamide moiety were not detectable due to the water peak, 11.99 (bs, 1H), 9.43–9.36 (m, 1H), 8.81 (d, *J* = 9.1 Hz, 1H), 8.05–7.98 (m, 1H), 7.52–7.45 (m, 1H), 6.96–6.89 (m, 1H), 6.33 (d, *J* = 3.1 Hz, 1H), 6.09–6.03 (m, 1H), 4.65 (t, *J* = 8.8 Hz, 1H), 2.28 (s, 3H), 1.44–1.30 (m, 1H), 0.67–0.55 (m, 2H), 0.52–0.43 (m, 1H), 0.38–0.30 (m, 1H); <sup>13</sup>C NMR (126 MHz, DMSO-*d*<sub>6</sub>,  $\delta$  [ppm]): 184.2, 180.2, 172.4, 168.4, 163.5, 152.0, 151.7, 151.2, 127.7, 125.2, 124.1, 119.0, 113.0, 107.8, 106.6, 55.2, 40.3, 15.6, 13.5, 10.3, 3.5, 3.1; HRMS *m/z* (ESI<sup>+</sup>) [found: 410.1706, C<sub>22</sub>H<sub>24</sub>N<sub>3</sub>O<sub>5</sub><sup>+</sup> requires [M+H]<sup>+</sup> 410.1638]; HPLC retention time 12.36 min, 98.0%. The obtained analytical data are in good agreement with literature values.<sup>[6]</sup>

**(R)-3-[(2-[(Cyclobutyl(5-methylfuran-2-yl)methyl]amino)-3,4-dioxocyclobut-1-en-1-yl)amino]-2-hydroxy-*N,N*-dimethylbenzamide (27g)**



2-Hydroxy-3-[(2-methoxy-3,4-dioxocyclobut-1-en-1-yl)amino]-*N,N*-dimethylbenzamide (**26**, 48 mg, 0.20 mmol, 1.0 eq.) and (*R*)-cyclobutyl(5-methylfuran-2-yl)methanamine hydrochloride (**22g**, 80 mg, 0.40 mmol, 2.0 eq.) were dissolved in methanol (10 mL). After the addition of *N,N*-diisopropylethylamine (59  $\mu$ L, 0.40 mmol, 2.0 eq.), the reaction was stirred for 3 days. Extraction between water and ethyl acetate (3  $\times$  50 mL), drying over sodium sulfate, filtration, and evaporation of the solvent gave the crude product which was purified by preparative HPLC (acetonitrile/water (0.1% TFA): gradient 5–95%) to obtain the title compound as a white-brown amorphous solid (31 mg, 44%) <sup>1</sup>H NMR (500 MHz, DMSO-*d*<sub>6</sub>,  $\delta$  [ppm]): The two CH<sub>3</sub> groups of the dimethylamide moiety were not detectable due to the water peak, 12.01 (bs, 1H), 9.37 (s, 1H), 8.63 (d, *J* = 9.4 Hz, 1H), 8.03 (dd, *J* = 8.1, 1.5 Hz, 1H), 7.48 (dd, *J* = 8.0, 1.6 Hz, 1H), 6.92 (t, *J* = 8.0 Hz, 1H), 6.23 (d, *J* = 3.2 Hz, 1H), 6.06–6.02 (m, 1H), 5.21 (t, *J* = 9.2 Hz, 1H), 2.91–2.78 (m, 1H), 2.25 (s, 3H), 2.14–1.68 (m, 6H); <sup>13</sup>C NMR (126 MHz, DMSO-*d*<sub>6</sub>,  $\delta$  [ppm]): 184.2, 180.1, 172.2, 168.6, 163.1, 151.5, 151.0, 150.9, 127.5, 124.9, 123.8, 118.8, 112.8, 107.8, 106.4, 55.6, 40.1, 38.1, 24.9, 24.2, 17.2, 13.3; HRMS *m/z* (ESI<sup>+</sup>) [found: 427.1775, C<sub>23</sub>H<sub>26</sub>N<sub>3</sub>O<sub>5</sub><sup>+</sup> requires [M+H]<sup>+</sup> 424.1794]; HPLC retention time 12.65 min, 98.0%.

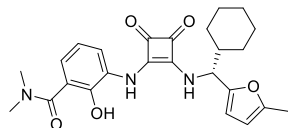
**(R)-3-[(2-[(Cyclopentyl(5-methylfuran-2-yl)methyl]amino)-3,4-dioxocyclobut-1-en-1-yl)amino]-2-hydroxy-*N,N*-dimethylbenzamide (27h)**



2-Hydroxy-3-[(2-methoxy-3,4-dioxocyclobut-1-en-1-yl)amino]-*N,N*-dimethylbenzamide (**26**, 75 mg, 0.30 mmol, 1.0 eq.) and (*R*)-cyclopentyl(5-methylfuran-2-yl)methanamine hydrochloride (**22h**, 0.13 g, 0.60 mmol, 2.0 eq.) were dissolved in methanol (10 mL). After the addition of *N,N*-diisopropylethylamine (0.11 mL, 0.60 mmol, 2.0 eq.), the reaction was stirred for 3 days. Extraction between water and ethyl acetate (3  $\times$  50 mL), drying over sodium sulfate, filtration, and evaporation of the solvent gave the crude product which was purified by preparative HPLC (acetonitrile/water (0.1% TFA): gradient 5–95%) to obtain the title compound as a white-brown amorphous solid (35 mg, 31%) <sup>1</sup>H NMR (500 MHz, DMSO-*d*<sub>6</sub>,  $\delta$  [ppm]): 9.93 (s, 1H), 9.29 (s, 1H), 8.70 (d, *J* = 9.5 Hz, 1H), 7.79 (dd, *J* = 7.2, 2.4 Hz, 1H), 6.92–6.83 (m, 2H), 6.24 (d, *J* = 3.1 Hz, 1H), 6.03 (dd, *J* = 3.0, 1.2 Hz, 1H), 5.08 (t, *J* = 9.2 Hz, 1H), 3.04–2.84 (m, 7H), 2.50–2.39 (m, 1H), 2.26 (s, 3H), 1.83–1.73 (m, 1H), 1.70–1.48 (m, 4H), 1.45–1.35 (m, 1H), 1.26–1.16 (m, 1H); <sup>13</sup>C NMR (126 MHz, DMSO-*d*<sub>6</sub>,  $\delta$

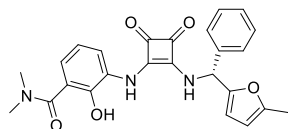
[ppm]): 184.0, 180.1, 168.4, 168.3, 163.3, 152.2, 151.2, 143.3, 128.6, 124.3, 122.2, 120.8, 119.8, 107.7, 106.4, 55.4, 43.3, 40.1, 29.4, 29.0, 24.9, 13.3; HRMS  $m/z$  (ESI<sup>+</sup>) [found: 438.2023, C<sub>24</sub>H<sub>28</sub>N<sub>3</sub>O<sub>5</sub><sup>+</sup> requires [M+H]<sup>+</sup> 438.1951]; HPLC retention time 12.81 min, 98.0%.

**(*R*)-3-[(2-[(Cyclohexyl(5-methylfuran-2-yl)methyl]amino)-3,4-dioxocyclobut-1-en-1-yl)amino]-2-hydroxy-*N,N*-dimethylbenzamide (27i)**



2-Hydroxy-3-[(2-methoxy-3,4-dioxocyclobut-1-en-1-yl)amino]-*N,N*-dimethylbenzamide (**26**, 0.11 g, 0.40 mmol, 1.0 eq.) and (*R*)-cyclohexyl(5-methylfuran-2-yl)methanamine hydrochloride (**22i**, 0.17 g, 0.80 mmol, 2.0 eq.) were dissolved in methanol (10 mL). After the addition of *N,N*-diisopropylethylamine (0.14 mL, 0.80 mmol, 2.0 eq.), the reaction was stirred for 3 days. Extraction between water and ethyl acetate (3 × 50 mL), drying over sodium sulfate, filtration, and evaporation of the solvent gave the crude product which was purified by preparative HPLC (acetonitrile/water (0.1% TFA): gradient 5–95%) to obtain the title compound as a white-brown amorphous solid (35 mg, 20%) <sup>1</sup>H NMR (600 MHz, DMSO-*d*<sub>6</sub>, δ [ppm]): 9.94 (s, 1H), 9.34 (s, 1H), 8.67 (d, *J* = 9.6 Hz, 1H), 7.78 (dd, *J* = 7.3, 2.3 Hz, 1H), 6.92 – 6.84 (m, 2H), 6.21 (d, *J* = 3.1 Hz, 1H), 6.04 (dd, *J* = 3.0, 1.3 Hz, 1H), 5.09 – 5.03 (m, 1H), 2.94 (s, 6H), 2.27 (s, 3H), 1.89 – 1.57 (m, 5H), 1.54 – 1.49 (m, 1H), 1.28 – 1.18 (m, 2H), 1.16 – 0.92 (m, 3H); <sup>13</sup>C NMR (151 MHz, DMSO-*d*<sub>6</sub>, δ [ppm]): 184.0, 180.2, 168.6, 168.3, 163.3, 151.3, 151.2, 143.3, 128.6, 124.4, 122.2, 120.9, 119.8, 107.9, 106.4, 56.4, 41.3, 40.1, 29.3, 28.4, 25.7, 25.3, 13.3; HRMS  $m/z$  (ESI<sup>+</sup>) [found: 452.2181, C<sub>25</sub>H<sub>30</sub>N<sub>3</sub>O<sub>5</sub><sup>+</sup> requires [M+H]<sup>+</sup> 452.2107]; HPLC retention time 13.23 min, 98.0%.

**(*R*)-2-Hydroxy-*N,N*-dimethyl-3-[(2-[(5-methylfuran-2-yl)phenyl]methylamino)-3,4-dioxocyclobut-1-en-1-yl)amino]benzamide (27j)**



2-Hydroxy-3-[(2-methoxy-3,4-dioxocyclobut-1-en-1-yl)amino]-*N,N*-dimethylbenzamide (**26**, 0.13 g, 0.5 mmol, 1.0 eq.) and (*R*)-(5-methylfuran-2-yl)(phenyl)methanamine hydrochloride (**22j**, 0.22 g, 1.0 mmol, 2.0 eq.) were dissolved in methanol (10 mL). After the addition of *N,N*-diisopropylethylamine (0.18 mL, 1.0 mmol, 2.0 eq.), the reaction was stirred for 3 days. Extraction between water and ethyl acetate (3 × 50 mL), drying over sodium sulfate, filtration, and evaporation of the solvent gave the crude product which was purified by preparative HPLC (acetonitrile/water (0.1% TFA): gradient 5–95%) to obtain the title compound as a white-brown amorphous solid (20 mg, 10%) <sup>1</sup>H NMR (600 MHz, DMSO-*d*<sub>6</sub>, δ [ppm]): 9.95 (s, 1H), 9.37 (s, 1H), 9.19 (d, *J* = 9.1 Hz, 1H), 7.77 (dd, *J* = 7.0, 2.6 Hz, 1H), 7.46 – 7.41 (m, 2H), 7.40 – 7.33 (m, 3H), 6.92 – 6.85 (m, 2H), 6.48 (d, *J* = 8.8 Hz, 1H), 6.16 (d, *J* = 3.1 Hz, 1H), 6.08 (d, *J* = 3.1 Hz, 1H), 2.94 (s, 6H), 2.26 (s, 3H); <sup>13</sup>C NMR (151 MHz, DMSO-*d*<sub>6</sub>, δ [ppm]): 183.9, 180.5, 168.3, 168.0, 163.9, 152.1, 151.4, 143.4, 139.5, 128.8, 128.5, 128.0, 126.8, 124.4, 122.4, 120.9, 119.8, 108.9, 106.6, 54.9, 40.1, 13.3; HRMS  $m/z$  (ESI<sup>+</sup>) [found: 446.1713, C<sub>25</sub>H<sub>24</sub>N<sub>3</sub>O<sub>5</sub><sup>+</sup> requires [M+H]<sup>+</sup> 446.1638]; HPLC retention time 12.41 min, 98.0%.

## Biological tests

**Cell culture:** HEK293T cells were cultured as previously described.<sup>[1]</sup> In brief, HEK293T cells (gift from Chair of Physiology, Prof. Dr. Alzheimer, FAU Erlangen-Nürnberg) were grown on 10 cm culture dishes at 37 °C and 5% CO<sub>2</sub>. As growth medium, Dulbecco's Modified Eagle Medium (DMEM)/F12 (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Gibco™ Fetal Bovine Serum, qualified, Brazil), L-glutamine (final concentration: 2 mM; from Gibco™ L-glutamine 200 mM, 100X), penicillin (final concentration: 100 units/mL), and streptomycin (final concentration: 100 µg/mL; from Gibco™ Penicillin-Streptomycin 10,000 U/mL) was used. Cells were split every three to four days and regularly confirmed to be free of mycoplasma contamination using the luminescence-based MycoAlert Plus Kit (Lonza).

**Transient transfection using polyethylenimine:** For transfection, we used a previously published procedure.<sup>[1]</sup> In brief, HEK293T cells were plated onto culture dishes (Ø 10 cm or Ø 15 cm) and grown to a confluence of approximately 50% at 37 °C and 5% CO<sub>2</sub>. The growth medium was renewed one-hour before transfection. The transfection mix was prepared, as described in the following. Solution A (2.1-2.2% total DNA in Gibco™ phosphate buffered saline, pH 7.4 [5.5 µg in 250 µL, 10.5 µg in 500 µL]) and solution B (3% PEI (linear, 25 kDa, from Polysciences) solution prepared from a PEI stock solution (1 µg/µL) in PBS without MgCl<sub>2</sub> and CaCl<sub>2</sub>) were mixed one to one, the resulting mixture was vortexed for 5 s and incubated for 30 min at room temperature. The pre-incubated transfection mix was added dropwise to the cells and cell cultivation was continued at 37 °C and 5% CO<sub>2</sub>.

**Membrane preparation:** Membranes were prepared as previously reported.<sup>[1]</sup> In brief, membranes from HEK293T cells transiently expressing the respective GPCR were prepared as follows. The medium of the transfected cells was refreshed after 24 h before cells were harvested 48 h post-transfection. The growth medium was removed, the cells were carefully washed with cold phosphate buffered saline (10 mL per Ø 15 cm dish). The cells were detached with 15 mL of ice-cold Tris-EDTA buffer (10 mM Tris, 0.5 mM EDTA, 5.4 mM KCl, 140 mM NaCl, pH 7.4) and subsequently centrifuged with 218 g for 8 minutes. The supernatant was removed, and the cells were resuspended in 10 mL Tris-EDTA buffer. The cells were lysed with an Ultraturrax (20,000 rpm) used five times for 5 seconds with a 25-second break on ice in between. The lysate was centrifuged at 50,830 g for 18 min at 4 °C. The supernatant was discarded, and the pellet was homogenized in membrane buffer (50 mM Tris, 1 mM EDTA, 5 mM MgCl<sub>2</sub>, 100 µg/mL bacitracin, 5 µg/mL soybean trypsin inhibitor, pH 7.4) with a glass-teflon homogenizer. Aliquots of 250 µL were shock frozen in liquid nitrogen and directly stored at -80 °C. Finally, the protein concentration was determined using the Lowry method.<sup>[9]</sup>

**cDNA constructs:** The CCR6-Nluc (CCR6\_Nluc and CCR6\_GSSG\_Nluc) and the AVPR2-Nluc fusion constructs (AVPR2\_Nluc) in pcDNA3.1 were generated using the Gibson Assembly<sup>[10]</sup> (New England Biolabs) method. Therefore, the sequences of the Nluc enzyme<sup>[11]</sup> (pNLF1-C, Promega), (3xHA)-tagged CCR6 (CCR6, cdna.org, #CCR060TN00) and (3xHA)-tagged AVPR2 (AVPR2, cdna.org, #AVR020TN00) were amplified by polymerase chain reaction and were directly fused in frame with different linker sequences (no linker and GSSG). The FLAG-Nluc-CCR9 (FLAG\_Nluc\_CCR9) and FLAG-Nluc-AVPR2 (FLAG\_Nluc\_AVPR2) fusion constructs in pcDNA3.1 were generated using the Gibson Assembly<sup>[10]</sup> (New England Biolabs) method. Therefore, the sequences of (3xHA)-tagged CCR9 (CCR9, cdna.org, #CCR090TN00) or (3xHA)-tagged AVPR2 (AVPR2, cdna.org, #AVR020TN00) were amplified by polymerase chain reaction and were directly fused in frame of the linearized vector of FLAG\_Nluc\_TAS2R14 obtained by restriction digestion with XhoI and XbaI. The FLAG\_Nluc\_CCR9 and FLAG\_Nluc\_AVPR2 fusion proteins have a 4 AA (GSSG) linker between nanoluciferase and the receptor. DNA sequencing was performed to verify sequence integrity (Eurofins Genomics). Plasmids were cloned into *E.coli* DH5- $\alpha$  (New England Biolabs) and purified using a Maxiprep DNA purification kit (Invitrogen). For studies with CXCR1 and CXCR2, we modified the procedure described above. Instead of (3xHA)-tagged CCR6 (cdna.org, #CCR060TN00), we used (3xHA)-tagged CXCR1 (cdna.org, #CXCR10TN00) or respectively (3xHA)-tagged CXCR2 (cdna.org, #CXCR20TN00). For CXCR1 and CXCR2, we inserted no linker between the C-terminus of the respective receptor and the Nluc-tag. The constructs for the Nluc-labeled CXCR1, and CXCR2 were already published.<sup>[1]</sup>

**ELISA:** ELISA-based experiments were performed as previously reported.<sup>[1-3]</sup> For confirmation of CCR6 expression, HEK293T cells were transfected with the plasmid encoding 3xHA-CCR6, 3xHA-tagged CCR1\_Nluc or 3xHA-tagged CCR6\_GSSG\_Nluc construct using polyethylenimine in suspension. Therefore, HEK293T cells were detached from their culture plates and diluted to a density of  $3 \times 10^5$  cells/mL in growth medium. This cell suspension was mixed with the preformed transfection mix (PEI/DNA ratio 2.5:1) consisting of 1.2 µg of receptor cDNA plasmid and 1.2 µg of single stranded salmon sperm DNA (ssDNA, Sigma Aldrich) in phosphate buffered saline (PBS) per 2.4 mL of cell suspension. Subsequently, cells were transferred to a 48-well plate ( $7.5 \times 10^4$  cells/well), which was pretreated with poly-D-lysine (0.1 mg/mL, dissolved in water). Cells were incubated for 48 h at 37 °C and 5% CO<sub>2</sub>. On the day of the assay, the medium was removed, and cells were incubated with 200 µL/well of ROTI@Histofix 4% fixation solution (Carl Roth) for 10 min at room temperature. Cells were washed once with 300 µL washing buffer for two minutes (150 mM NaCl, 25 mM Tris, pH 7.5) and blocked for one hour using 800 µL blocking buffer (30 g/L skim milk powder in washing buffer). After removal of the blocking solution, 200 µL/well of anti-HA rabbit IgG antibody (Sigma Aldrich, catalog # H6908, 1:4,000 in blocking solution) were added. After 60 min of incubation, wells were washed twice for two minutes (300 µL/well) and blocked again for one hour at room temperature, before 200 µL/well anti-rabbit IgG-HRP antibody (Invitrogen by Thermo Fisher Scientific, catalog # G-21234, 1:1,000 in blocking solution) was added. After incubation for one hour, cells were washed three times

for two minutes (300  $\mu$ L/well), before the substrate reaction was initiated by the addition of substrate buffer (6 mM o-phenylenediamine in 35 mM citric acid, 66 mM  $\text{Na}_2\text{HPO}_4$ , pH 5.0). After 15 minutes incubation in the dark, the reactions were terminated by addition of 1 M  $\text{H}_2\text{SO}_4$  (200  $\mu$ L/well). For each well, 2 x 150  $\mu$ L of the resulting mixture were transferred to a clear, flat bottom 96-well plate and absorption was measured at 492 nm in a microplate reader. The measured absorbance values were baseline-corrected using cells transfected with a non-tagged muscarinic receptor (M3R, *cdna.org*) as negative control. These baseline-corrected values were normalized to 3xHA-CCR6 expression. For ELISA experiments to validate the FLAG-Nluc-CCR9 expression an anti-FLAG mouse antibody (Sigma Aldrich, product number: F3165, 1:4000 in blocking solution) as first antibody and an anti-mouse IgG peroxidase antibody (Sigma Aldrich, product number: A9044, 1:20,000 in blocking solution) as second antibody were used. The measured absorbance values were baseline-corrected using cells transfected with a 3xHA-tagged AVPR2\_Nluc as negative control. These baseline-corrected values were normalized to FLAG-Nluc-AVPR2 expression. The remaining steps were carried out as described above.

**Emission and excitation spectra of the fluorescent ligands:** The emission and excitation spectra of the fluorescent ligands have been previously reported.<sup>[1]</sup>

**Emission spectra of Nluc-labeled CCR6 (CCR6\_Nluc) protein:** Furimazine (Promega, Mannheim, Germany 1:2,000) was added to the membrane preparations (5  $\mu$ g protein/well). After 5 minutes incubation in the dark, the emission spectra were measured ranging from 350 to 700 nm using a CLARIOstar (BMG Labtech, Ortenberg, Germany) microplate reader. The emission spectra of CXCR1\_Nluc and CXCR2\_Nluc have already been reported by Huber *et al.*<sup>[1]</sup>

**NanoBRET binding assays: Membrane-based NanoBRET saturation assay:** For the establishment of our NanoBRET binding assay, we referred to recently published protocols.<sup>[1, 12]</sup> The fluorescent ligands Mz438 (**3**), LT220 (**4**), and LT221 (**5**) were dissolved in DMSO (1 mM) and further diluted to varying concentrations in assay buffer (50 mM  $\text{Na}_2\text{HPO}_4$ , 50 mM  $\text{KH}_2\text{PO}_4$ , pH 7.4, 1 mg/mL saponin, 5% FBS) and 5  $\mu$ L of these dilutions were pipetted to a 384-well plate. To determine total binding, 5  $\mu$ L of assay buffer were added to the corresponding wells, while 5  $\mu$ L of a solution of the reported non-fluorescent *cmpd24* (**10**, final assay concentration: 10  $\mu$ M) for CCR6 or navarixin (**1**, final assay concentration: 10  $\mu$ M) for CXCR1 in assay buffer were used to determine non-specific binding. Then, 20  $\mu$ L of the membrane preparations (CCR6\_Nluc or CXCR1\_Nluc) diluted in assay buffer (4  $\mu$ g total protein/well) were added and the plates were incubated for 90 min at 37 °C. Subsequently, 5  $\mu$ L of a furimazine solution (Promega, Mannheim, Germany, final assay dilution: 1:5,000) were added to each well (final assay volume: 35  $\mu$ L) before measuring luminescence with a CLARIOstar microplate reader using 620/10 nm and 475/30 nm emission filters after 5 min of incubation in the dark. Bioluminescence resonance energy transfer (BRET) was determined as the ratio of acceptor fluorescence and donor luminescence. The algorithms for one-site saturation binding from PRISM10.2.1 (GraphPad, USA) were utilized to analyze total, non-specific and specific binding. Specific binding signals were calculated as a difference of total and non-specific binding. If required, netBRET values were calculated as the difference between total BRET values and the values obtained in the absence of a fluorescent ligand.

**Membrane-based NanoBRET competition assay:** The fluorescent ligand **5** was dissolved in assay buffer and 5  $\mu$ L of this solution (final assay concentration: 250 nM) were pipetted to a 384-well plate, followed by the addition of 5  $\mu$ L of varying dilutions of the competing ligand dissolved in assay buffer. Then, 20  $\mu$ L of the membrane preparations (CCR6\_Nluc or CXCR1\_Nluc, diluted in assay buffer, 4  $\mu$ g total protein/well) were added and the plates were incubated for 90 min at 37 °C. Subsequently, 5  $\mu$ L of a furimazine solution (final assay dilution: 1:5,000 in assay buffer) were added to each well (final assay volume: 35  $\mu$ L). Plates were read on a CLARIOstar microplate reader using 620/10 nm and 475/30 nm emission filters after 5 min of incubation in the dark. To determine the inhibition constants ( $K_i$ ) of the non-labeled ligands, data were analyzed using the one site-fit  $K_i$  equation in PRISM10.2.1 (GraphPad, USA). For compounds that showed more than 50% competition at the highest concentration tested, we manually set a constraint for the curve fitting to approach the value detected for non-specific binding (0% specific BRET). For compounds that showed less than 50% competition at the highest competitor concentration tested, only the values for percentual inhibition of tracer binding at a given concentration are provided.

**Membrane-based NanoBRET association kinetic assay:** 5  $\mu$ L of a solution of the fluorescent ligand (**5**) diluted to varying concentrations (final assay concentrations: 50–500 nM) in assay buffer and 5  $\mu$ L of assay buffer were transferred to a 384-well plate. For the determination of non-specific binding, we added a solution of a non-labeled competitor dissolved in assay buffer (**10**, final assay concentration: 10  $\mu$ M for CCR6 or **1**, final concentration: 10  $\mu$ M for CXCR1) instead. After the addition of 5  $\mu$ L of a furimazine solution (final assay dilution: 1:630 in assay buffer), plates were incubated for 3 minutes in the dark at ambient temperature. Subsequently, 20  $\mu$ L of the membrane preparations (CCR6\_Nluc or CXCR1\_Nluc 4  $\mu$ g total protein/well) were added (final assay volume: 35  $\mu$ L). BRET ratios were measured with a CLARIOstar microplate reader using 620/10 nm and 475/30 nm emission filters over time at ambient temperature. The obtained data were analyzed using the association kinetics (one ligand concentration) algorithm in PRISM10.2.1 (GraphPad, USA) to determine association kinetics using a pre-determined  $k_{\text{off}}$  as a constraint.

**Membrane-based NanoBRET dissociation kinetic assay:** 5  $\mu$ L of a solution of the fluorescent ligand (**5**) diluted to varying concentrations (final assay concentrations: 50–500 nM) in assay buffer and 5  $\mu$ L of assay buffer were transferred to a



384-well plate. In order to determine non-specific binding, we added a solution of a non-labeled competitor dissolved in assay buffer (**10**, final assay concentration: 10  $\mu$ M for CCR6 or **1**, final concentration: 10  $\mu$ M for CXCR1) instead of the 5  $\mu$ L of assay buffer. Then, 20  $\mu$ L of the membrane preparation (4  $\mu$ g total protein/well) were added, and plates were incubated for 1.5-2 h at ambient temperature in the dark. Subsequently, 5  $\mu$ L of a furimazine solution (final assay dilution: 1:630 in assay buffer) were added. Plates were incubated for further 5 minutes in the dark at ambient temperature. Thereafter, 1  $\mu$ L of a solution of a unlabeled competitor dissolved in assay buffer (**10**, final assay concentration: 10  $\mu$ M for CCR6 or **1**, final concentration: 10  $\mu$ M for CXCR1) was added. For control experiments, we added 1  $\mu$ L of assay buffer instead of the competitor solution. BRET ratios were measured with a CLARIOstar microplate reader using 620/10 nm and 475/30 nm emission filters over time at ambient temperature. Specific BRET ratios were calculated as a difference of total and non-specific binding. The obtained data were analyzed using the dissociation - one phase exponential decay algorithm in PRISM10.2.1 (GraphPad, USA) to determine dissociation kinetics.

*Live cell NanoBRET:* HEK293T cells were transfected with 5.5  $\mu$ g of the plasmid (CXCR1\_Nluc or CCR6\_GSSG\_Nluc) using polyethylenimine (PEI; 7.5  $\mu$ g) as transfection reagent. After 24 h at 37 °C and 5% CO<sub>2</sub>, the cells were detached with DMEM and transferred to a white F-bottom assay plate which was coated with poly-D-lysine (0.1 mg/mL, dissolved in water) (CXCR1: 384-well plate [10,000 cells/well], coated with 5  $\mu$ L/well poly-D-lysine; CCR6: 96-well plate [25,000 cells/well], with 20  $\mu$ L/well) and incubated for further 24 h at 37 °C and 5% CO<sub>2</sub>. Subsequently, cells were washed with phosphate-buffered saline (Gibco™ DPBS, with CaCl<sub>2</sub> and MgCl<sub>2</sub>). Assay medium (Gibco™ DMEM/F-12, 15 mM HEPES, no phenol red supplemented with 5% FBS) was added and cells were incubated at 37 °C for 30 minutes. Then, 5  $\mu$ L of a solution containing the fluorescent ligand (**5**), diluted in assay medium at varying concentrations, were added in case of saturation binding experiments. To determine non-specific binding, 5  $\mu$ L of a solution of **10** dissolved in assay medium (final assay concentration: 10  $\mu$ M) were added. For competition binding experiments, 5  $\mu$ L of a solution of the fluorescent ligand (**5**) diluted in assay medium (final assay concentration: 250 nM) and 5  $\mu$ L of a solution of the potential competitor (diluted from 10 mM DMSO-stock solutions with assay medium) at varying concentrations were added to the corresponding wells. After 90 min of incubation at 37 °C, 5  $\mu$ L of a furimazine solution (final assay dilution: 1:2,500 – 1:3,000, diluted with assay medium) were added. At the 96-well plates the double volume was added. After a further incubation of 5 min in the dark at 37 °C, BRET ratios were measured with a CLARIOstar microplate reader using 620/10 nm and 475/30 nm emission filters. Total, non-specific and specific binding, which was calculated as a difference of total and non-specific binding, were analyzed using the algorithms for one-site saturation binding in PRISM10.2.1 (GraphPad, USA). To determine the inhibition constants ( $K_i$ ) of the potential competitors, data were normalized to total and non-specific binding and analyzed using the one site-fit  $K_i$  equation in PRISM10.2.1 (GraphPad, USA).

**Cellular NanoBiT  $\beta$ -arrestin recruitment assays:** Chemokine-induced  $\beta$ -arrestin-2 recruitment to chemokine receptor CCR6 was monitored using a nanoluciferase complementation-based assay (NanoBiT, Promega).<sup>[13]</sup> 5 x 10<sup>6</sup> HEK293T cells were plated in 10 cm dishes and cultured for 24 h before transfection with vectors encoding for the human chemokine receptor CCR6 C-terminally fused with SmBiT and  $\beta$ -arrestin 2 N-terminally fused with LgBiT. 24 h after transfection cells were harvested, distributed into white 96-well plates (5 x 10<sup>4</sup> cells per well) and incubated for 1 h at 37 °C with compound **10** at concentrations ranging from 0.1 nM to 3.2  $\mu$ M. The endogenous chemokine ligand CCL20 (10 nM) was then added, and luminescence generated upon nanoluciferase complementation in the presence of coelenterazine H was measured with a Mithras LB940 luminometer (Berthold Technologies) for 20 min.

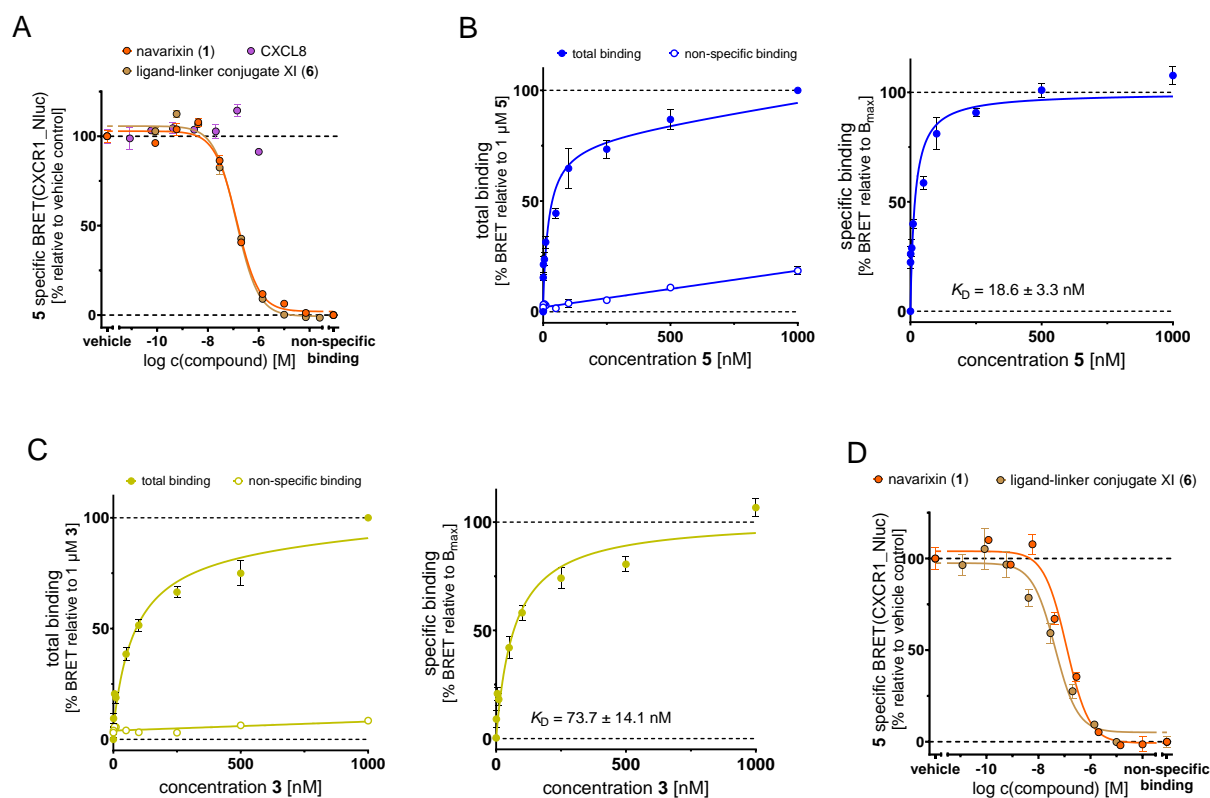
## Computational Methods

### Molecular docking:

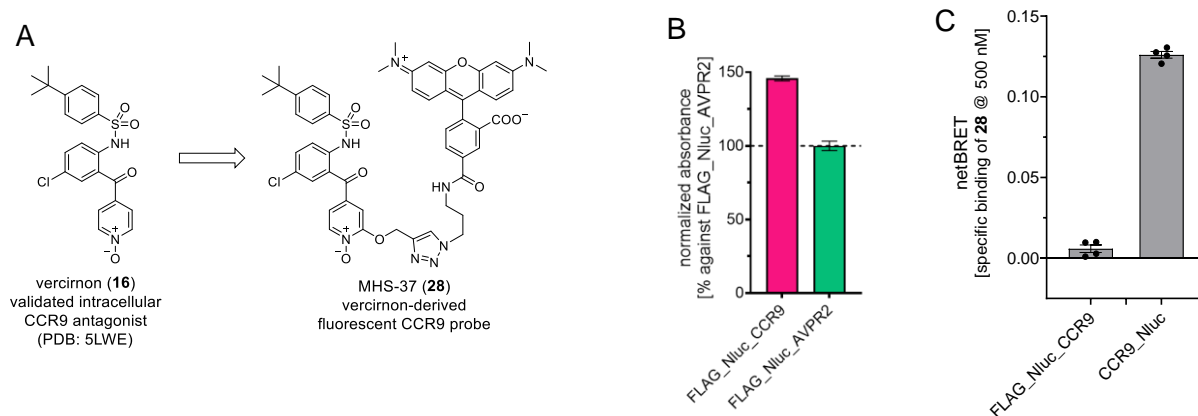
Modeling studies were performed with the programs of the Schrödinger Small-Molecule Drug Discovery Suite 2021-4 (Schrödinger LLC, NY, USA). The 2D structures of the ligands were converted to valid 3D Lewis structures using LigPrep (Schrödinger LLC, NY, USA). Their ionization and tautomeric states (pH 7.4) were generated using Epik,<sup>[14]</sup> and geometrically optimized with the OPLS4 force field.<sup>[15]</sup> Homology models of both CCR6 and CXCR1 inactive states were built with Prime (Schrödinger LLC, NY, USA), using as template a solved inactive CXCR2 structure in complex with EBX/00767013 (PDB ID: 6LFL).<sup>[16]</sup> Then, the CCR6 homology model in complex with EBX was prepared with the Protein PrepWizard.<sup>[17]</sup> This involves adding missing hydrogen atoms, adjusting the ionization state of polar amino acids at neutral pH, and meanwhile adjusting bond orders and formal charges of the ligand, optimizing the H-bond network of the protein–ligand complex, and, finally, energetically minimizing the complex using the OPLS4 force field.<sup>[15]</sup> The geometric center of the bound ligand was considered as the grid centroid, and template docking was carried out using Glide with the SP scoring function.<sup>[18]</sup>

## Supplementary Figures

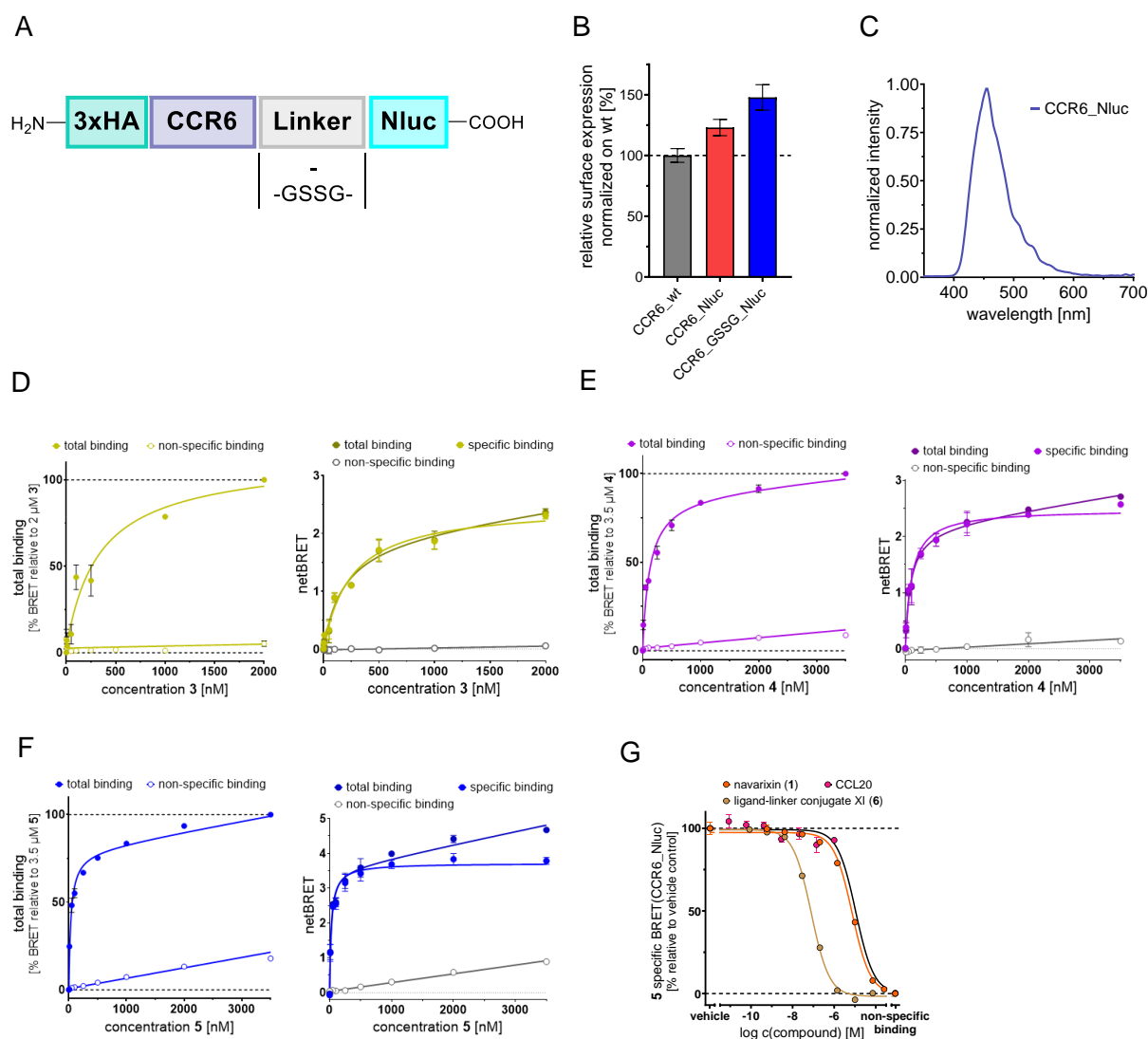
**Figure S1.** Development of a cell-free and cellular NanoBRET-based binding assay for CXCR1. A) Representative competition binding curves from single experiments (triplicate measurement) with the known CXCR1/CXCR2 antagonists navarixin (**1**),<sup>[6]</sup> the ligand-linker conjugate XI (**6**)<sup>[1]</sup> and the extracellular orthosteric CXCR1 agonist CXCL8,<sup>[19]</sup> obtained with LT221 (**5**) (250 nM) and CXCR1\_Nluc membranes. B) Binding curves (total and non-specific binding on the left, specific binding on the right) with fluorescent ligand **5** in a cellular NanoBRET-based experiment (quadruplicate measurement,  $n = 4$ ) using HEK293T cells transiently overexpressing CXCR1\_Nluc. C) Binding curves (total and non-specific binding on the left, specific binding on the right) with fluorescent ligand **3** in a cellular NanoBRET-based experiment (quadruplicate measurement,  $n = 5$ ) using HEK293T cells transiently overexpressing CXCR1\_Nluc. D) Representative competition binding curves from single NanoBRET-based experiments (quadruplicate measurement) with navarixin (**1**) (orange) and the ligand-linker conjugate **6** (beige) obtained with **5** (250 nM) and HEK293T cells transiently overexpressing CXCR1\_Nluc.



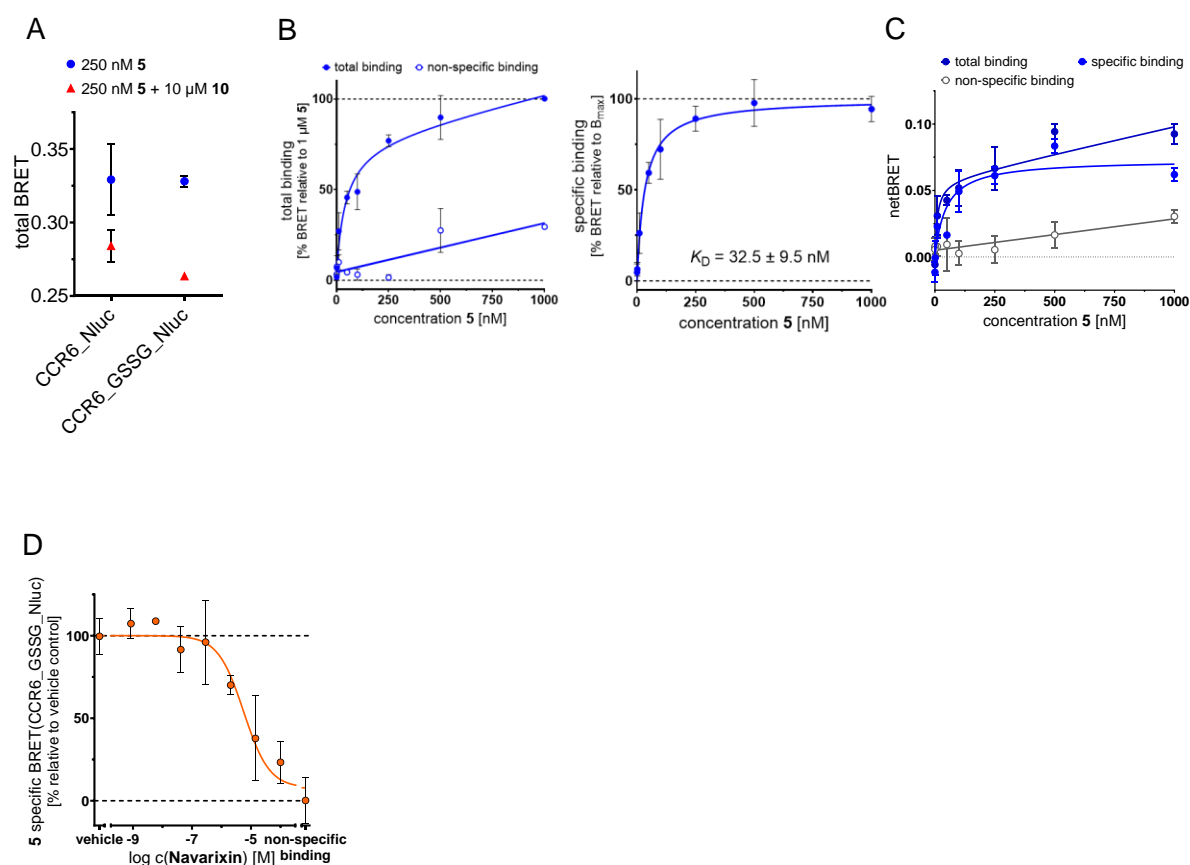
**Figure S2.** Resonance energy transfer from the Nluc (donor) to the fluorescent tracer (acceptor) cannot happen across the cell membrane. A) Chemical structure of vercirnon (**16**) and the vercirnon-derived fluorescent tracer MHS-37 (**28**),<sup>[3, 20]</sup> which are known to bind to the intracellular allosteric binding site of CCR9. B) Expression level of N-terminally Nluc-tagged CCR9 construct FLAG\_Nluc\_CCR9 detected via ELISA. FLAG\_Nluc\_CCR9 was normalized to the expression of FLAG\_Nluc\_AVPR2, which was used for comparison. Bar diagram represents the mean values  $\pm$  SEM (n = 3) with each test performed in quadruplicate. The experiment indicates that the FLAG\_Nluc\_CCR9 construct is well-expressed in HEK293T cells. C) Representative bar diagram of a quadruplicate measurement with HEK293T cells expressing the given CCR9 construct showing specific binding of **28** (500 nM) determined as the difference between total and non-specific binding. A signal indicating specific binding was only detected for C-terminally (intracellularly) tagged CCR9\_Nluc (n = 5) but not for the N-terminally (extracellularly) tagged construct FLAG\_Nluc\_CCR9 (n = 3).



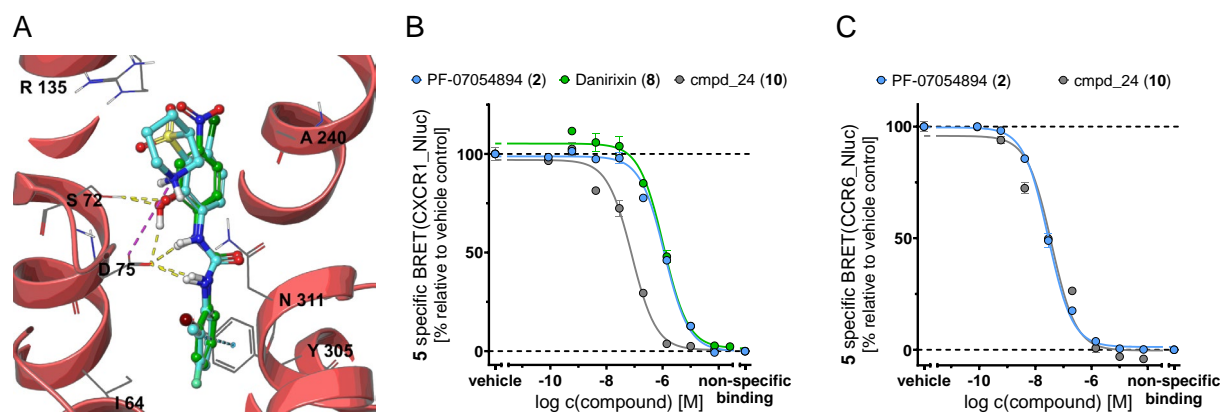
**Figure S3.** Development of a cell-free NanoBRET-based binding assay for CCR6. A) Schematic representation of the genetic C-terminally Nluc-labeled constructs of CCR6 that were investigated in the course of assay development. B) Expression level of different 3xHA-CCR6-Nluc constructs detected via ELISA and normalized to the expression of wild-type 3xHA-CCR6 (CCR6\_wt). Bar diagram represents the mean values  $\pm$  SEM ( $n = 3$ ) with each test performed in quadruplicate. The experiment indicates that all CCR6-Nluc constructs are well-expressed in HEK293T cells. C) Exemplary emission spectra of a membrane preparation from HEK293T cells transiently overexpressing the CCR6\_Nluc construct, which was used for the further development of our membrane based NanoBRET assay. D-F) Binding curves for total and non-specific binding on the left (triplicate measurement,  $n = 3$ ), and one representative example for total, specific, and non-specific binding curves from a single triplicate measurement on the right using fluorescent ligands Mz438 (**3**, see D), LT220 (**4**, see E), and LT221 (**5**, see F) in a cell-free NanoBRET-based experiment with membranes from HEK293T cells transiently overexpressing CCR6\_Nluc. G) Representative competition binding curves from single experiments (triplicate measurement) with the known CXCR1/CXCR2 antagonists navarixin (**1**),<sup>[6]</sup> the ligand-linker conjugate XI (**6**)<sup>[1]</sup> and the extracellular orthosteric CCR6 agonist CCL20,<sup>[21]</sup> obtained with **5** (250 nM) and CCR6\_Nluc membranes.



**Figure S4.** Development of a cellular NanoBRET-based binding assay for CCR6. A) Representative NanoBRET assay windows for LT221 (**5**), generated with live HEK293T cells transiently expressing different CCR6-Nluc constructs. Tests were performed at least in triplicates (diagram representing the mean values  $\pm$  SD;  $n = 2$ ). The signals for non-specific binding (full displacement of **5**, red triangles) were detected in the presence of **5** (250 nM) and the high affinity competitor **10** (10  $\mu$ M), whereas the signals obtained for the vehicle controls represent total binding of **5** at a concentration of 250 nM (blue circles). As the application of CCR6\_GSSG\_Nluc resulted in a larger and more robust assay window than CCR6\_Nluc, we used the CCR6\_GSSG\_Nluc construct for further cell-based studies. B) Binding curves (total and non-specific binding on the left, specific binding on the right) with fluorescent ligands **5** in a cellular NanoBRET-based experiment (triplicate measurement,  $n \geq 2$ ) using HEK293T cells transiently overexpressing CCR6\_GSSG\_Nluc. C) Representative specific binding curve from a single experiment (triplicate measurement) with fluorescent ligands **5** in a cellular NanoBRET-based experiment using HEK293T cells transiently overexpressing CCR6\_GSSG\_Nluc. D) Representative competition binding curve from single NanoBRET-based experiments (triplicate measurement) with navarixin (**1**) obtained with **5** (250 nM) and HEK293T cells transiently overexpressing CCR6\_GSSG\_Nluc.

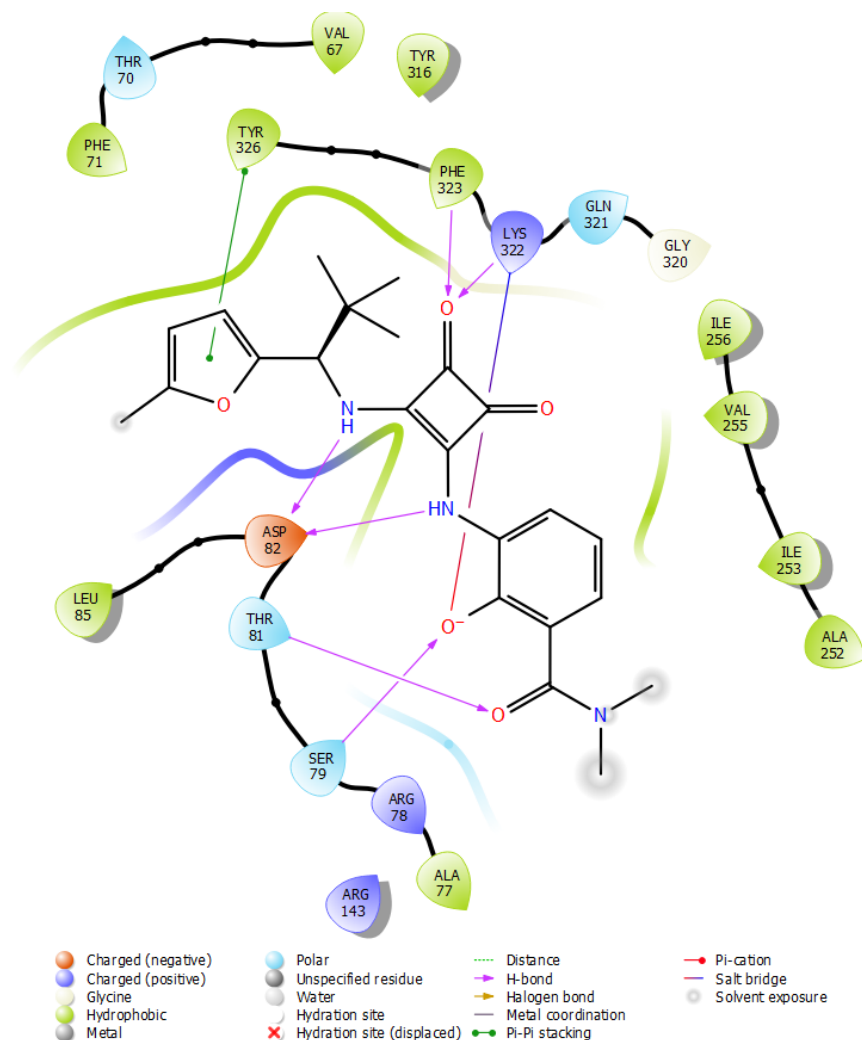


**Figure S5.** Application of LT221 (**5**) as a fluorescent tool for mapping known and potential CXCR1 and/or CCR6 ligands. A) Overlay of predicted CXCR1 (red) binding modes for the diarylurea-based compounds **8** (cyan) and **9** (green). B) Representative competition binding curves from single experiments (triplicate measurement) with the identified intracellular CXCR1 binders (**2**, **8**, and **10**). Tests were performed with **5** (250 nM) and CXCR1\_Nluc membranes. C) Representative competition binding curves from single experiments (triplicate measurement) with the identified intracellular CCR6 binders. Tests were performed with **5** (250 nM) and CCR6\_Nluc membranes.



**Figure S6.** The small molecule antagonists **10** is predicted to be anchored to the IABS of CCR6 via hydrophobic interactions with an aromatic cage formed by F71<sup>1x57</sup>, Y316<sup>7x53</sup>, F323<sup>8x50</sup>, and Y326<sup>8x53</sup>. A) 2D illustration of the predicted binding mode of **10** to the IABS of CCR6. B) Sequence alignment of amino acids forming the IABS of the investigated chemokine receptors CXCR1, CCR6 and GPCRs with a previously reported IABS (i.e., CXCR2, CCR2, CCR7, CCR9, CCR6,  $\beta_2$ AR)<sup>[16, 20, 22]</sup> shows that the aromatic cage is a special feature of CCR6 and not present in the other investigated receptors, due to the lack of an aromatic amino acid in positions 1x57 and 8x53 (highlighted in red). For sequence identity (%) and sequence similarity (%) analyses we used CXCR1 as a template.

A

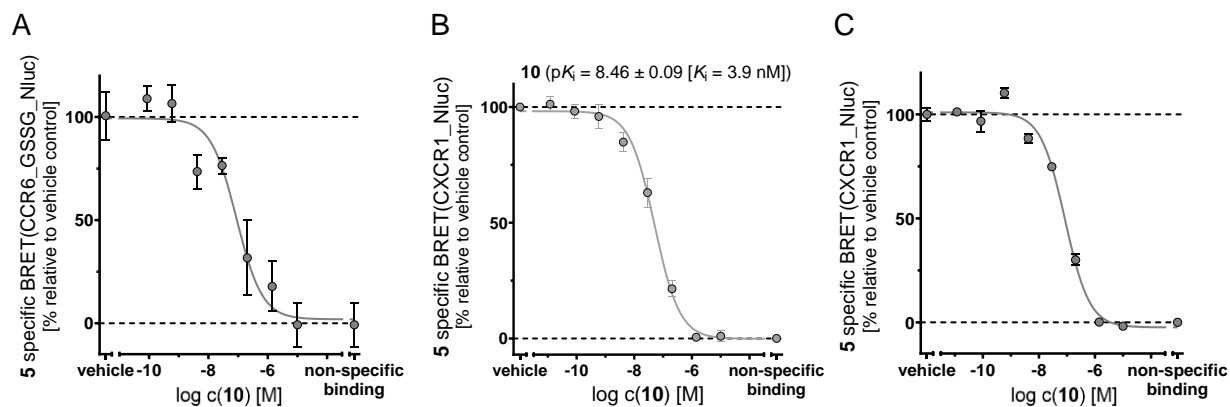


B

	TM1				ICL1		TM2				TM3				TM6						TM7	H8							
	1x53	1x56	1x57	1x60	12x49	12x51	2x37	2x39	2x40	2x43	3x45	3x46	3x50	3x53	6x29	6x32	6x33	6x34	6x36	6x37	6x40	7x53	8x47	8x48	8x49	8x50	8x53	%I	%S
CXCR1	V	V	I	S	V	R	S	T	D	L	C	I	R	A	Q	R	A	M	V	I	V	Y	G	Q	N	F	G	100	100
CXCR2	V	V	I	S	V	R	S	T	D	L	C	I	R	A	Q	R	A	M	V	I	V	Y	G	Q	K	F	G	96	96
CCR6	V	T	F	Y	K	R	S	T	D	L	C	I	R	A	K	K	A	I	V	I	V	Y	G	Q	K	F	Y	67	78
CCR2	V	I	L	C	K	K	C	T	D	L	L	L	R	A	K	R	A	V	V	I	I	Y	G	E	K	F	L	48	78
CCR7	V	T	Y	F	R	K	T	T	D	L	C	I	R	A	E	K	A	I	V	I	V	Y	G	V	K	F	D	56	74
CCR9	V	V	Y	C	R	K	T	T	D	L	C	I	R	A	S	K	A	L	V	T	V	Y	G	E	R	F	D	56	74
β <sub>2</sub> AR	V	A	I	F	R	Q	T	T	N	I	V	I	R	A	K	K	A	L	T	L	I	Y	S	P	D	F	A	33	70

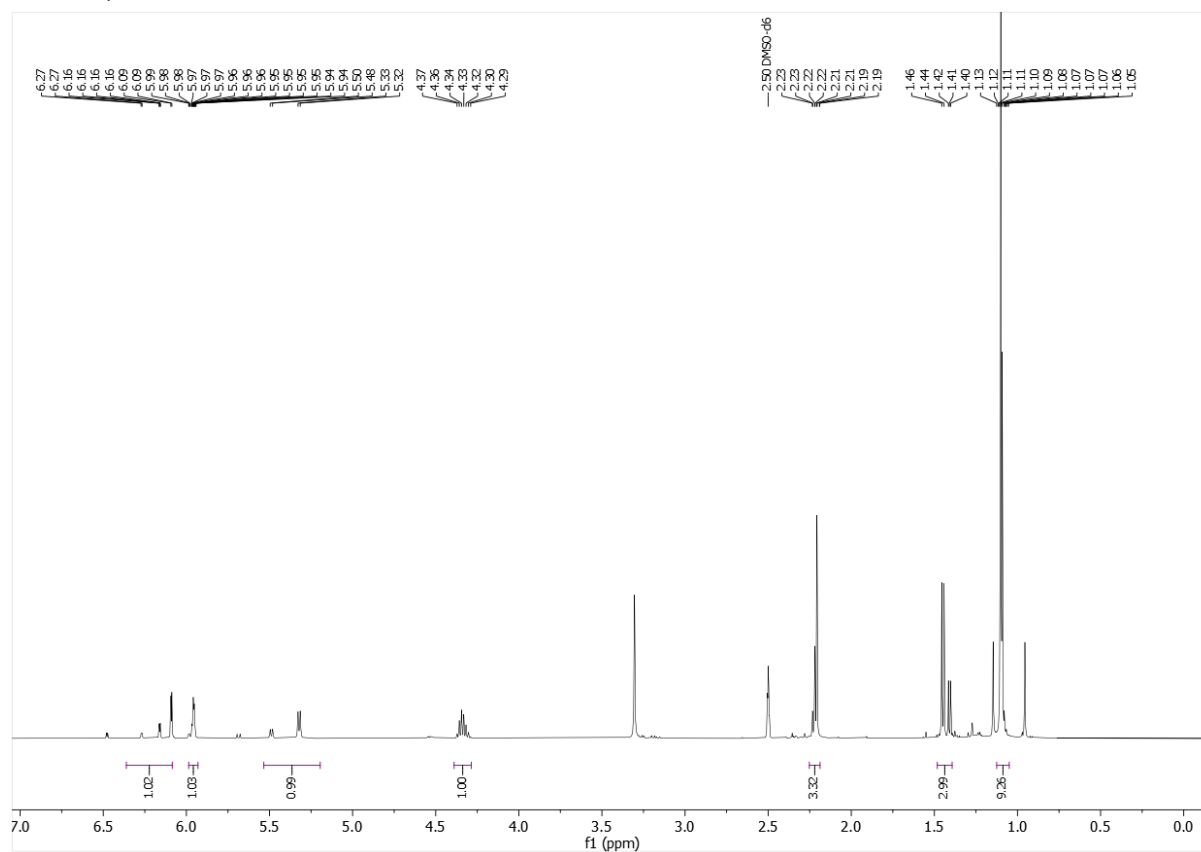


**Figure S7.** Cellular evaluation of **10** as an intracellular chemokine receptor antagonist. A) Representative competition binding curve from a single NanoBRET-based experiment (triplicate measurement) with **10** obtained with **5** (250 nM) and HEK293T cells transiently overexpressing CCR6\_GSSG\_Nluc. B) Cellular NanoBRET competition binding curve and  $pK_i$  value (mean  $\pm$  SEM, quadruplicate measurement) for **10** ( $n = 6$ ) obtained with **5** (250 nM) and HEK293T cells transiently overexpressing CXCR1\_Nluc.  $K_i$  values are given in square brackets. C) Representative competition binding curve from a single NanoBRET-based experiment (quadruplicate measurement) for **10** obtained with **5** (250 nM) and HEK293T cells transiently overexpressing CXCR1\_Nluc.

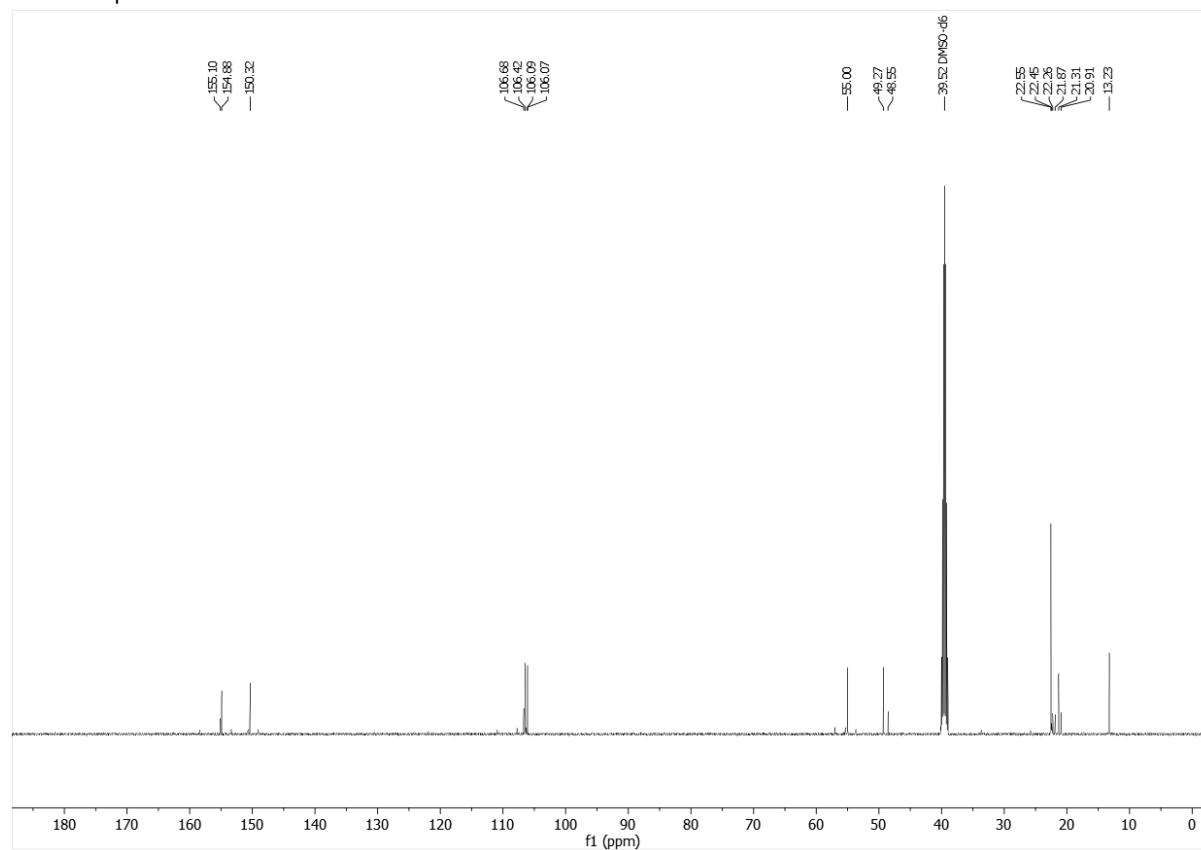


## Supplementary NMR spectra

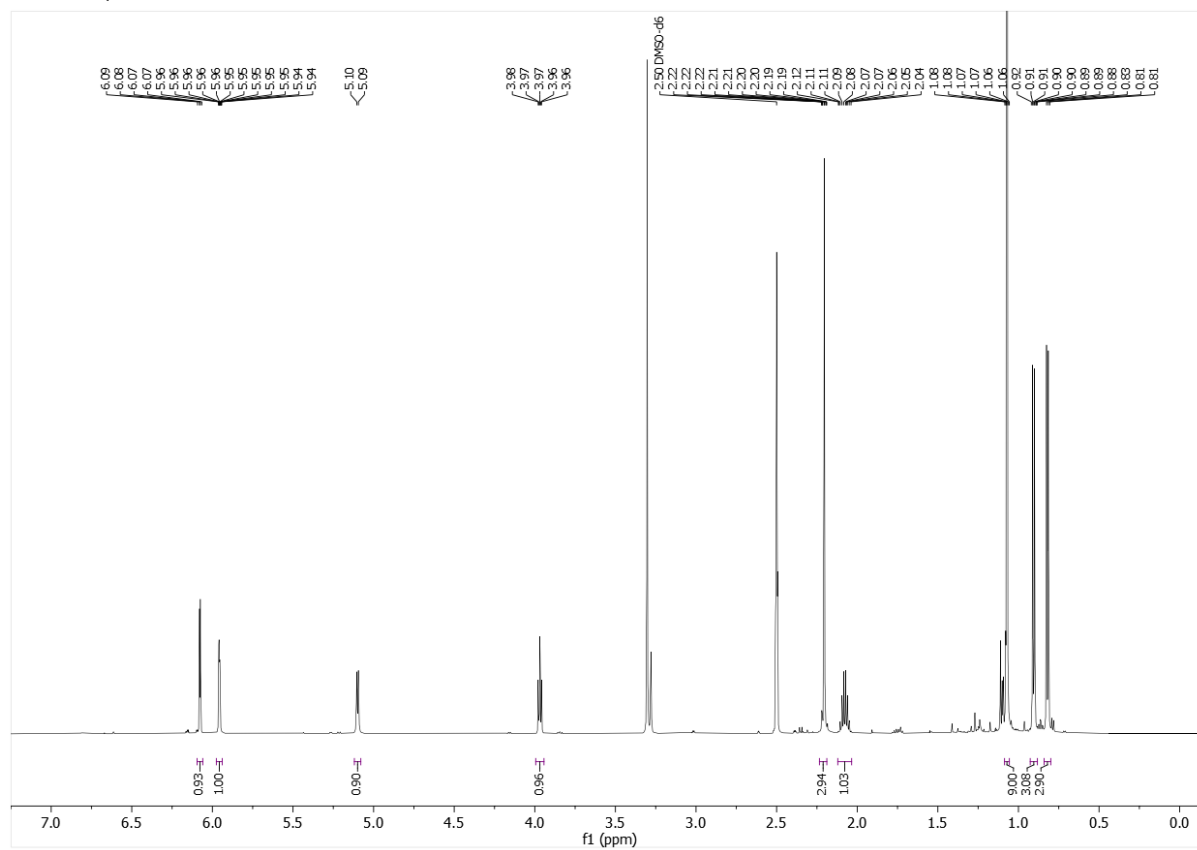
$^1\text{H}$ -NMR spectrum of **21b**



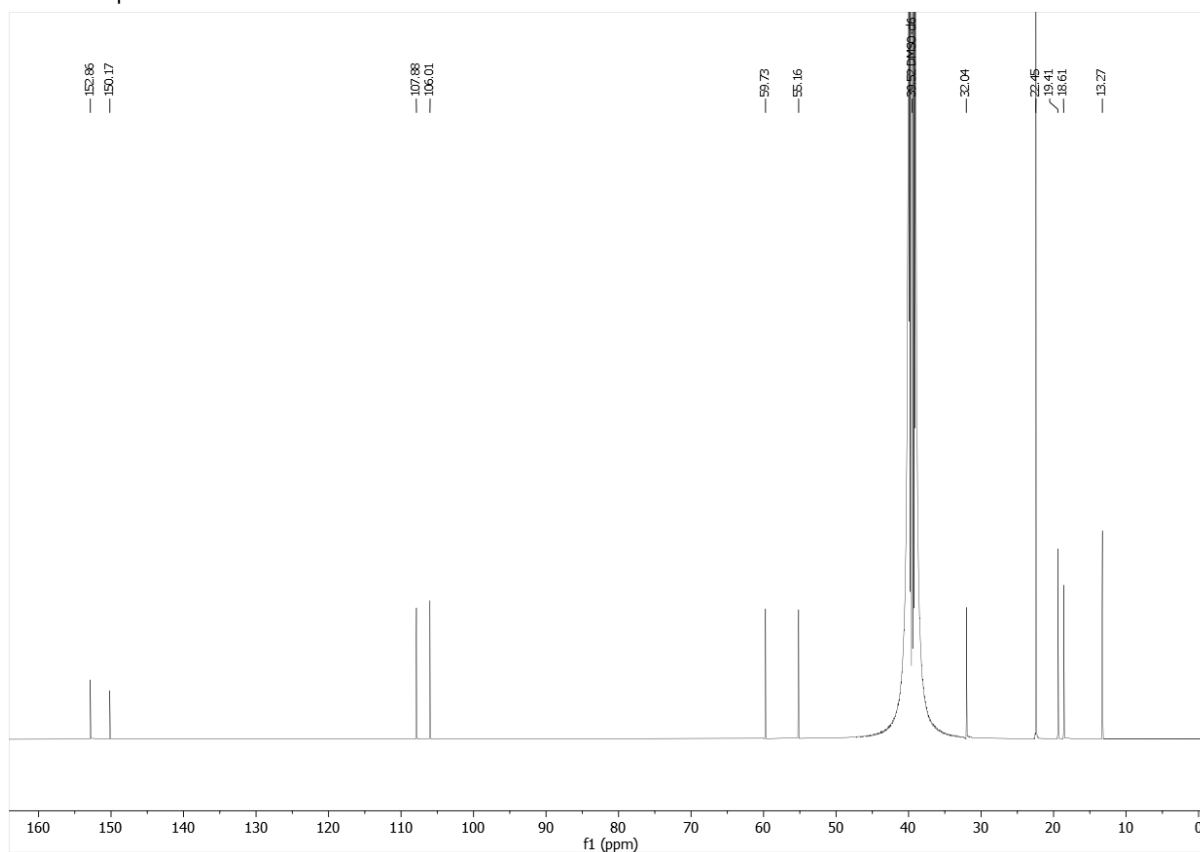
$^{13}\text{C}$ -NMR spectrum of **21b**



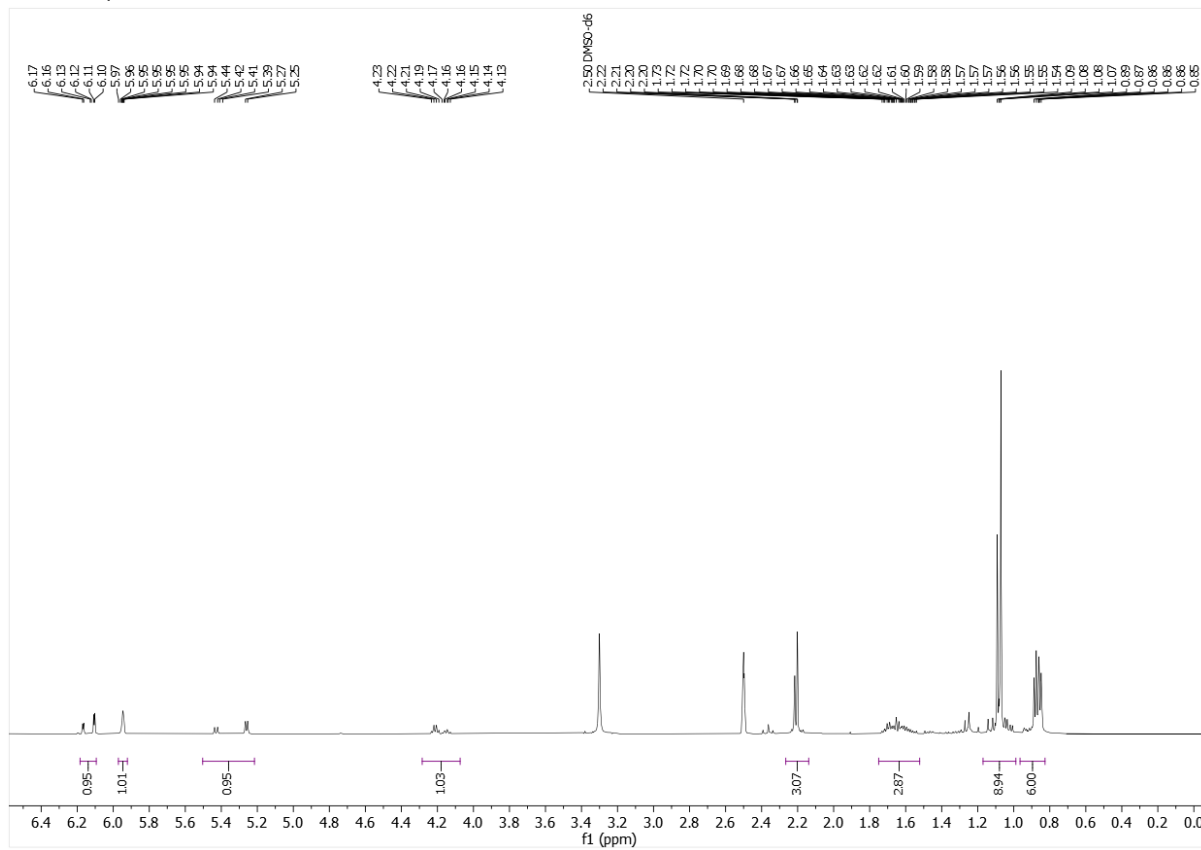
<sup>1</sup>H-NMR spectrum of **21c**



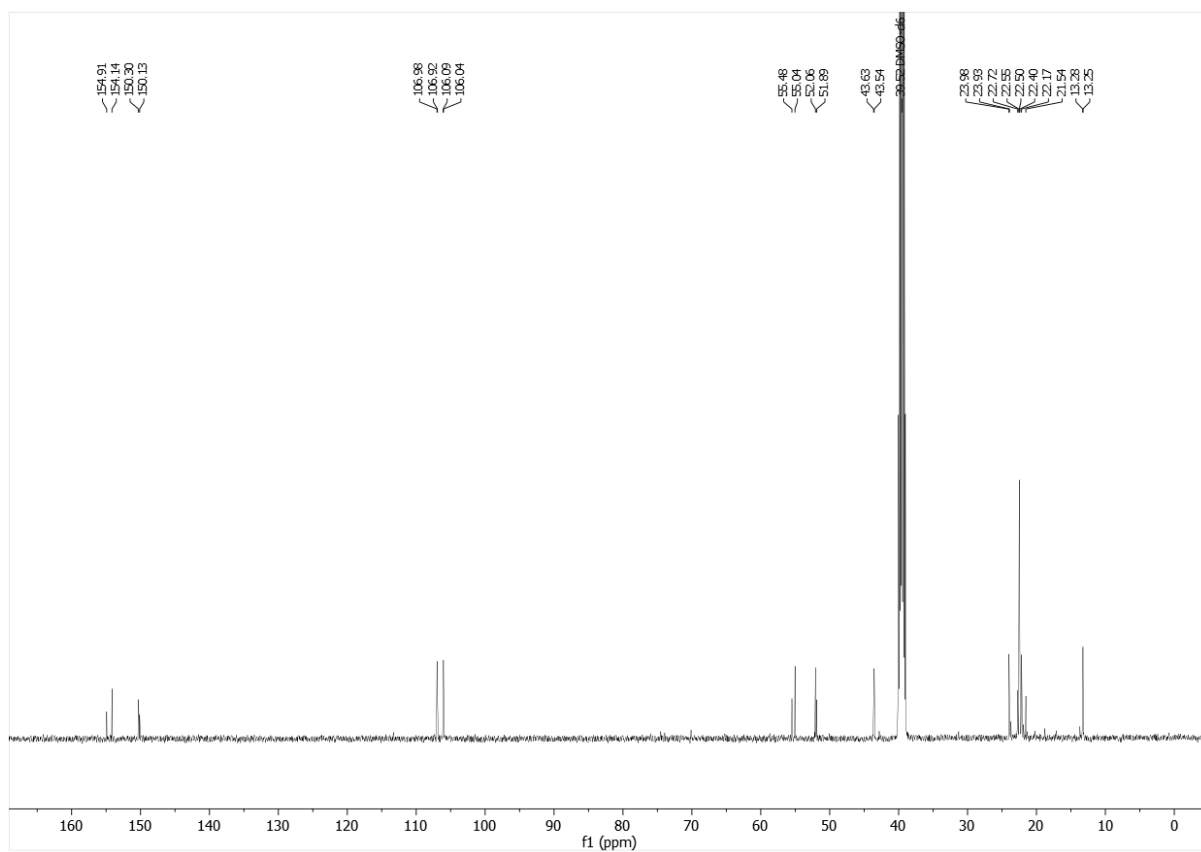
<sup>13</sup>C-NMR spectrum of **21c**



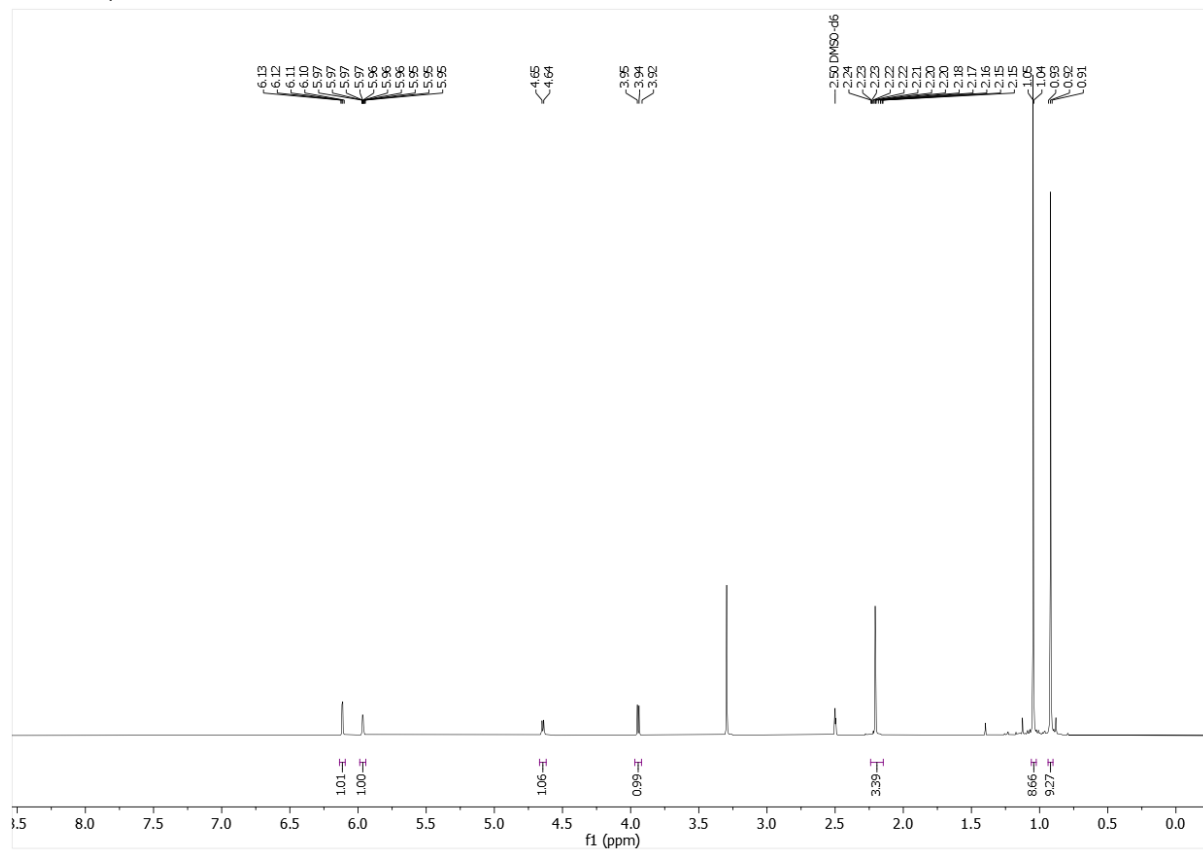
<sup>1</sup>H-NMR spectrum of **21d**



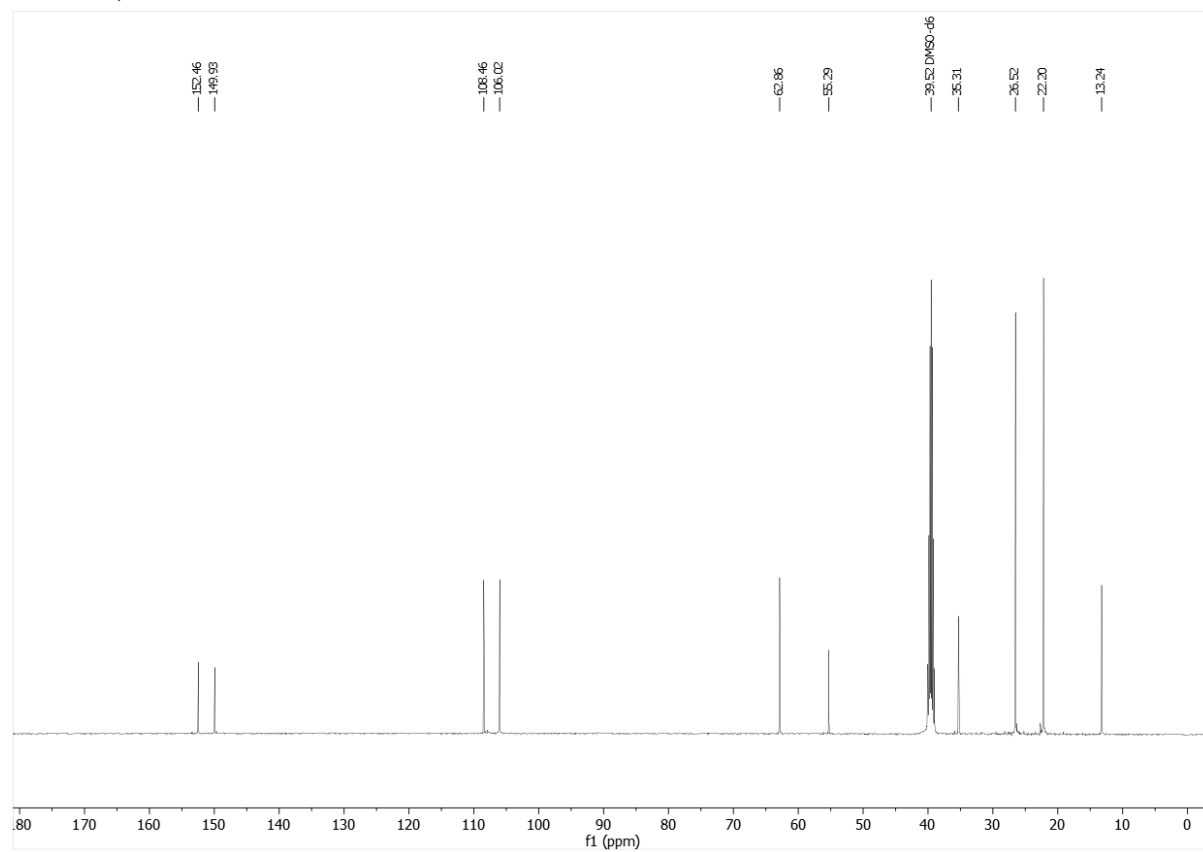
<sup>13</sup>C-NMR spectrum of **21d**



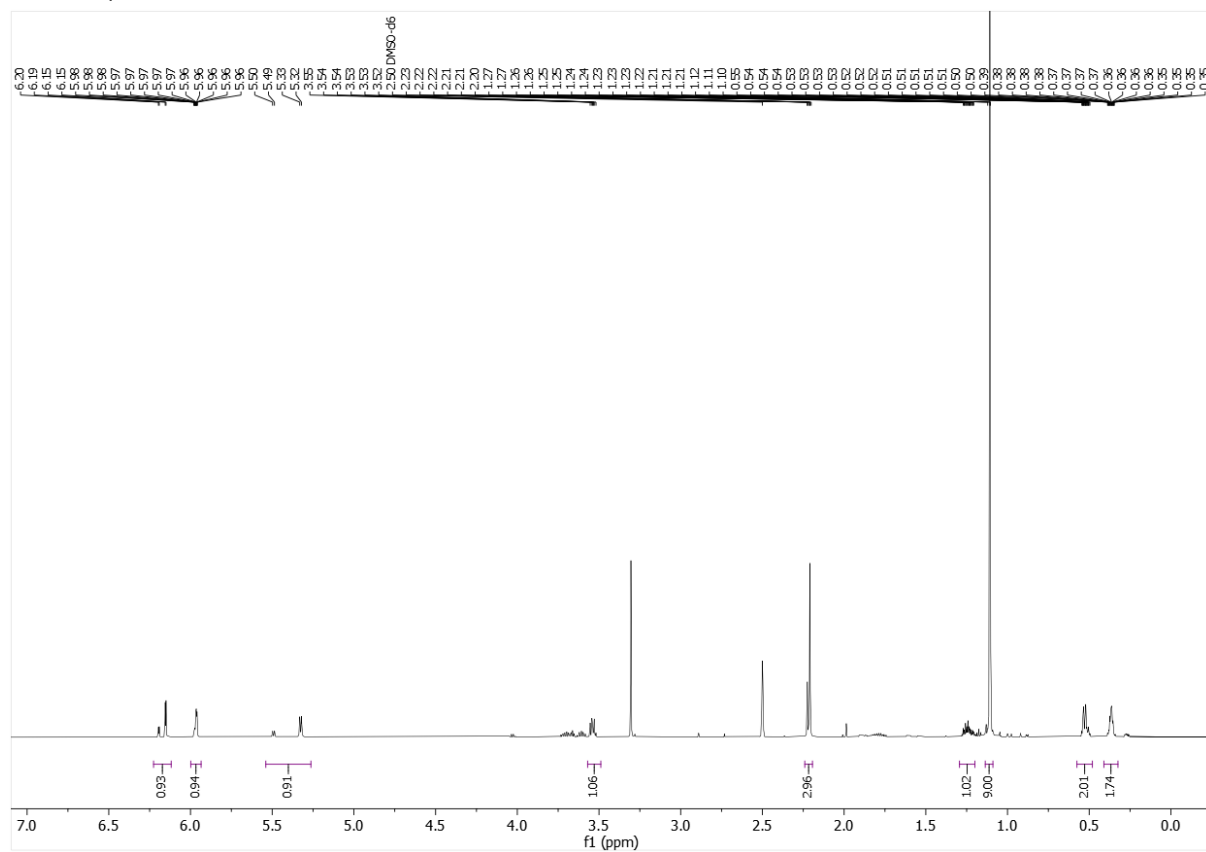
<sup>1</sup>H-NMR spectrum of **21e**



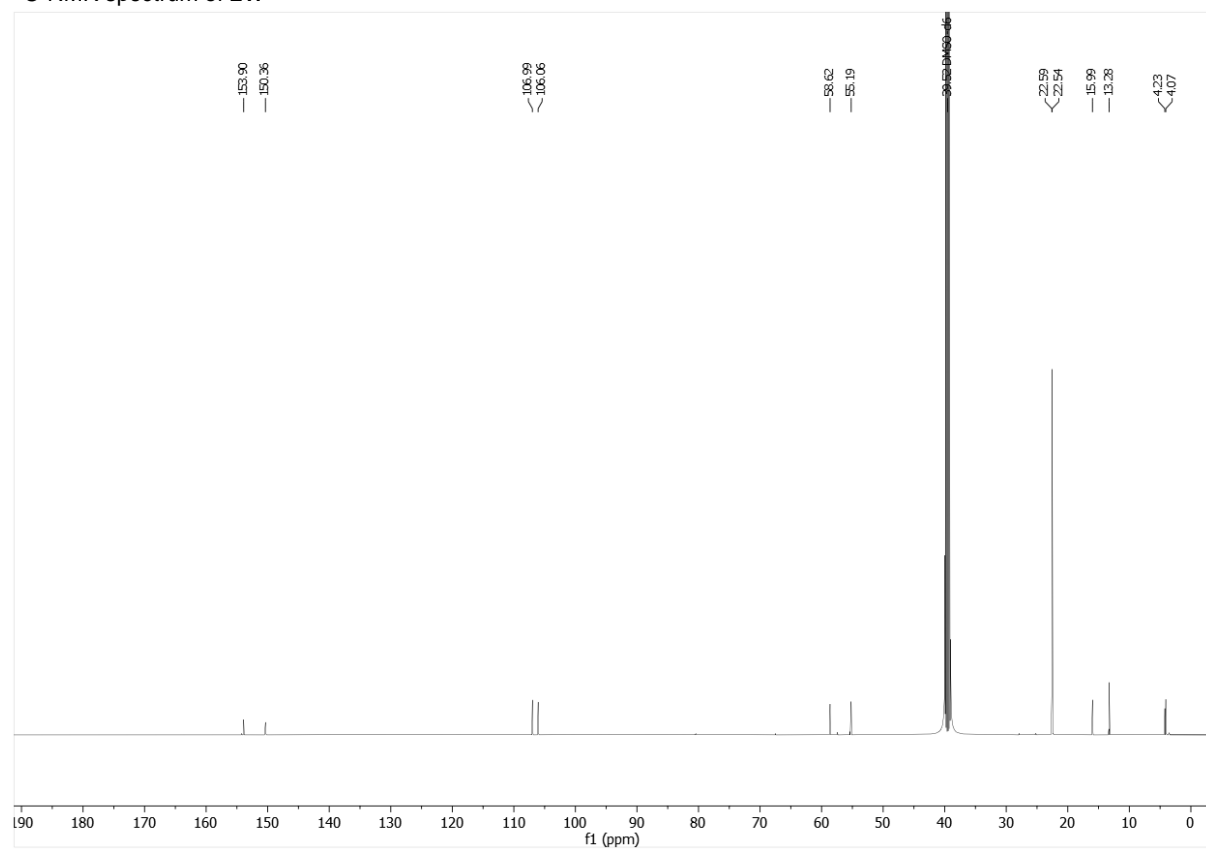
<sup>13</sup>C-NMR spectrum of **21e**



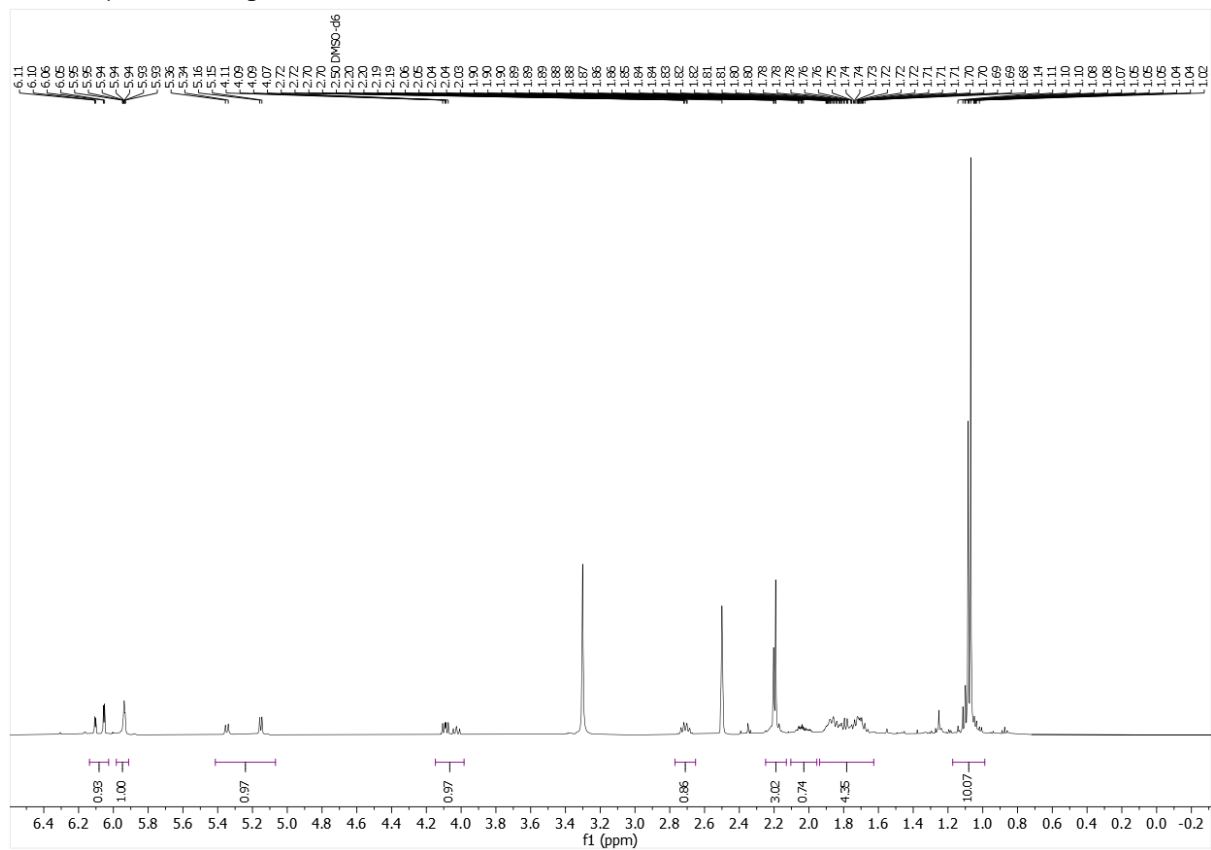
<sup>1</sup>H-NMR spectrum of **21f**



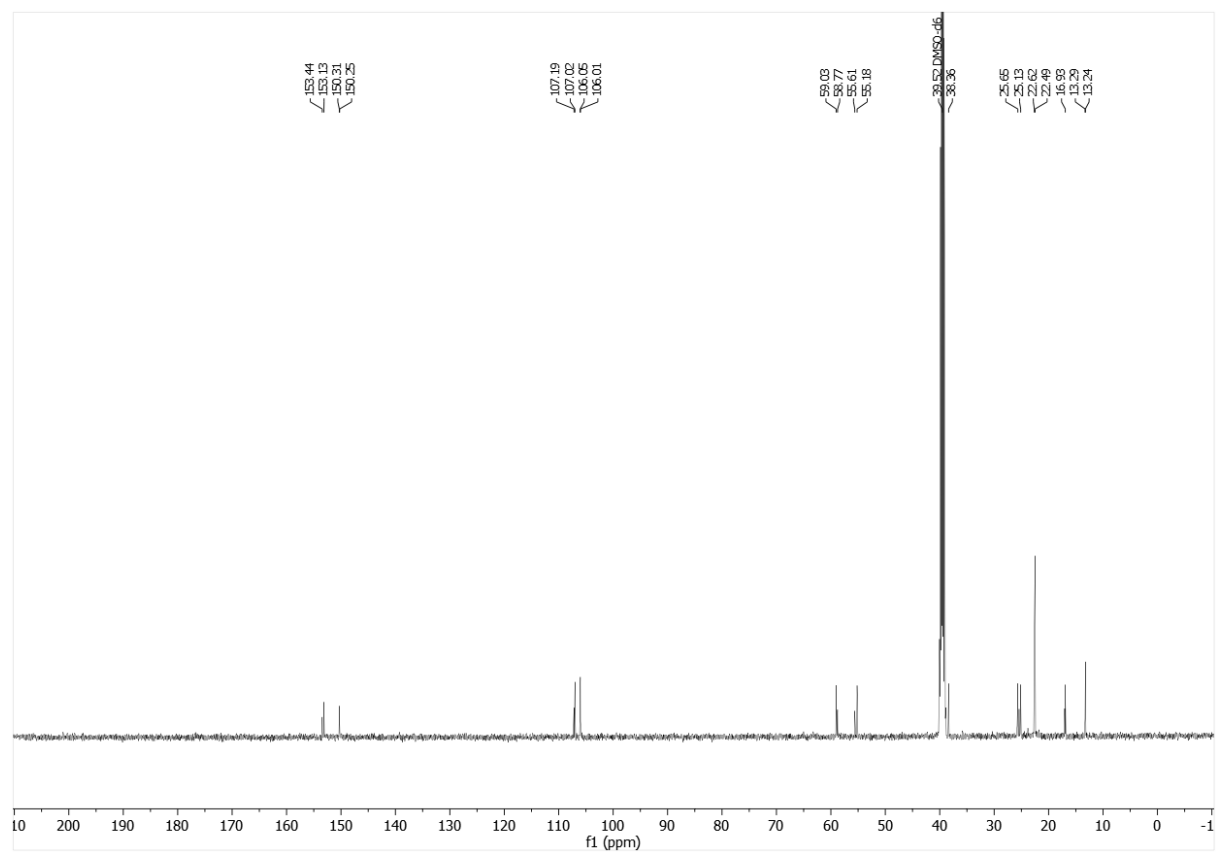
<sup>13</sup>C-NMR spectrum of **21f**



<sup>1</sup>H-NMR spectrum of **21g**



<sup>13</sup>C-NMR spectrum of **21g**



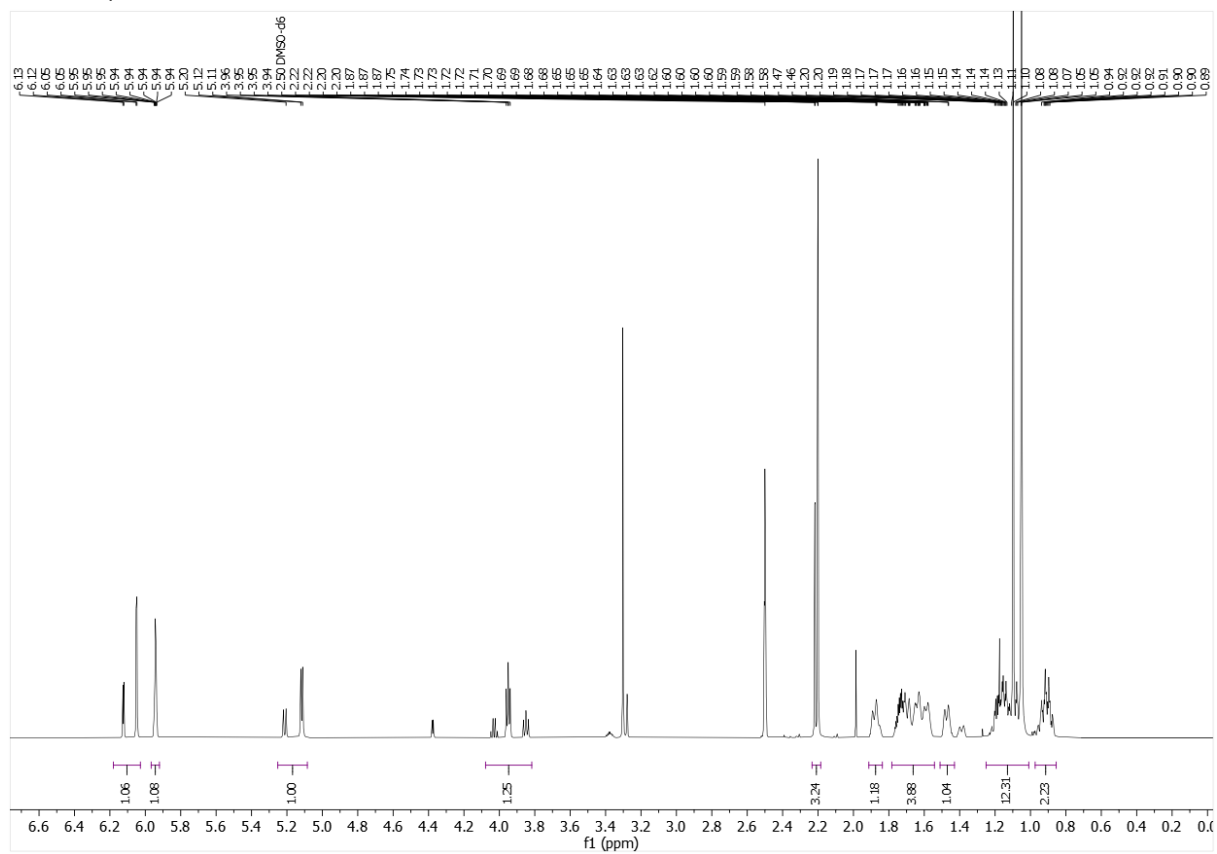
<sup>1</sup>H NMR spectrum of compound **1** in DMSO-d<sub>6</sub>. The spectrum shows peaks from 0.92 to 6.13 ppm. Key features include a broad peak at ~5.9 ppm (1.00H), a sharp peak at ~3.8 ppm (1.14H), a multiplet at ~2.5 ppm (0.95H), a multiplet at ~2.2 ppm (3.30H), a broad peak at ~1.5 ppm (16.58H), and a sharp peak at ~1.0 ppm (1.14H). Integration values are shown below the baseline.

<sup>13</sup>C NMR spectrum (CDCl<sub>3</sub>) of compound 10. The x-axis is labeled f1 (ppm) and ranges from 0 to 160. The spectrum shows several peaks in the aromatic region (149.95-154.72 ppm), a cluster of peaks between 55 and 60 ppm, a solvent triplet at 77.00 ppm, and aliphatic peaks between 13 and 35 ppm. A peak at 106.05 ppm is labeled CDCl<sub>3</sub>.

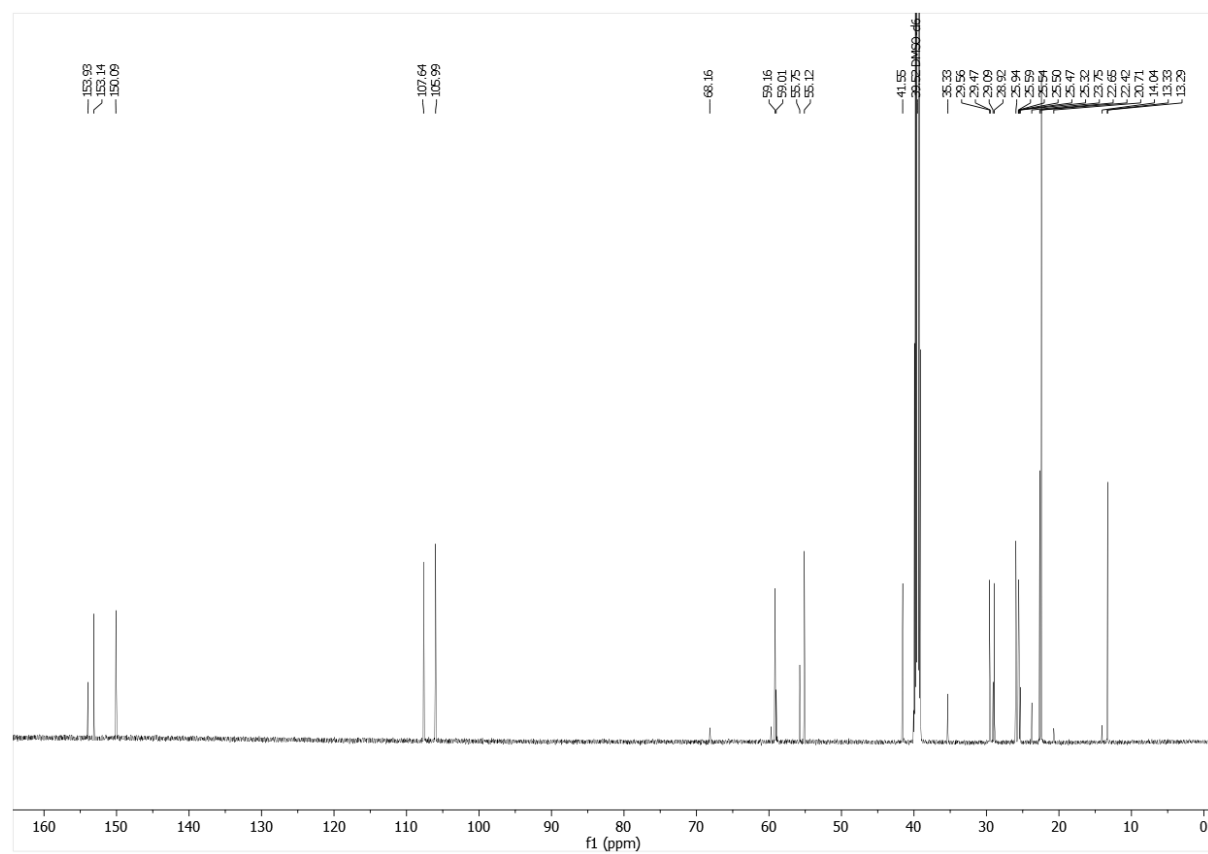
Chemical Shift (ppm)
154.72
154.01
150.07
149.95
107.26
107.15
106.05 (CDCl <sub>3</sub> )
105.99
77.70
58.41
57.23
56.03
55.29
55.12
43.99
43.56
39.50 (DMSO-d <sub>6</sub> )
34.96
28.87
28.61
28.54
29.24
24.95
24.87
24.74
22.89
22.02
17.42
13.50
13.25

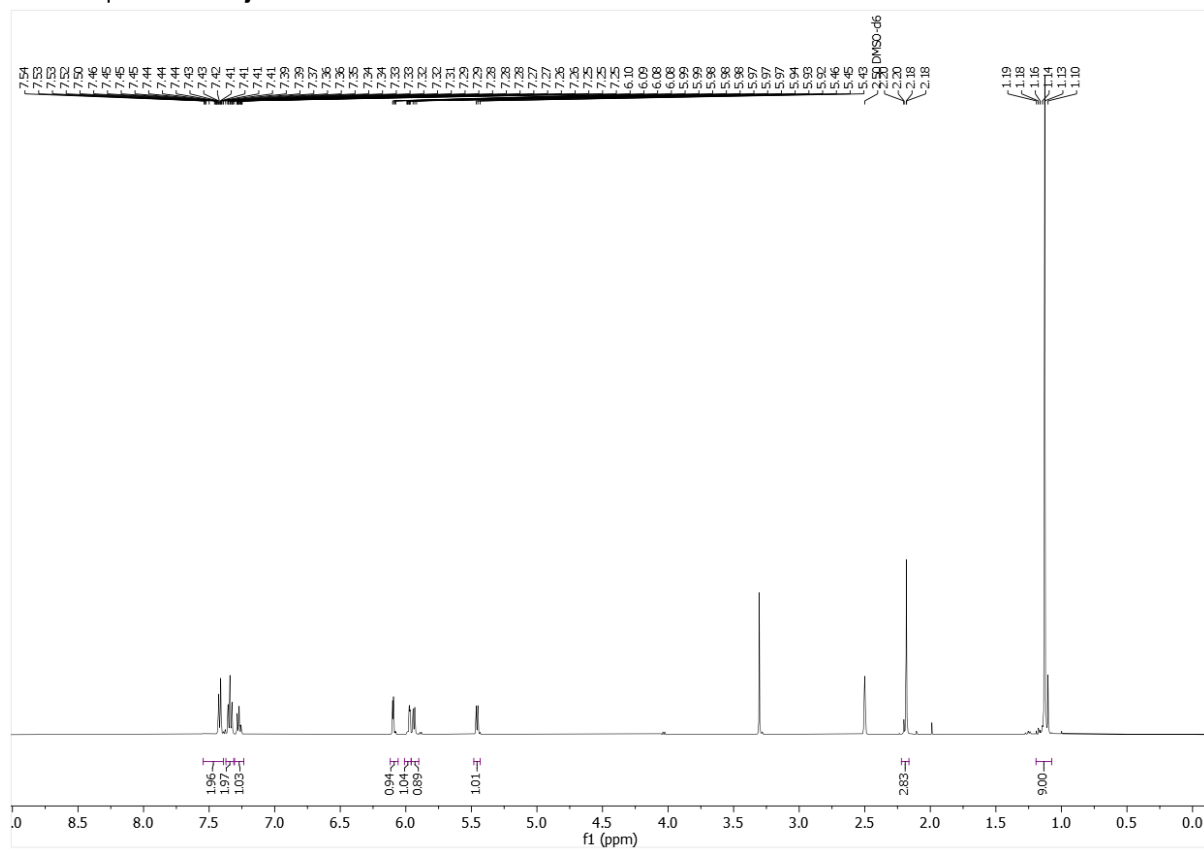
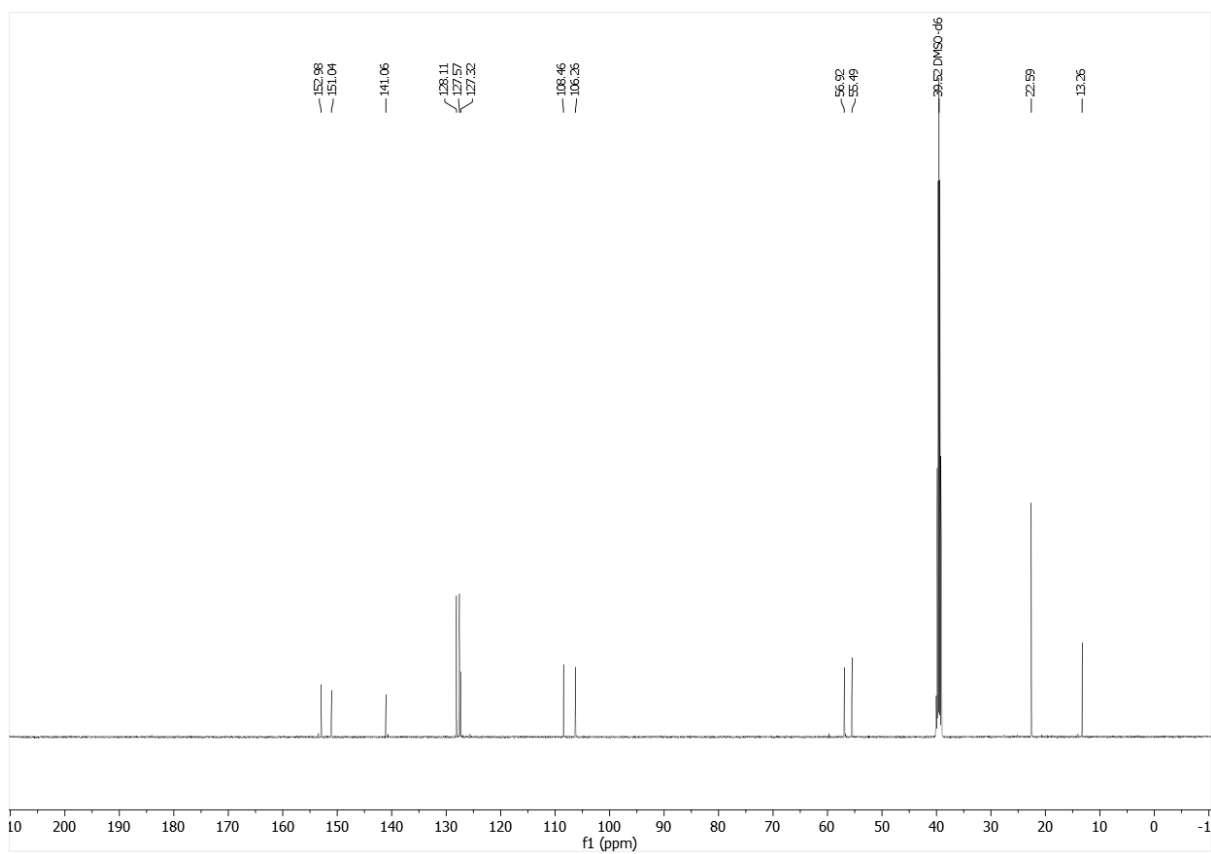


<sup>1</sup>H-NMR spectrum of **21i**

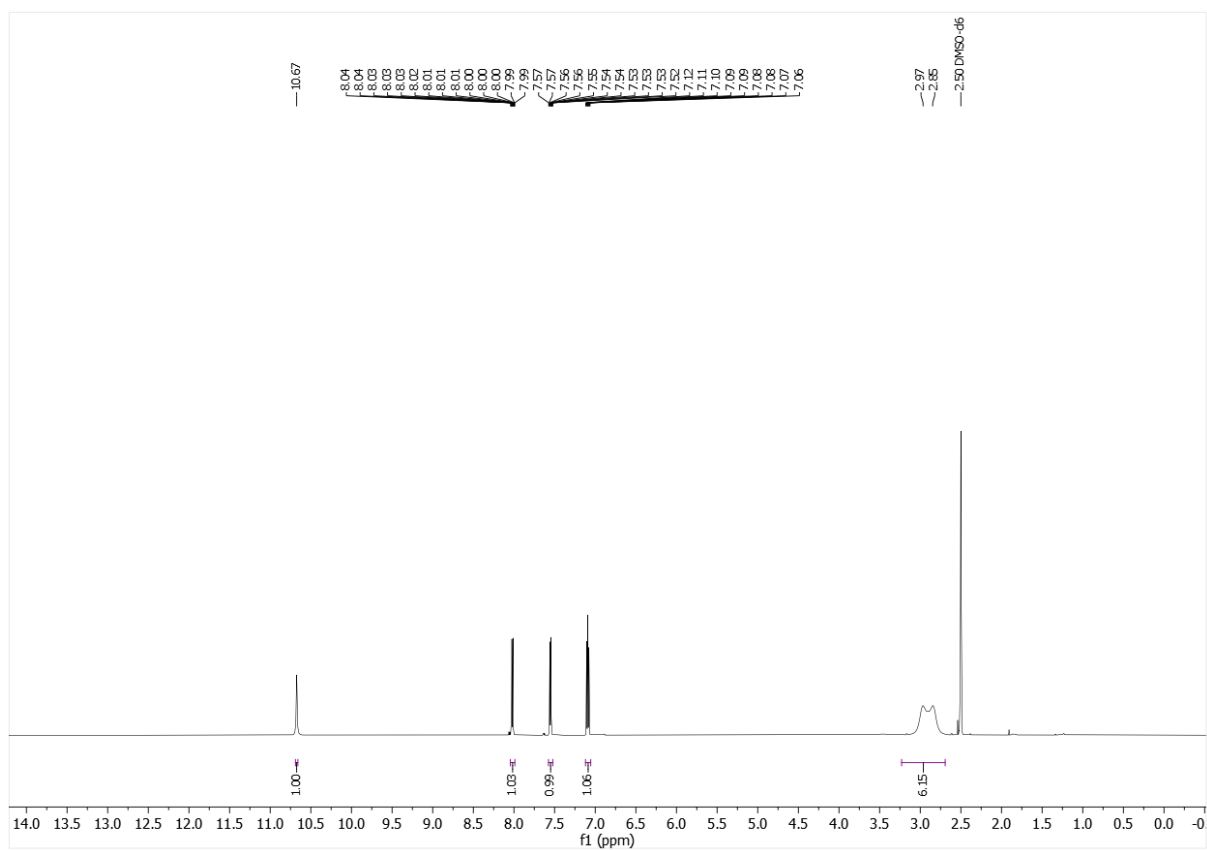


<sup>13</sup>C-NMR spectrum of **21i**

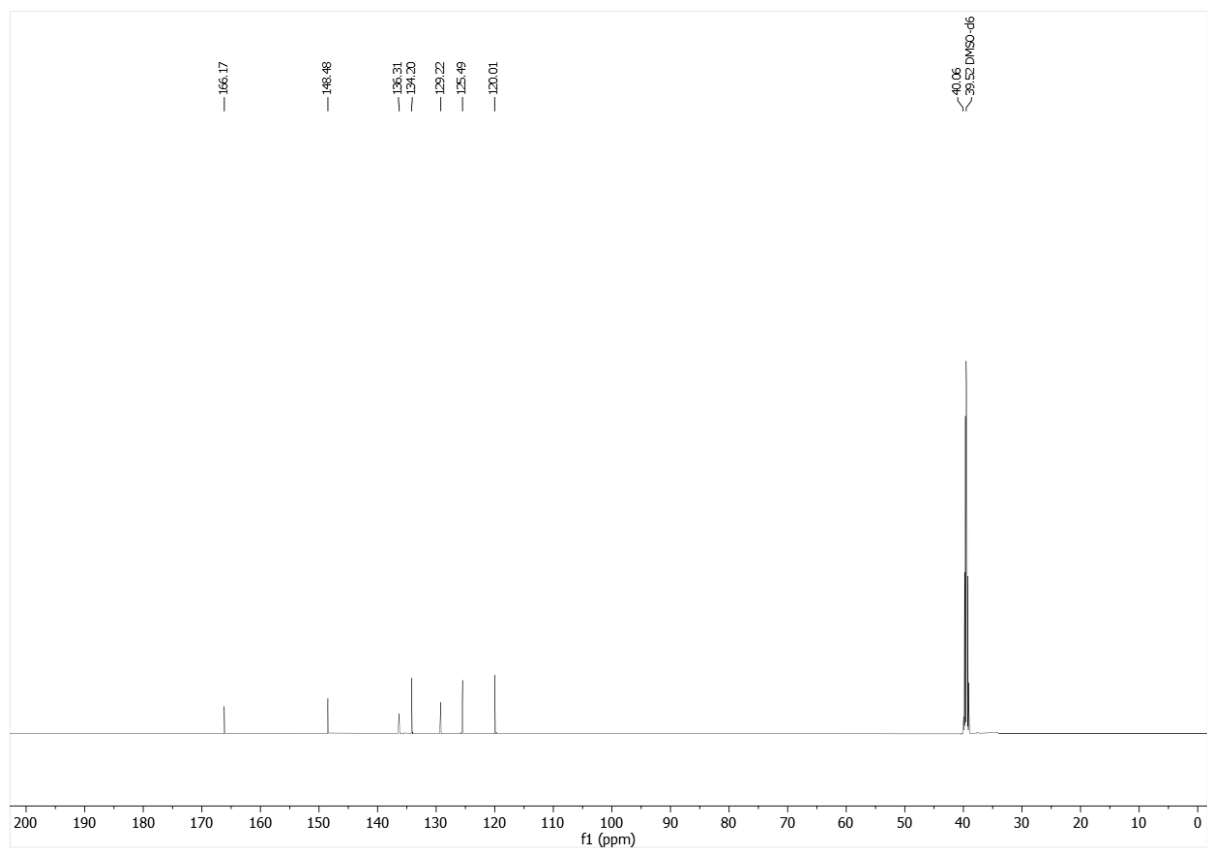


<sup>1</sup>H-NMR spectrum of **21j**<sup>13</sup>C-NMR spectrum of **21j**

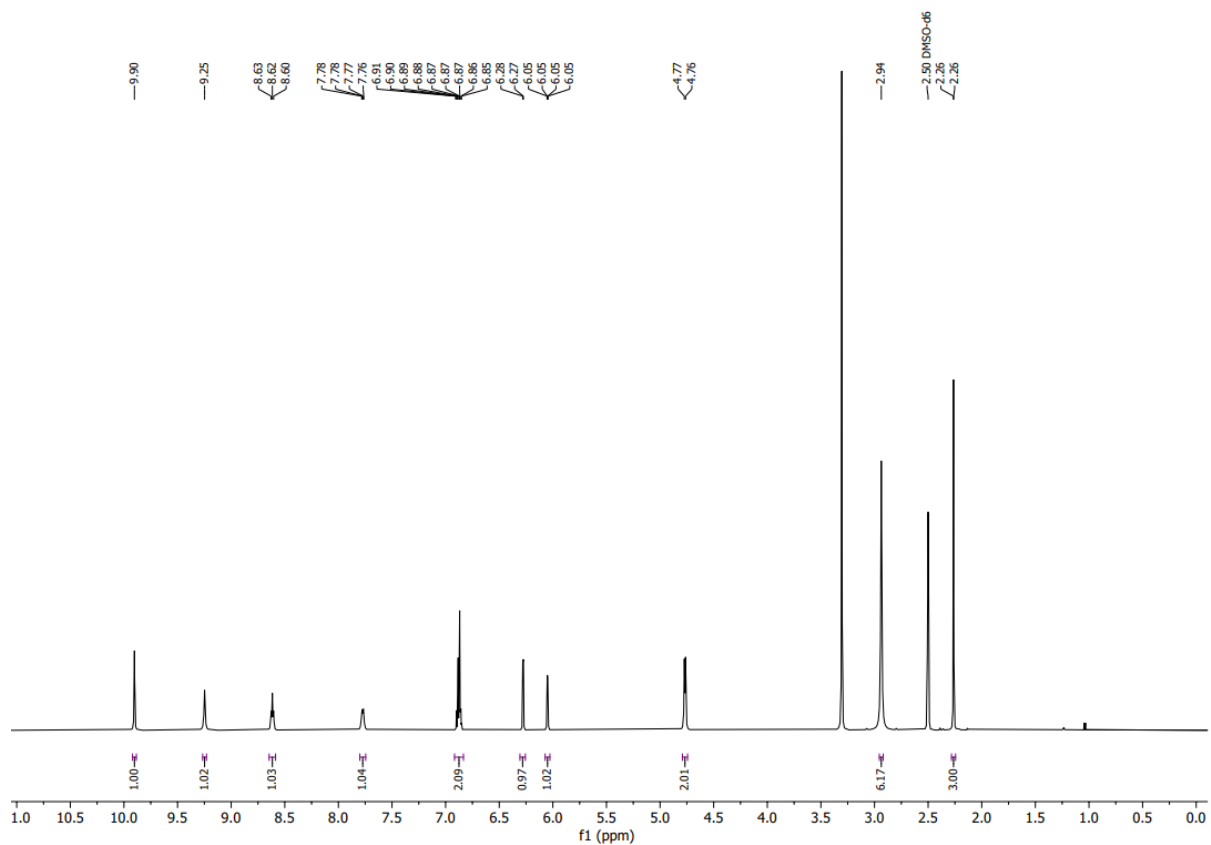
$^1\text{H}$ -NMR spectrum of **24**



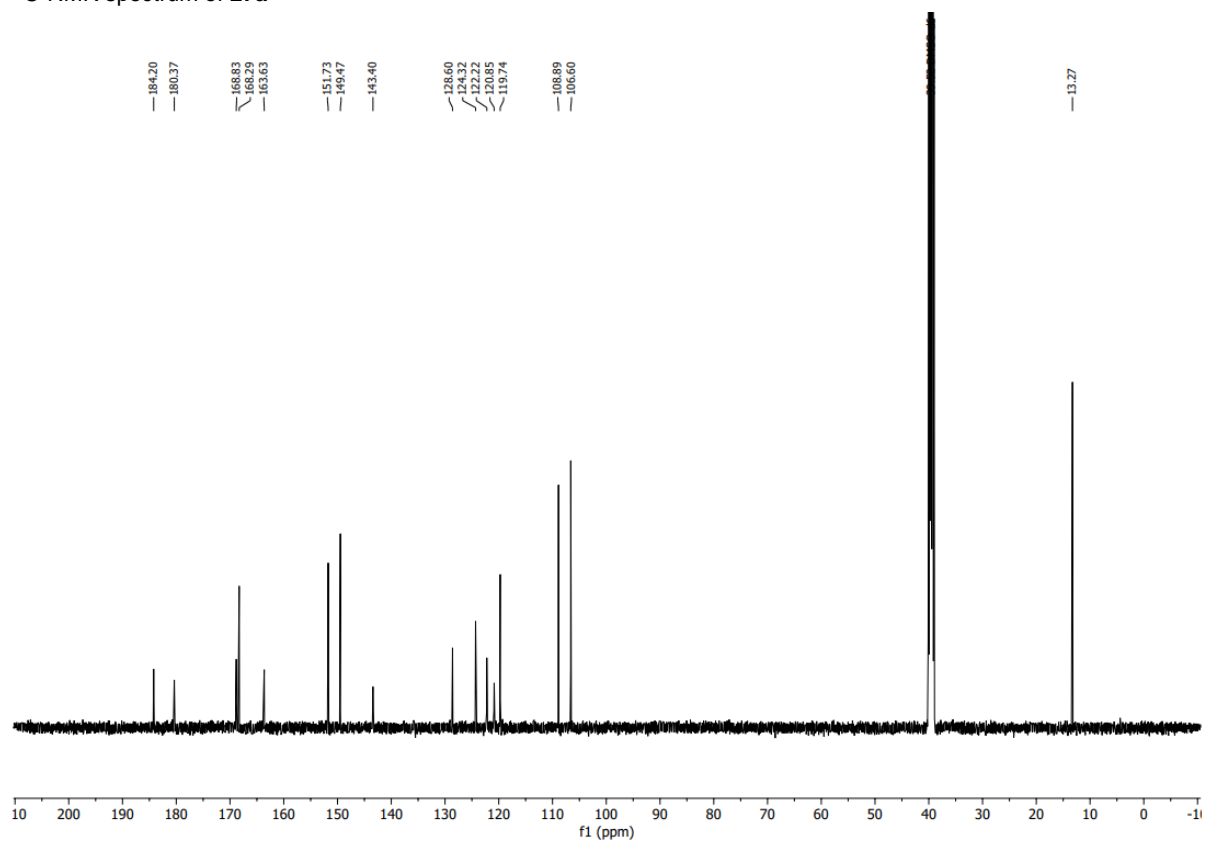
$^{13}\text{C}$ -NMR spectrum of **24**



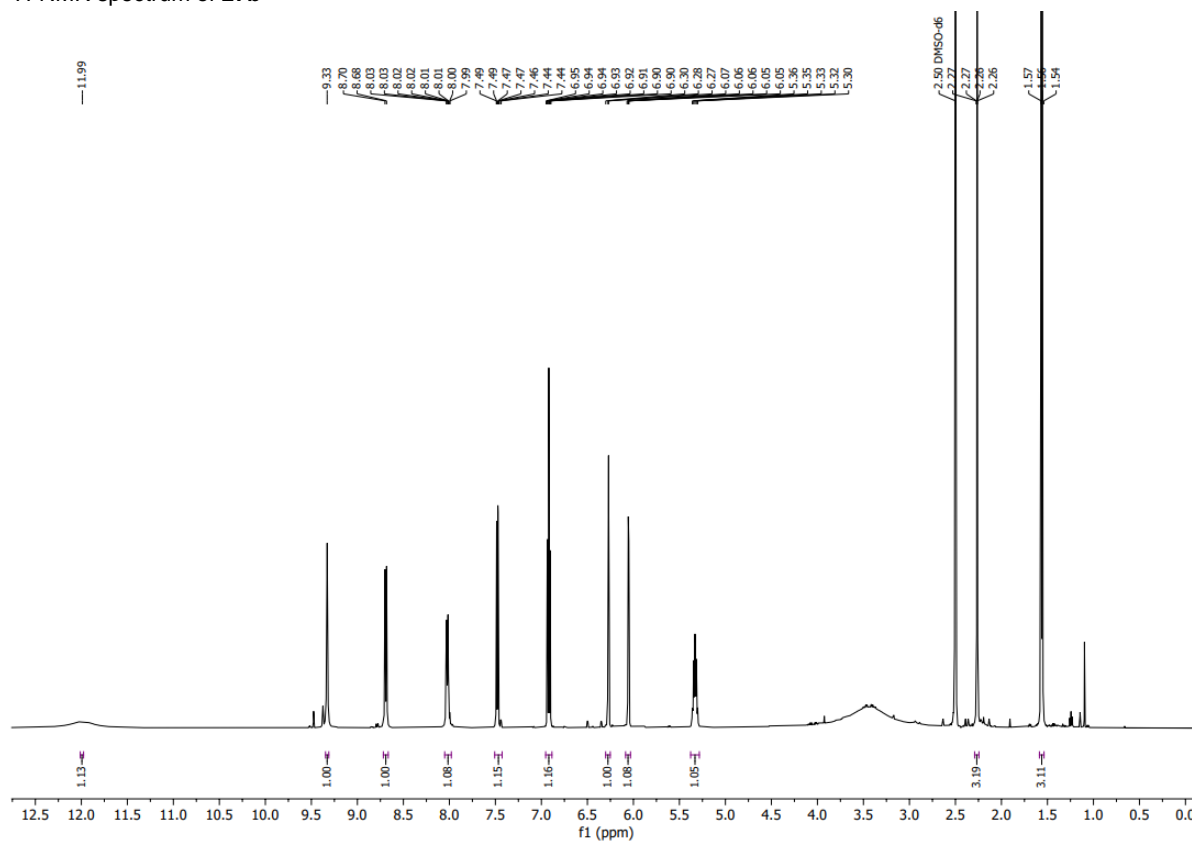
<sup>1</sup>H-NMR spectrum of **27a**



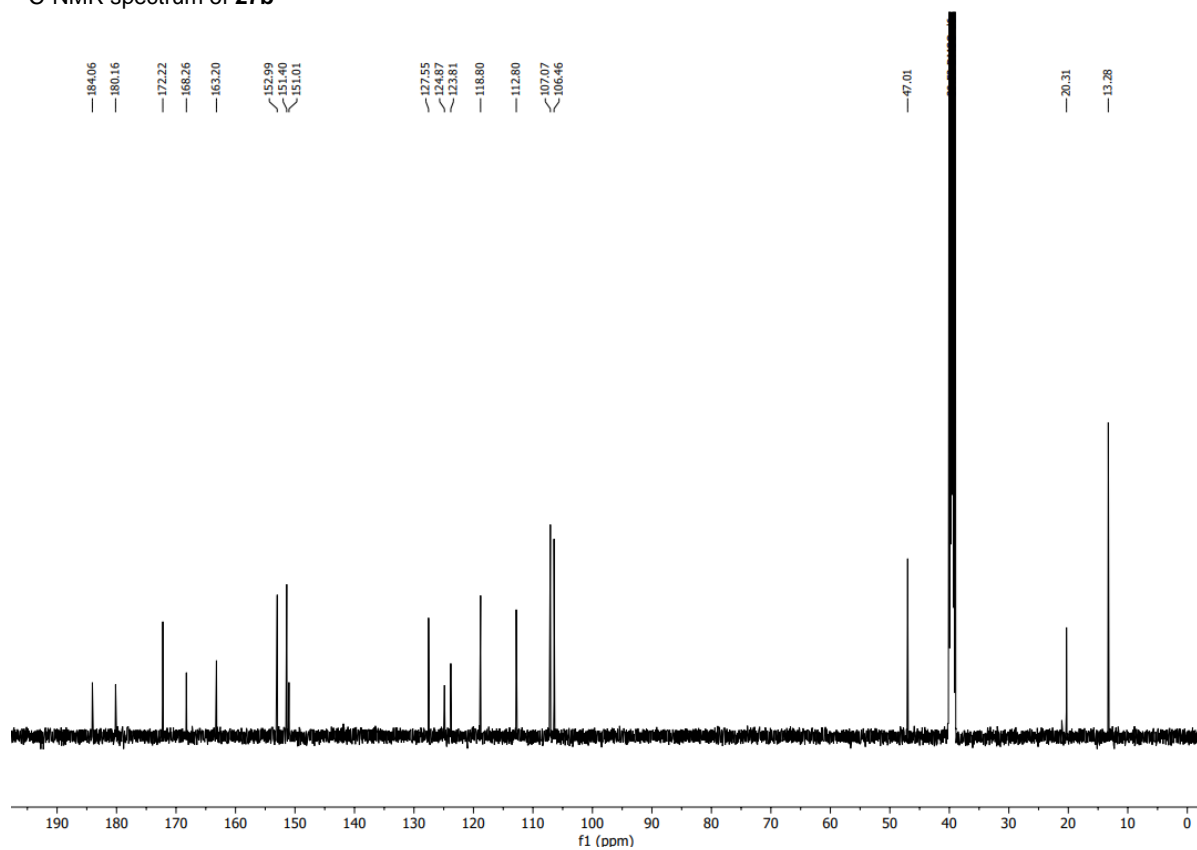
<sup>13</sup>C-NMR spectrum of **27a**



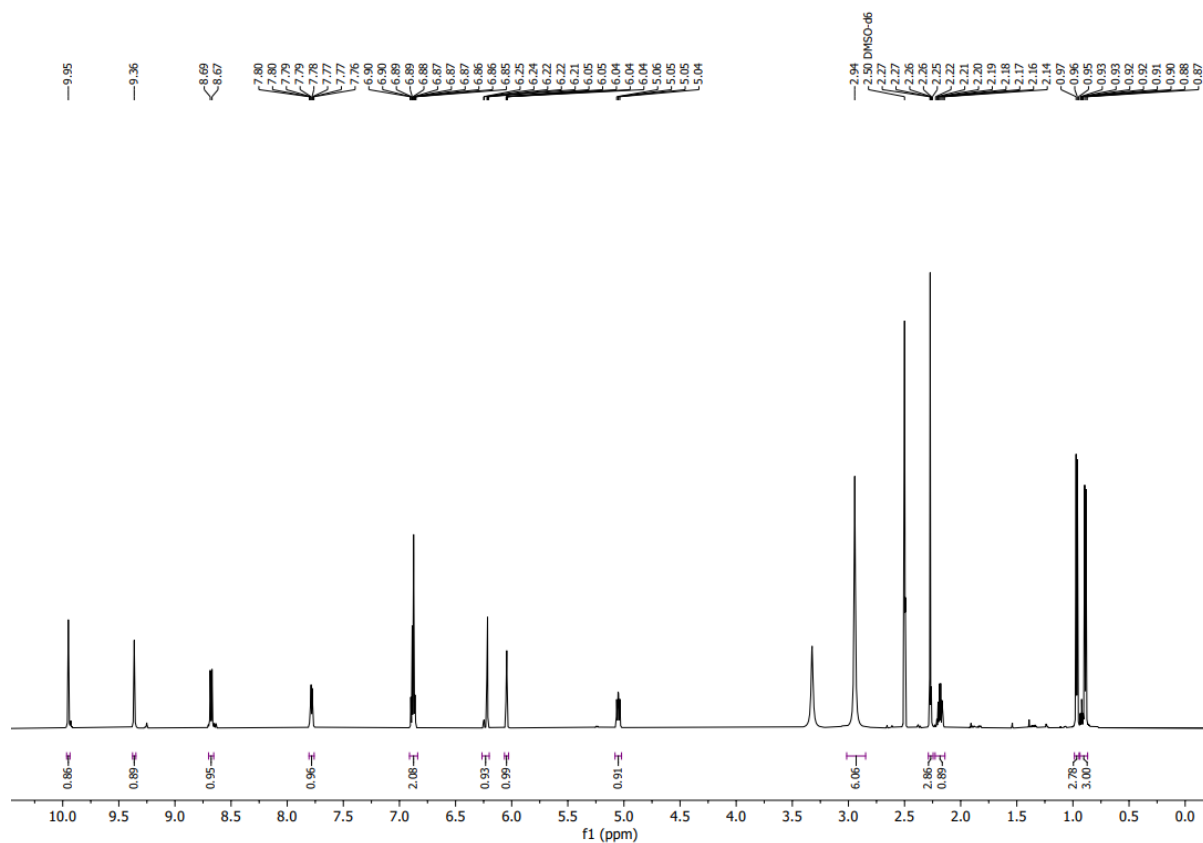
<sup>1</sup>H-NMR spectrum of **27b**



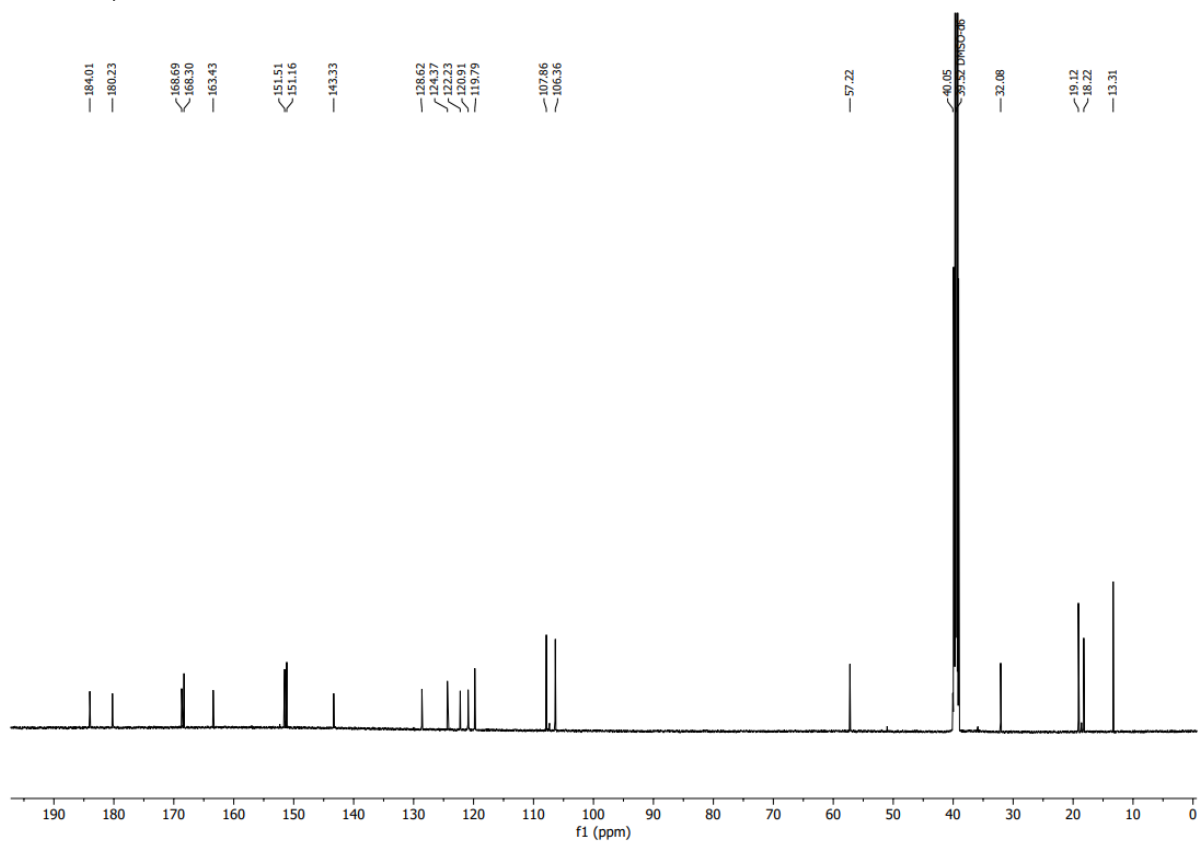
<sup>13</sup>C-NMR spectrum of **27b**



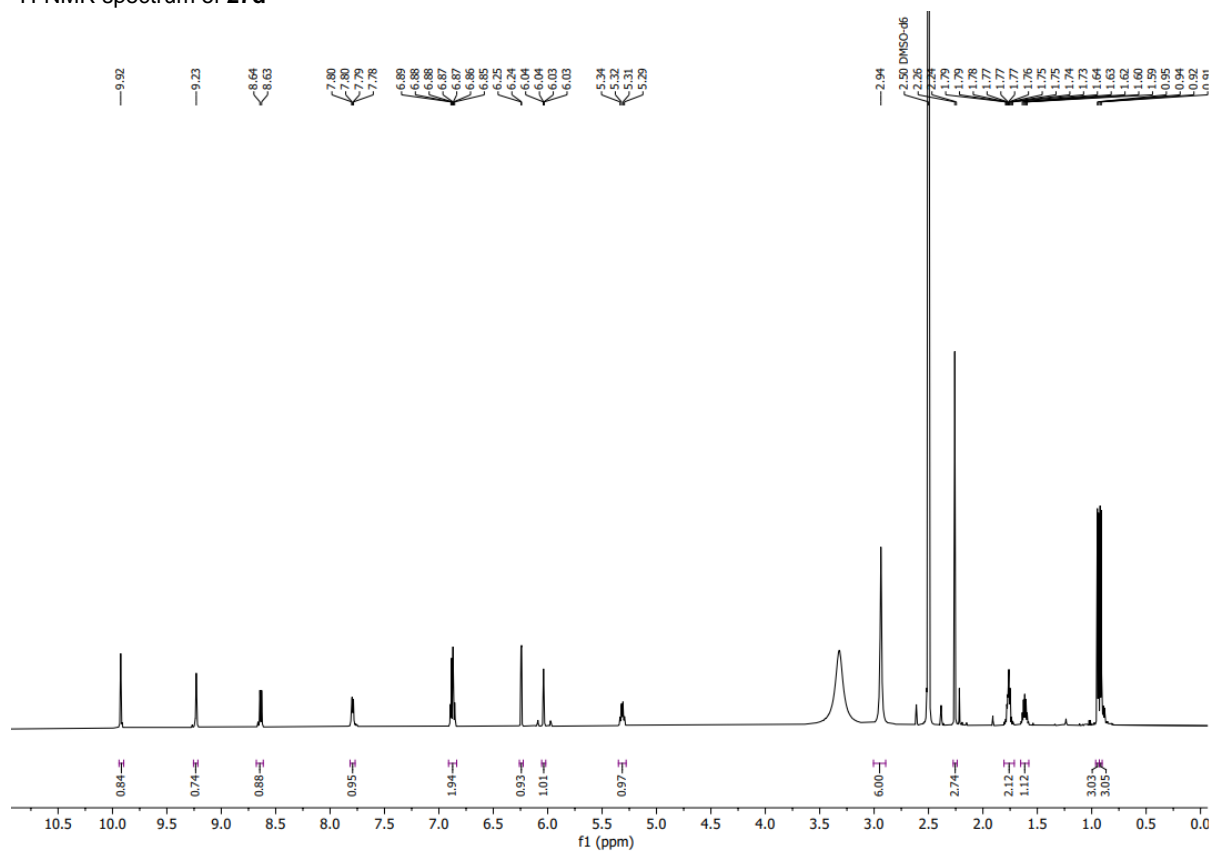
$^1\text{H}$ -NMR spectrum of **27c**



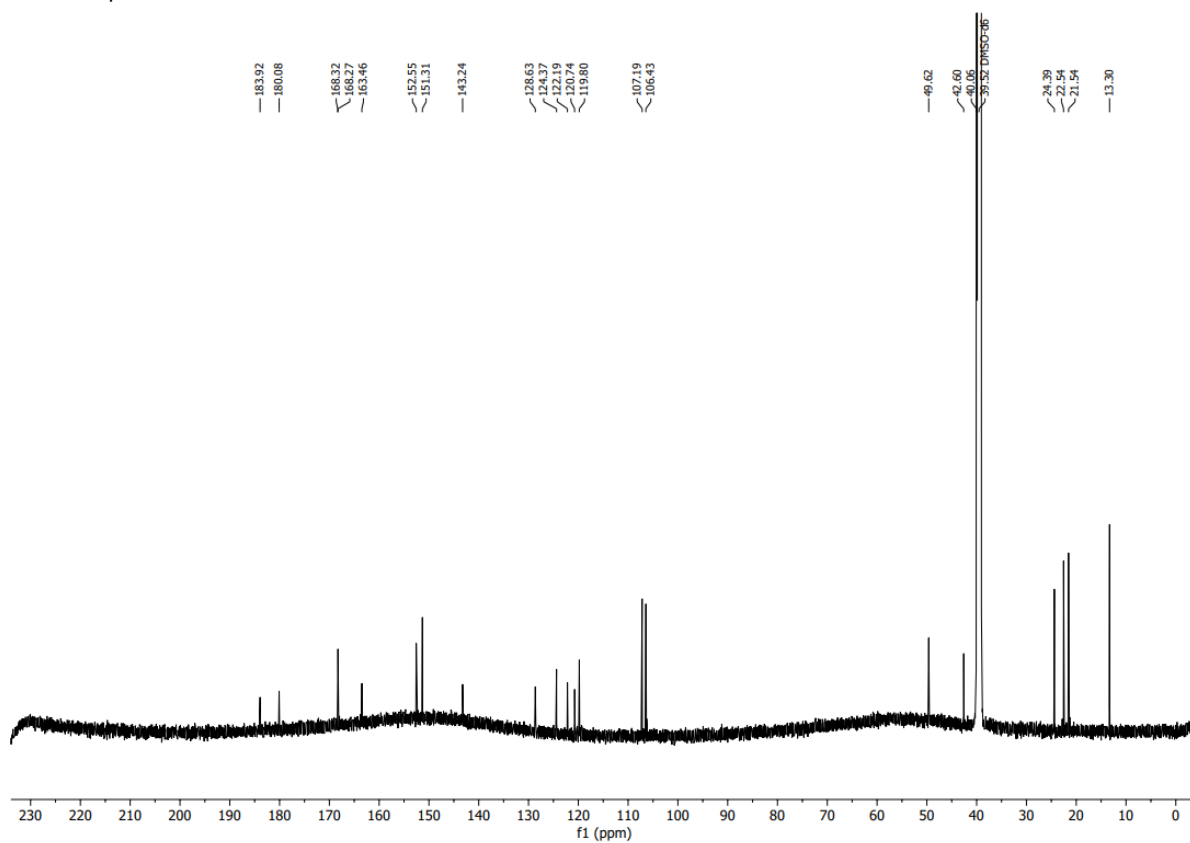
$^{13}\text{C}$ -NMR spectrum of **27c**



<sup>1</sup>H-NMR spectrum of **27d**



<sup>13</sup>C-NMR spectrum of **27d**

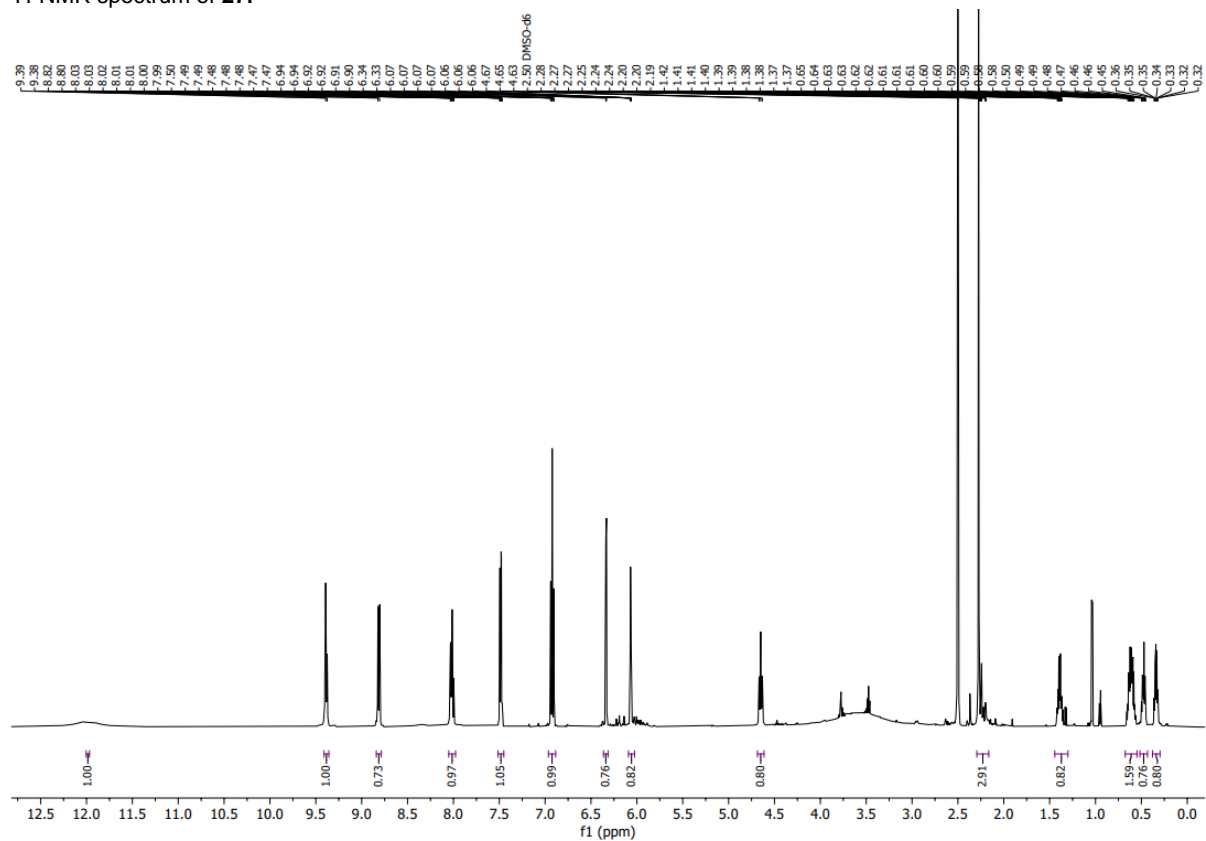


[illegible]

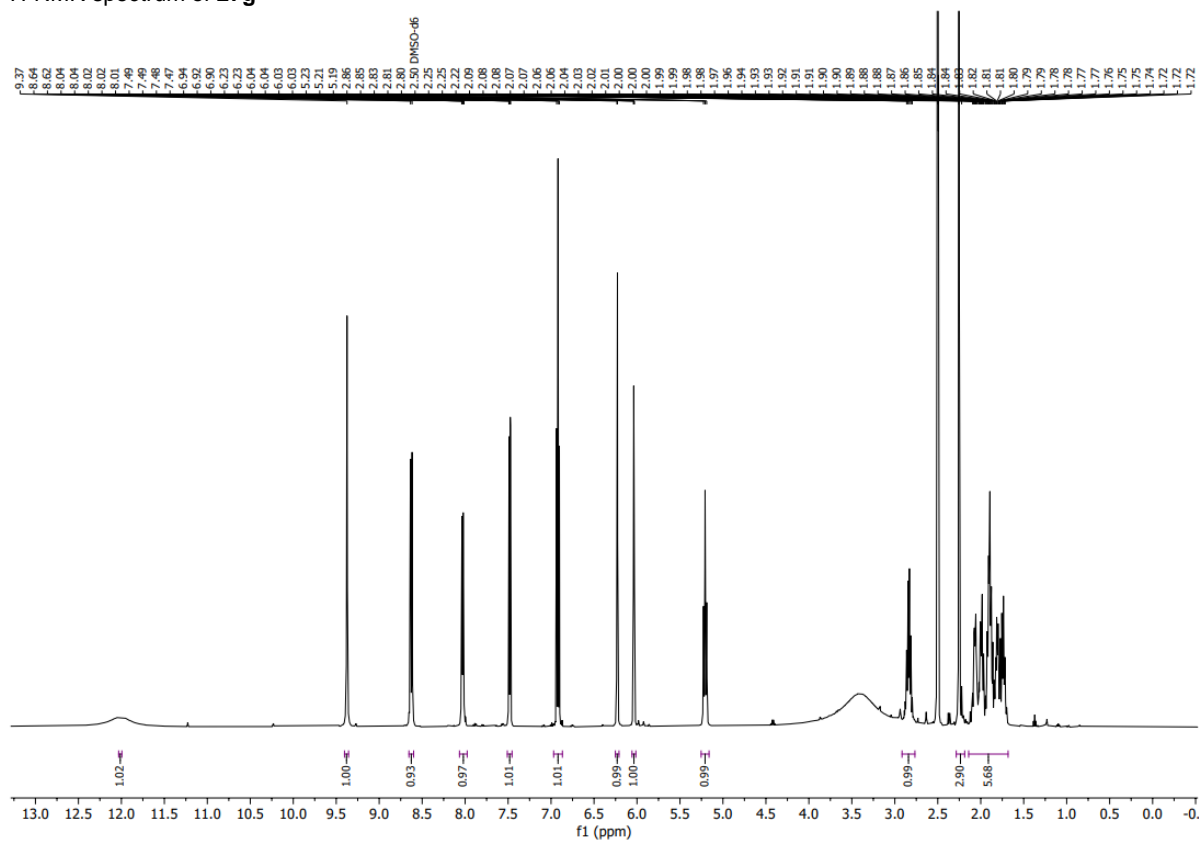
13C NMR spectrum (CDCl<sub>3</sub>) of compound 10b. The x-axis is labeled 'f1 (ppm)' and ranges from 0 to 190. The spectrum shows several sharp peaks. A large solvent triplet for CDCl<sub>3</sub> is centered at 77.0 ppm. Other peaks are labeled with their chemical shifts: 184.01, 180.23, 168.69, 168.30, 163.43, 151.51, 151.16, 143.33, 128.62, 128.57, 122.23, 120.91, 119.79, 107.86, 106.36, 57.22, 40.05, 39.52, 39.05, 32.08, 19.12, 18.22, and 13.31.



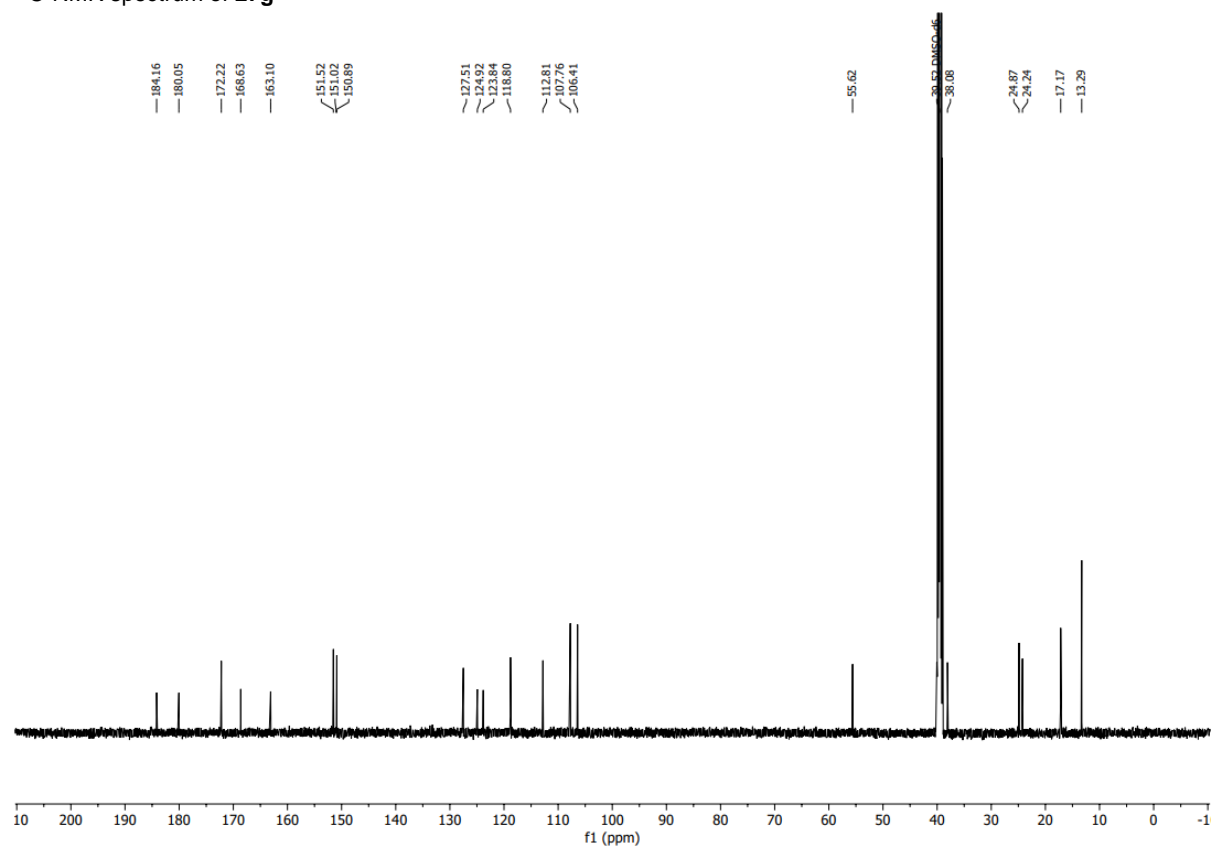
$^1\text{H}$ -NMR spectrum of **27f**



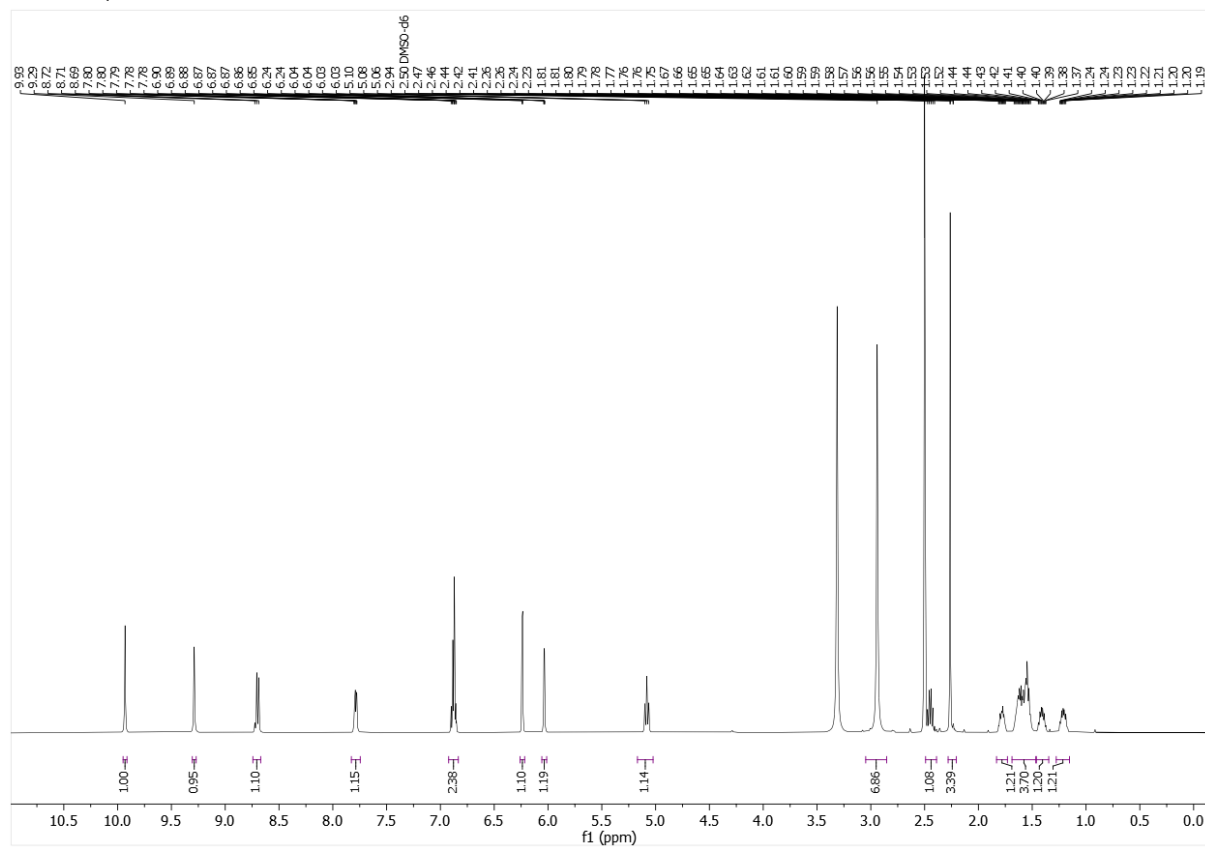
$^1\text{H}$ -NMR spectrum of **27g**



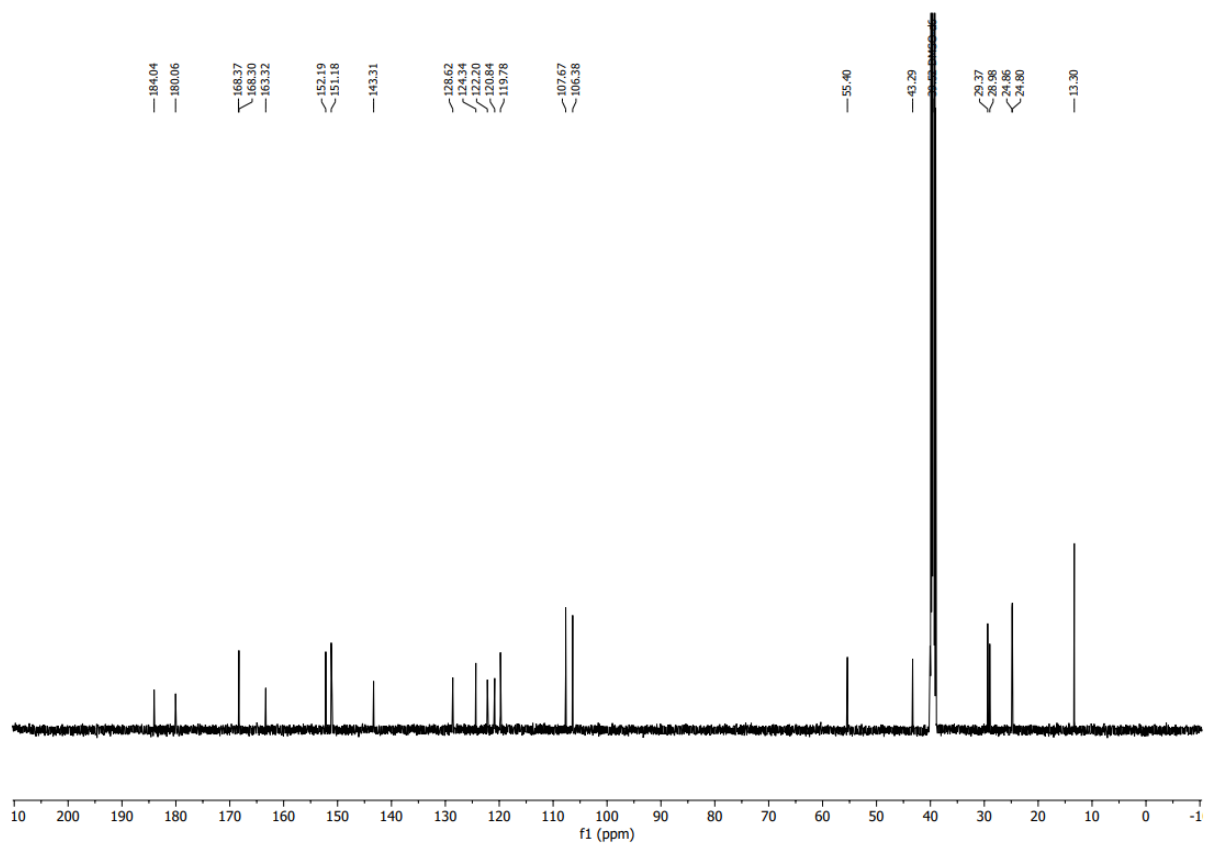
$^{13}\text{C}$ -NMR spectrum of **27g**



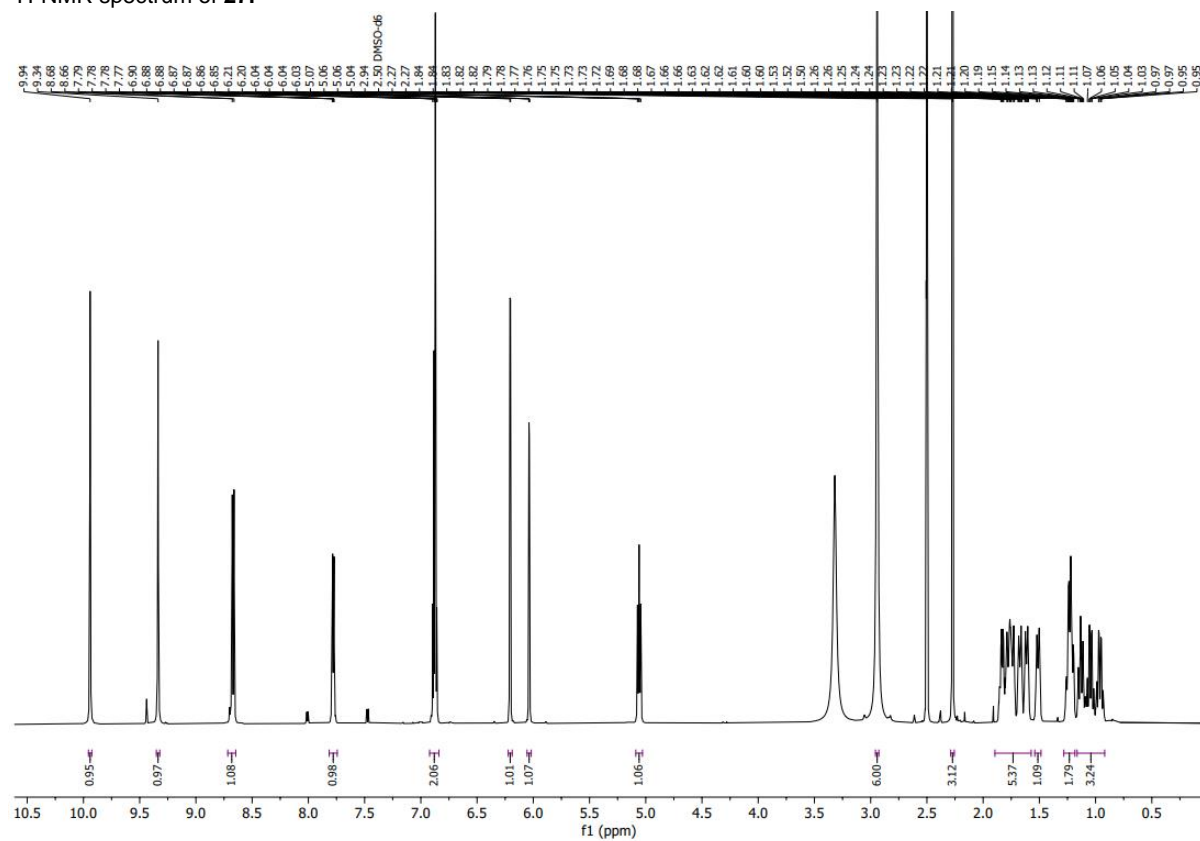
<sup>1</sup>H-NMR spectrum of **27h**



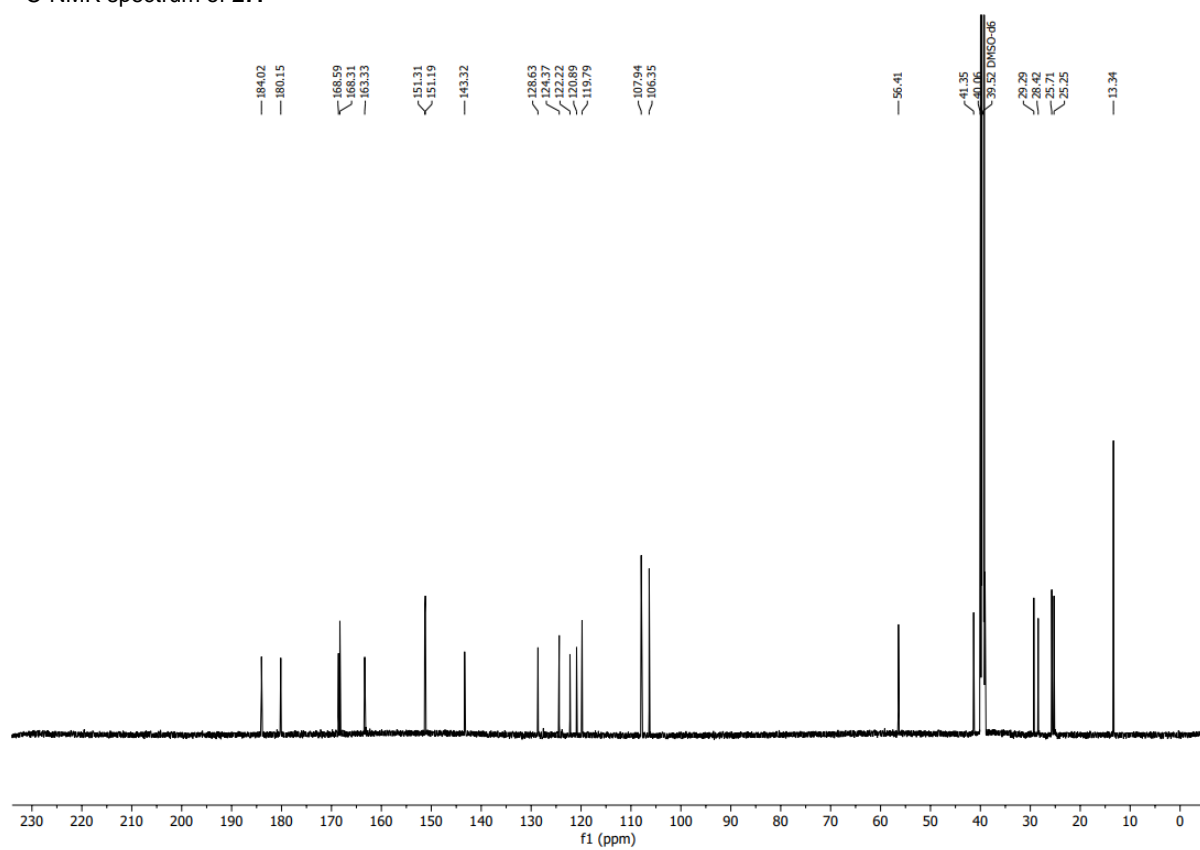
<sup>13</sup>C-NMR spectrum of **27h**



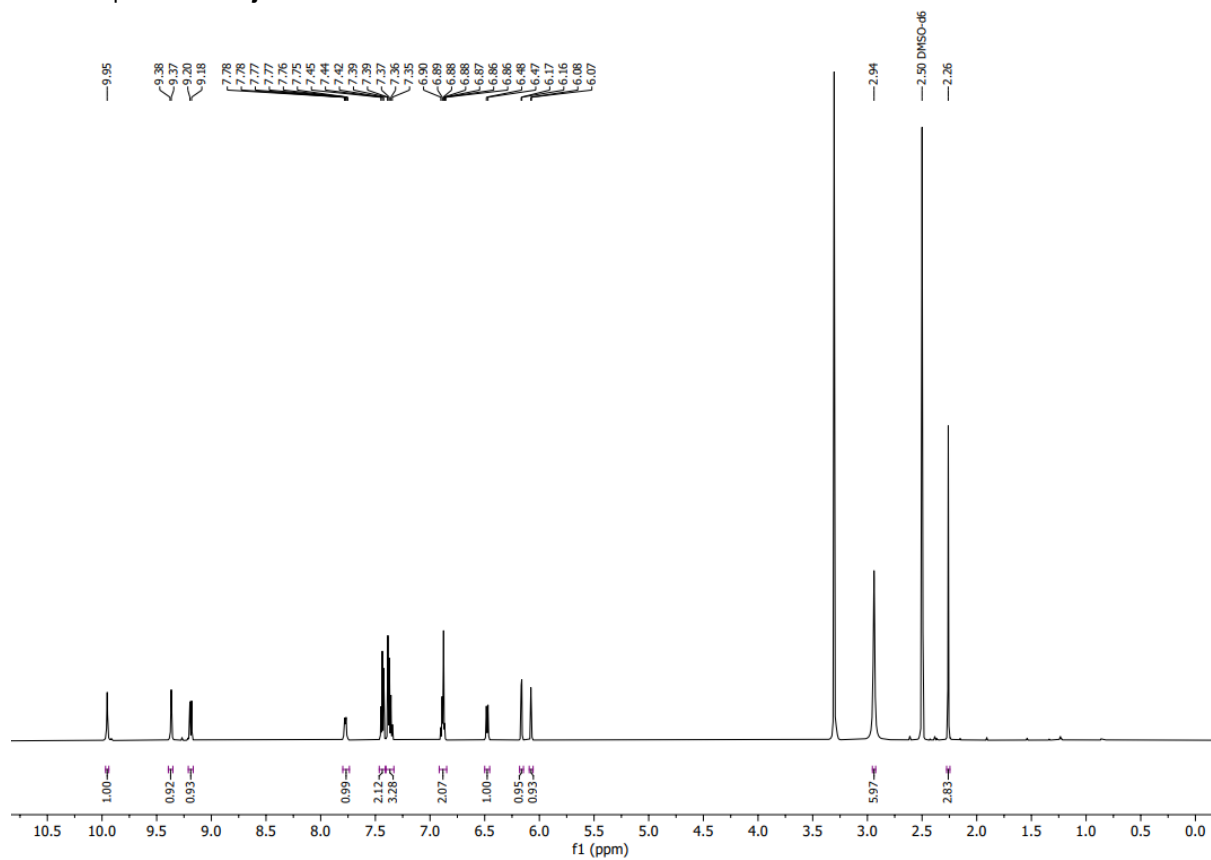
<sup>1</sup>H-NMR spectrum of **27i**



<sup>13</sup>C-NMR spectrum of **27i**

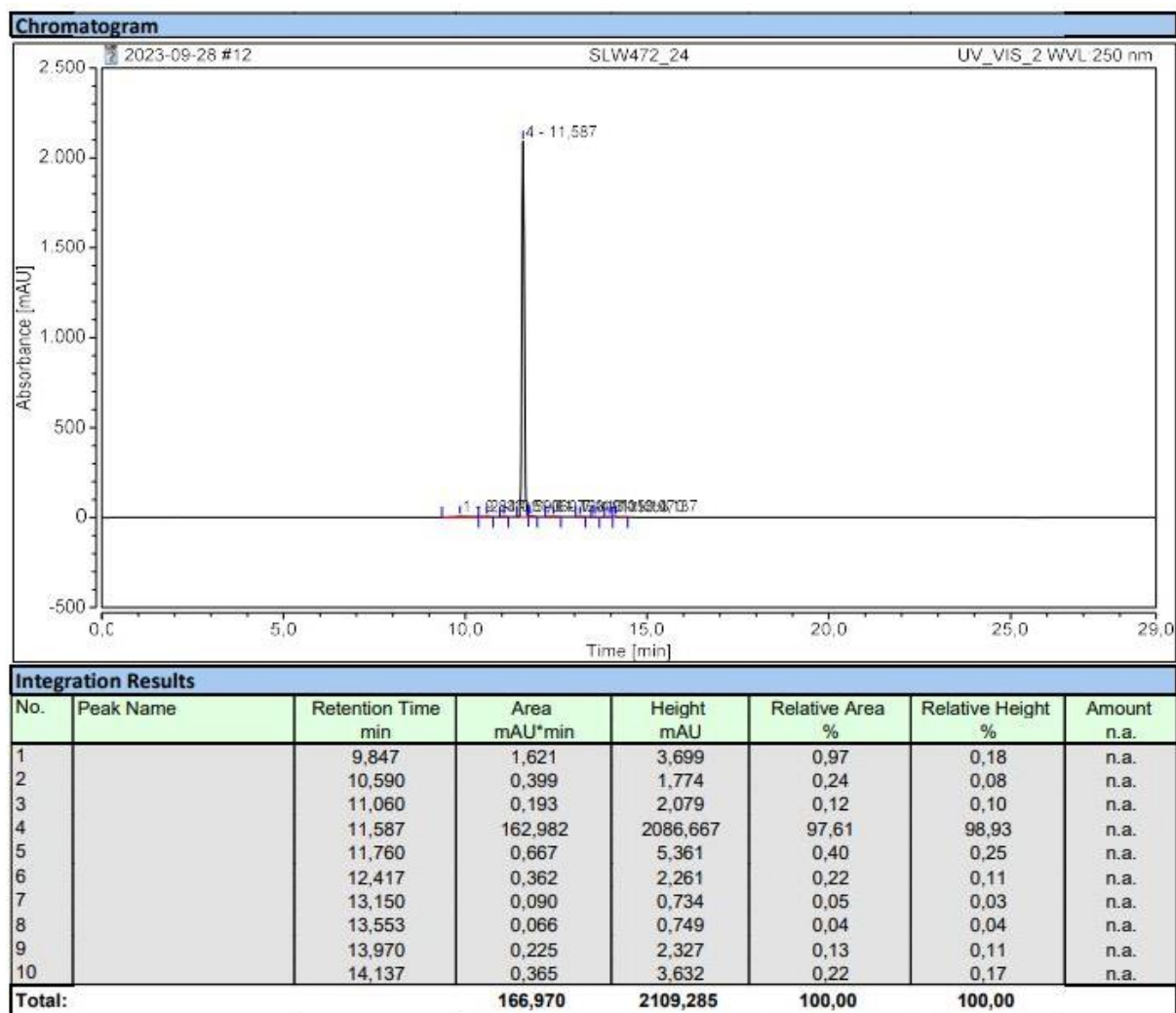


<sup>1</sup>H-NMR spectrum of **27j**

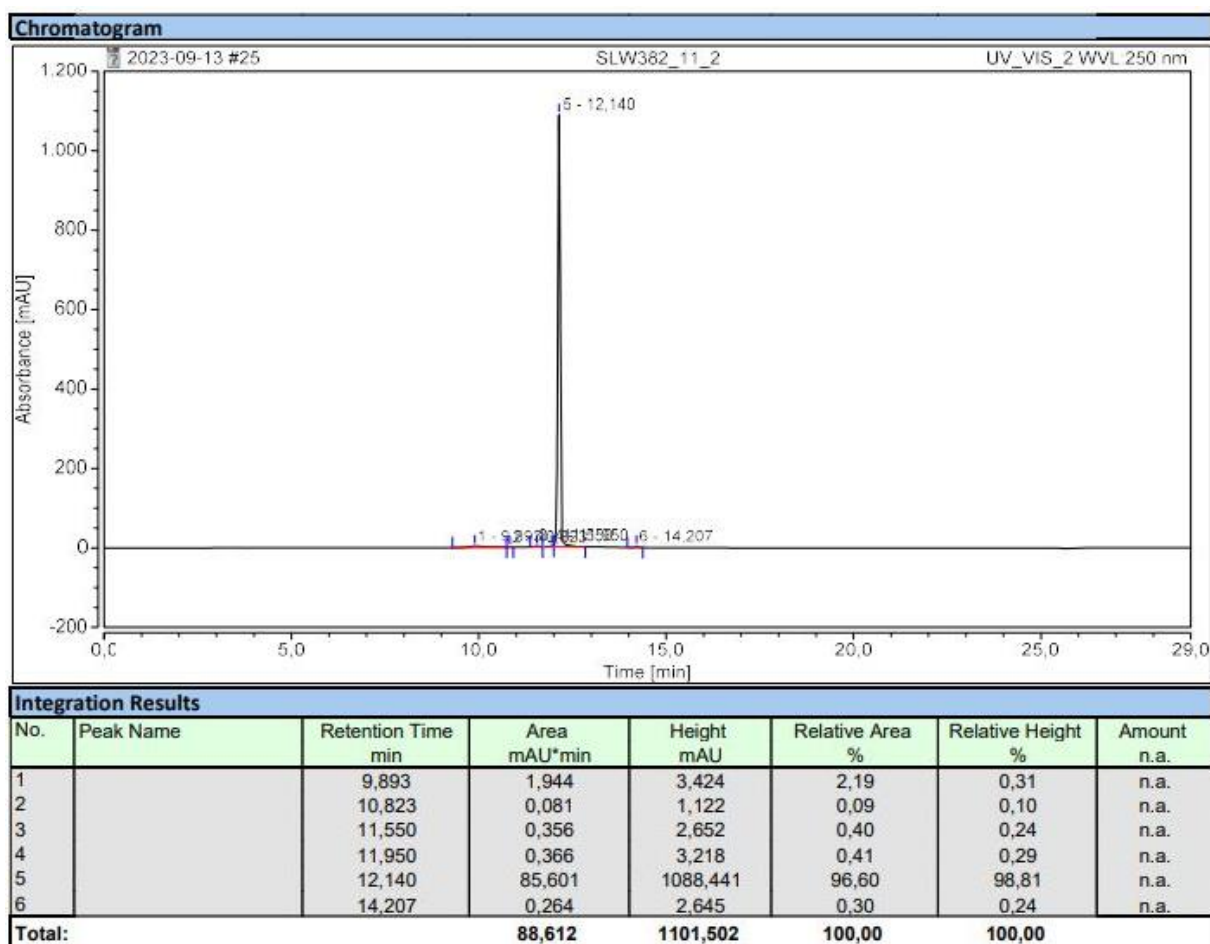


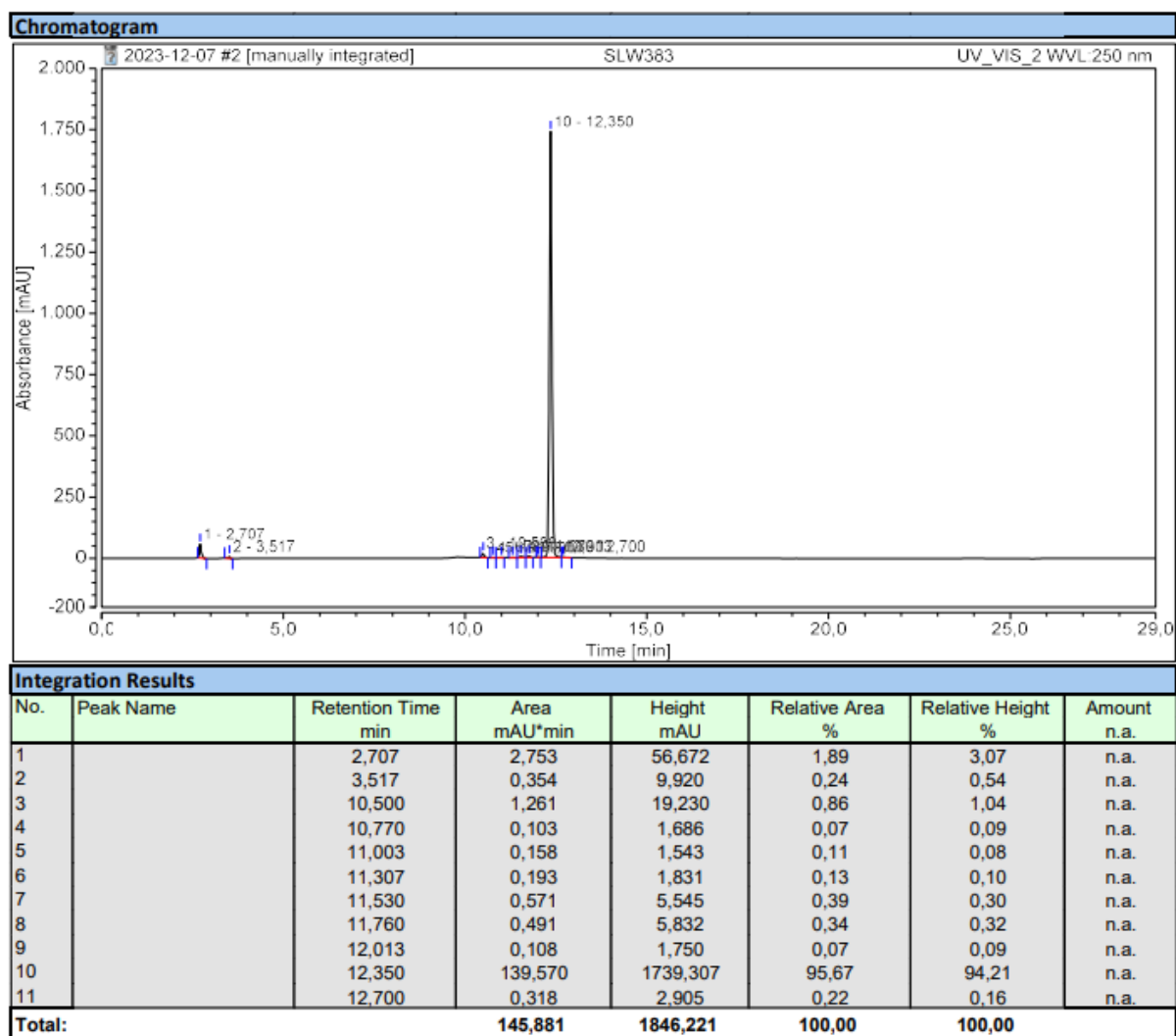
## Supplementary HPLC chromatograms

HPLC chromatogram of **27a**



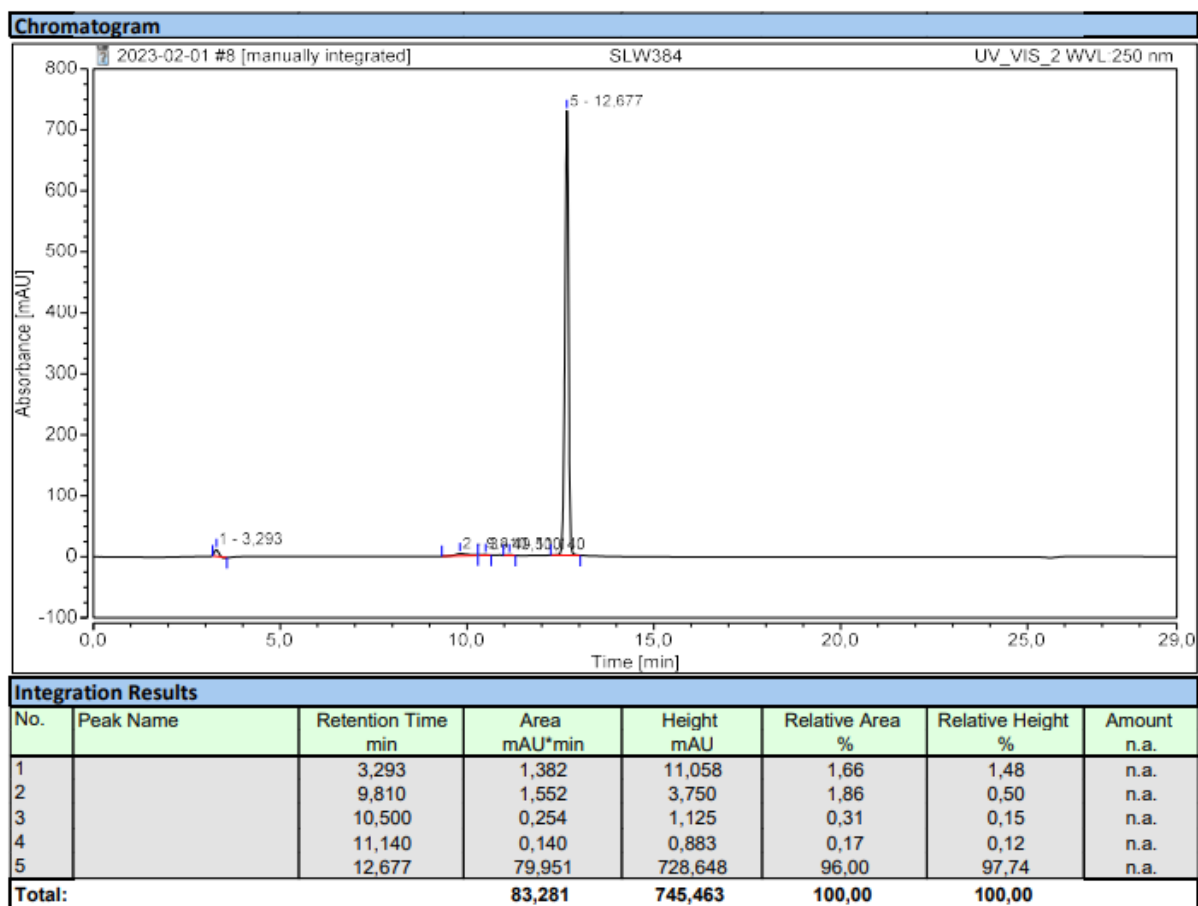
# HPLC chromatogram of 27b



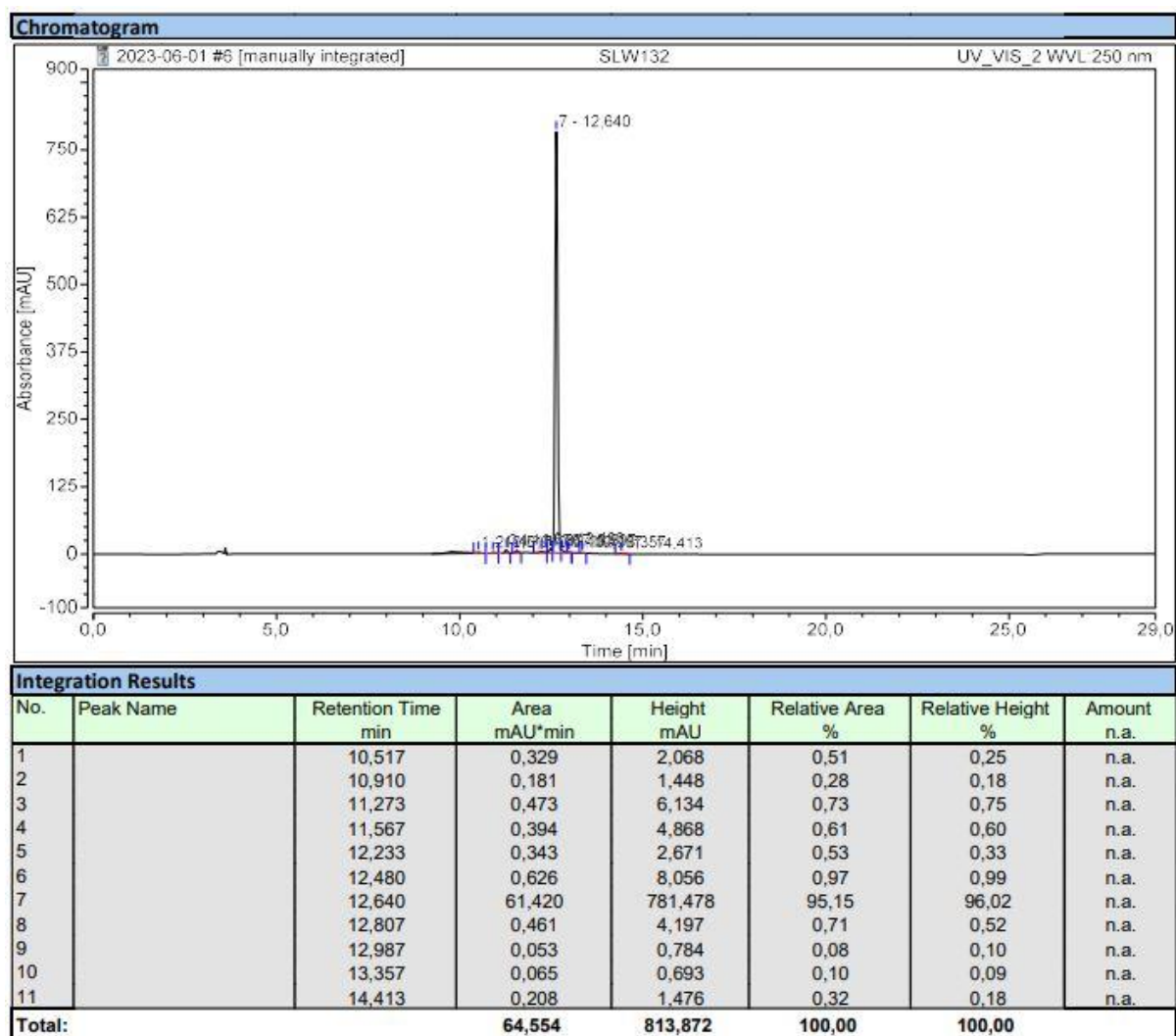
HPLC chromatogram of **27c**


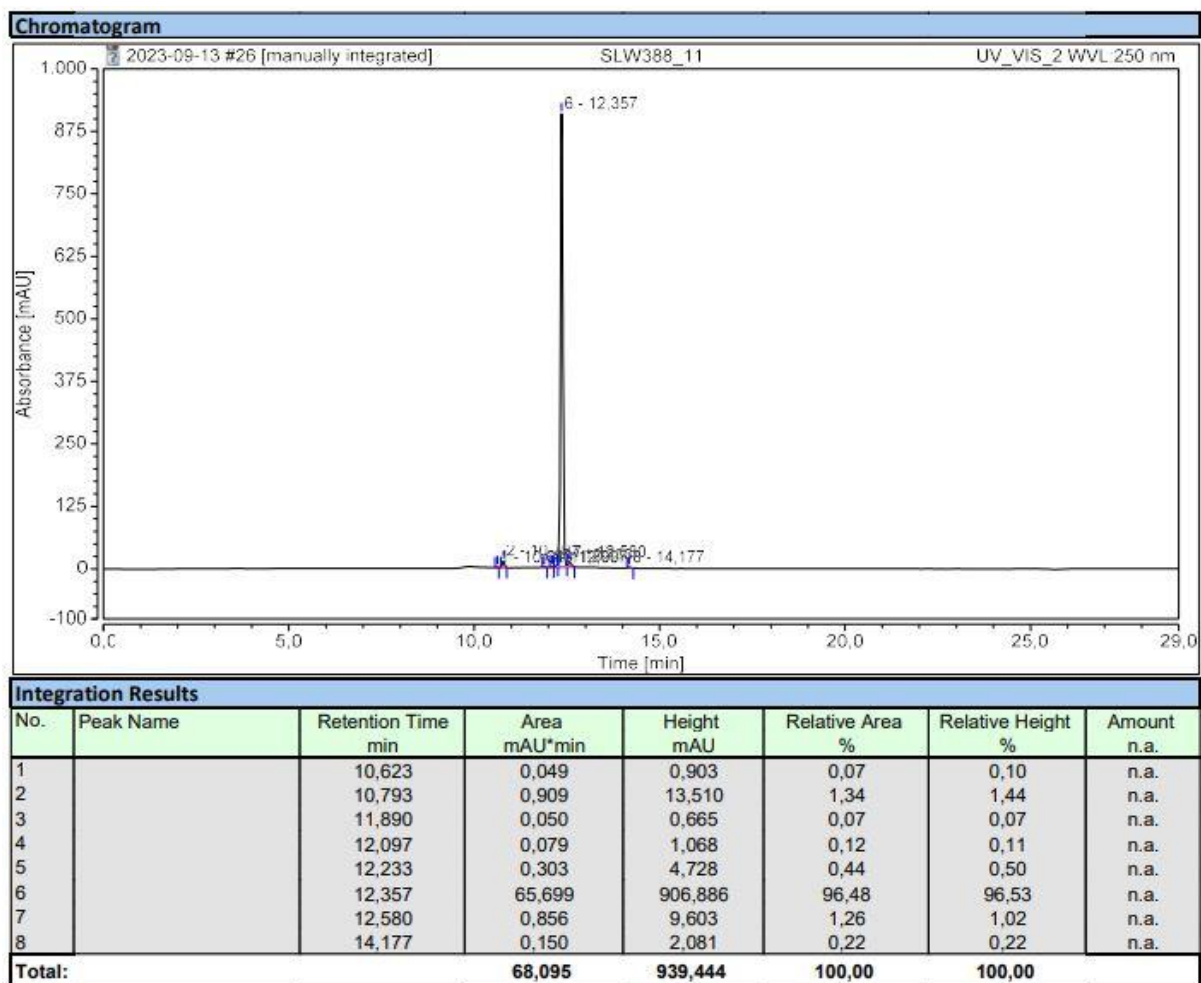


# HPLC chromatogram of 27d

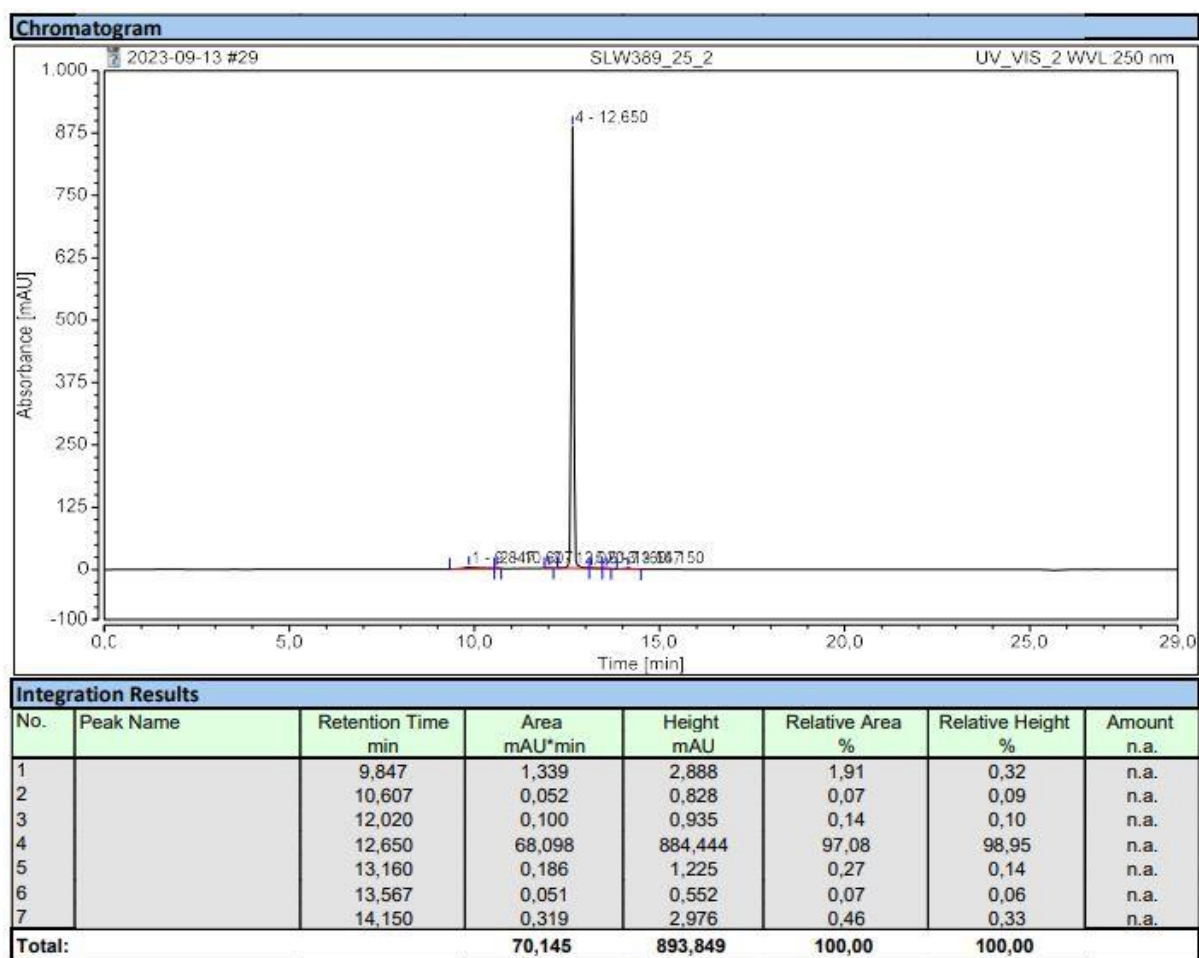


HPLC chromatogram of **27e** (= 10)

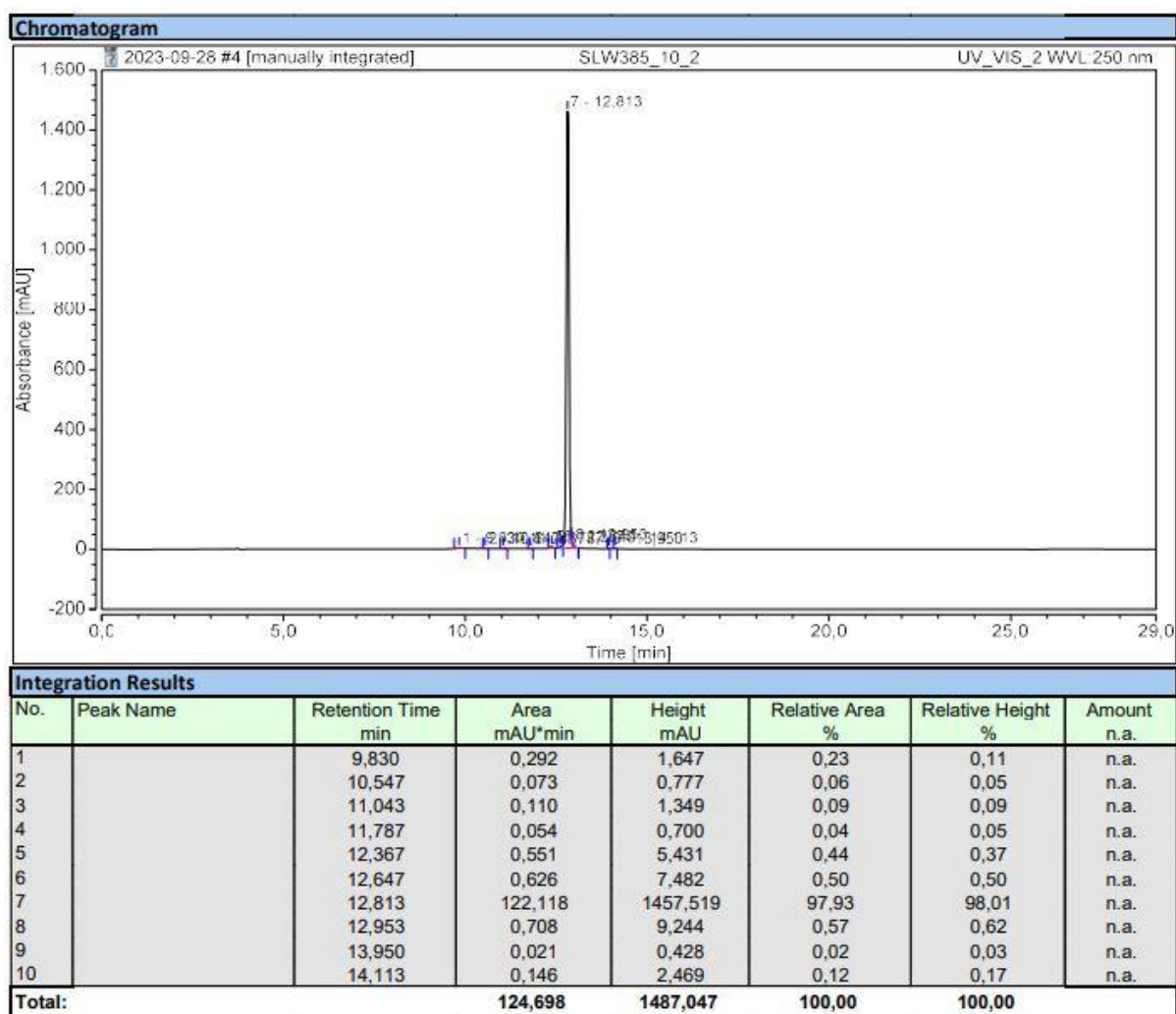


HPLC chromatogram of **27f**


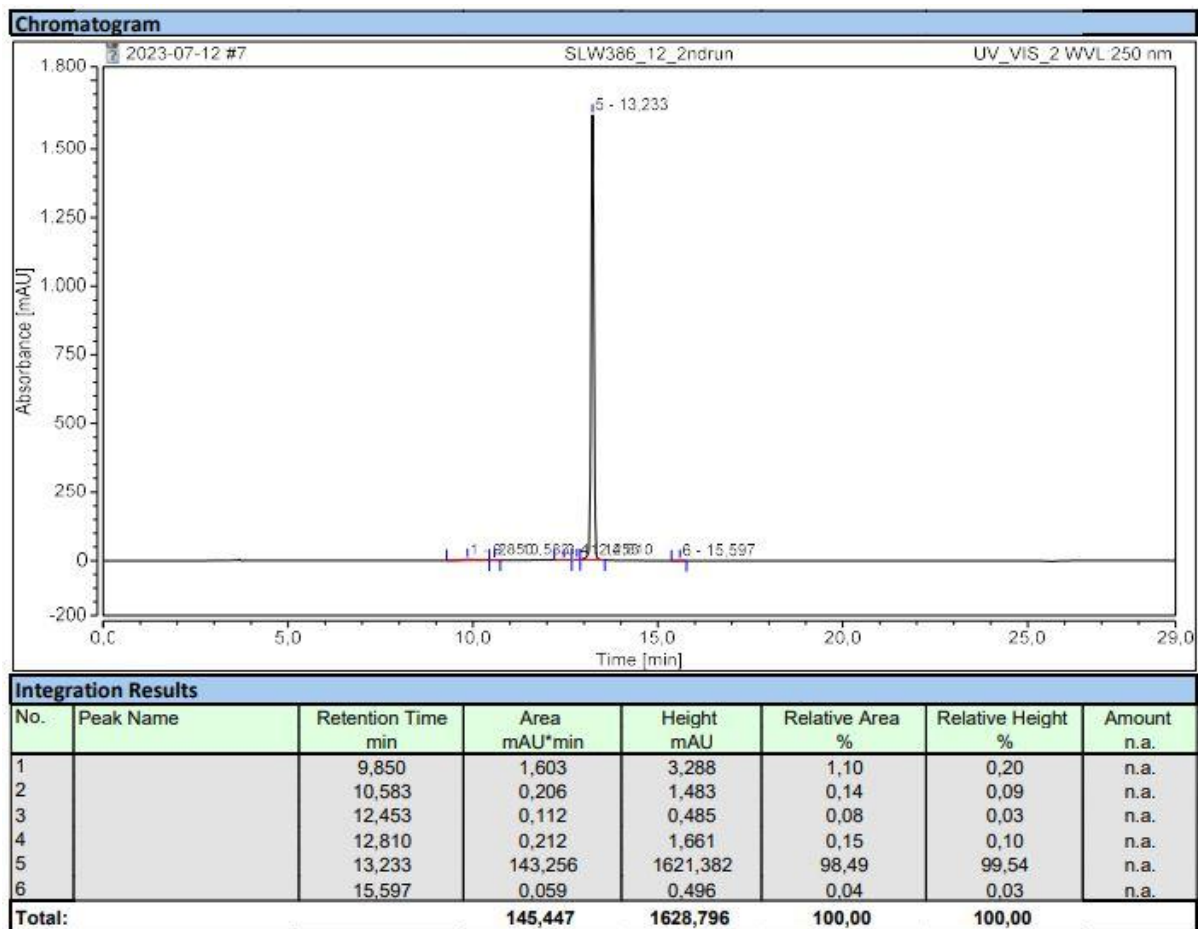
# HPLC chromatogram of 27g



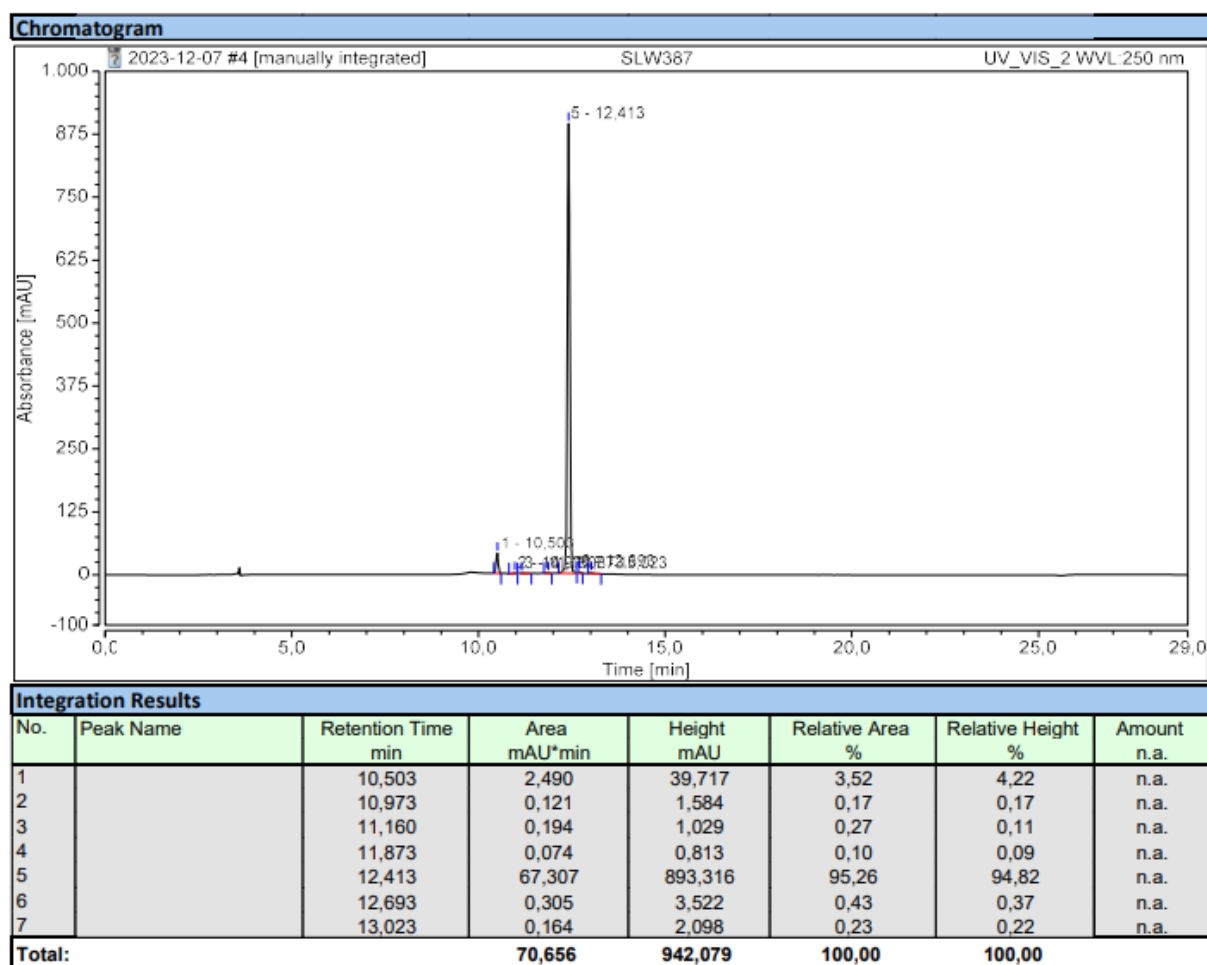
# HPLC chromatogram of 27h



# HPLC chromatogram of 27i



# HPLC chromatogram of 27j



## Supplementary References

- [1] M. E. Huber, S. Wurnig, L. Toy, C. Weiler, N. Merten, E. Kostenis, F. K. Hansen, M. Schiedel, *J. Med. Chem.* **2023**, *66*, 9916-9933.
- [2] L. Toy, M. E. Huber, M. F. Schmidt, D. Weikert, M. Schiedel, *ACS Chem. Biol.* **2022**, *17*, 2142-2152.
- [3] M. E. Huber, L. Toy, M. F. Schmidt, H. Vogt, J. Budzinski, M. F. J. Wiefhoff, N. Merten, E. Kostenis, D. Weikert, M. Schiedel, *Angew. Chem. Int. Ed.* **2022**, *61*, e202116782.
- [4] A. S. Hashmi, T. Haffner, W. Yang, S. Pankajakshan, S. Schafer, L. Schultes, F. Rominger, W. Frey, *Chem. - Eur. J.* **2012**, *18*, 10480-10486.
- [5] A. S. K. Hashmi, S. Schäfer, J. W. Bats, W. Frey, F. Rominger, *Eur. J. Org. Chem.* **2008**, *2008*, 4891-4899.
- [6] M. P. Dwyer, Y. Yu, J. Chao, C. Aki, J. Chao, P. Biju, V. Girijavallabhan, D. Rindgen, R. Bond, R. Mayer-Ezel, J. Jakway, R. W. Hipkin, J. Fossetta, W. Gonsiorek, H. Bian, X. Fan, C. Terminelli, J. Fine, D. Lundell, J. R. Merritt, L. L. Rokosz, B. Kaiser, G. Li, W. Wang, T. Stauffer, L. Ozgur, J. Baldwin, A. G. Taveras, *J. Med. Chem.* **2006**, *49*, 7603-7606.
- [7] J. R. Merritt, L. L. Rokosz, K. H. Nelson, Jr., B. Kaiser, W. Wang, T. M. Stauffer, L. E. Ozgur, A. Schilling, G. Li, J. J. Baldwin, A. G. Taveras, M. P. Dwyer, J. Chao, *Bioorg. Med. Chem. Lett.* **2006**, *16*, 4107-4110.
- [8] A. G. Taveras, C. J. Aki, J. Chao, M. Dwyer, J. Chao, Y. Yu, J. R. Merritt, P. Biju, J. Jakway, G. Lai, M. Wu, E. A. Hecker, D. Lundell, J. S. Fine, 3,4-Di-substituted cyclobutene-1,2-diones as CXC-chemokine receptor ligands. WO2004011418 A, **2004**.
- [9] O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **1951**, *193*, 265-275.
- [10] D. G. Gibson, L. Young, R. Y. Chuang, J. C. Venter, C. A. Hutchison, 3rd, H. O. Smith, *Nat. Methods* **2009**, *6*, 343-345.
- [11] M. P. Hall, J. Unch, B. F. Binkowski, M. P. Valley, B. L. Butler, M. G. Wood, P. Otto, K. Zimmerman, G. Vidugiris, T. Machleidt, M. B. Robers, H. A. Benink, C. T. Eggers, M. R. Slater, P. L. Meisenheimer, D. H. Klaubert, F. Fan, L. P. Encell, K. V. Wood, *ACS Chem. Biol.* **2012**, *7*, 1848-1857.
- [12] A. Allikalt, N. Purkayastha, K. Flad, M. F. Schmidt, A. Tabor, P. Gmeiner, H. Hübner, D. Weikert, *Sci. Rep.* **2020**, *10*, 21842.
- [13] a) R. Luis, G. D'Uonolo, C. B. Palmer, M. Meyrath, T. Uchanski, M. Wantz, B. Rogister, B. Janji, A. Chevigne, M. Szpakowska, *Methods Cell Biol.* **2022**, *169*, 279-294; b) M. Meyrath, M. Szpakowska, J. Zeiner, L. Massotte, M. P. Merz, T. Benkel, K. Simon, J. Ohnmacht, J. D. Turner, R. Kruger, V. Seutin, M. Ollert, E. Kostenis, A. Chevigne, *Nat. Commun.* **2020**, *11*, 3033; c) A. S. Dixon, M. K. Schwinn, M. P. Hall, K. Zimmerman, P. Otto, T. H. Lubben, B. L. Butler, B. F. Binkowski, T. Machleidt, T. A. Kirkland, M. G. Wood, C. T. Eggers, L. P. Encell, K. V. Wood, *ACS Chem. Biol.* **2016**, *11*, 400-408.
- [14] J. C. Shelley, A. Cholleti, L. L. Frye, J. R. Greenwood, M. R. Timlin, M. Uchimaya, *J. Comput. Aided Mol. Des.* **2007**, *21*, 681-691.
- [15] C. Lu, C. Wu, D. Ghoreishi, W. Chen, L. Wang, W. Damm, G. A. Ross, M. K. Dahlgren, E. Russell, C. D. Von Bargen, R. Abel, R. A. Friesner, E. D. Harder, *J. Chem. Theory Comput.* **2021**, *17*, 4291-4300.
- [16] K. Liu, L. Wu, S. Yuan, M. Wu, Y. Xu, Q. Sun, S. Li, S. Zhao, T. Hua, Z. J. Liu, *Nature* **2020**, *585*, 135-140.
- [17] G. M. Sastry, M. Adzhigirey, T. Day, R. Annabhimoju, W. Sherman, *J. Comput. Aided Mol. Des.* **2013**, *27*, 221-234.
- [18] R. A. Friesner, J. L. Banks, R. B. Murphy, T. A. Halgren, J. J. Klicic, D. T. Mainz, M. P. Repasky, E. H. Knoll, M. Shelley, J. K. Perry, D. E. Shaw, P. Francis, P. S. Shenkin, *J. Med. Chem.* **2004**, *47*, 1739-1749.
- [19] a) D. A. Hall, I. J. Beresford, C. Browning, H. Giles, *Br. J. Pharmacol.* **1999**, *126*, 810-818; b) S. Wilson, G. Wilkinson, G. Milligan, *J. Biol. Chem.* **2005**, *280*, 28663-28674.
- [20] C. Oswald, M. Rappas, J. Kean, A. S. Dore, J. C. Errey, K. Bennett, F. Deflorian, J. A. Christopher, A. Jazayeri, J. S. Mason, M. Congreve, R. M. Cooke, F. H. Marshall, *Nature* **2016**, *540*, 462-465.
- [21] D. R. Greaves, W. Wang, D. J. Dairaghi, M. C. Dieu, B. Saint-Vis, K. Franz-Bacon, D. Rossi, C. Caux, T. McClanahan, S. Gordon, A. Zlotnik, T. J. Schall, *J. Exp. Med.* **1997**, *186*, 837-844.
- [22] a) Y. Zheng, L. Qin, N. V. Zacarias, H. de Vries, G. W. Han, M. Gustavsson, M. Dabros, C. Zhao, R. J. Cherney, P. Carter, D. Stamos, R. Abagyan, V. Cherezov, R. C. Stevens, I. J. AP, L. H. Heitman, A. Tebben, I. Kufareva, T. M. Handel, *Nature* **2016**, *540*, 458-461; b) K. Jaeger, S. Bruenle, T. Weinert, W. Guba, J. Muehle, T. Miyazaki, M. Weber, A. Furrer, N. Haenggi, T. Tetaz, C. Y. Huang, D. Mattle, J. M. Vonach, A. Gast, A. Kuglstatter, M. G. Rudolph, P. Nogly, J. Benz, R. J. P. Dawson, J. Standfuss, *Cell* **2019**, *178*, 1222-1230; c) X. Liu, S. Ahn, A. W. Kahsai, K. C. Meng, N. R. Latorraca, B. Pani, A. J. Venkatakrishnan, A. Masoudi, W. I. Weis, R. O. Dror, X. Chen, R. J. Lefkowitz, B. K. Kobilka, *Nature* **2017**, *548*, 480-484.