Spy vs. Spy: Selecting the best reporter for $^{19}$F NMR competition experiments

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1. Supplementary figures

Supplementary Figure 1. Biophysical characterization of spy molecule 19 by $^{19}$F NMR and SPR. (a) Measurement of the transverse relaxation rate ($R_2$) of 19 at 100 μM in absence (blue) and in presence (red) of protein at 1 μM. (b) Measurement of the $K_D$ of 19 by SPR.

Supplementary Figure 2. Comparison of the assay window of spy molecules 6, 11 and 19 at the same conditions. Overlay of the main $^{19}$F CPMG peak (40 scans) of spy molecules 6, 11 and 19 at 50 μM in absence (blue) or in presence of VBC 1 μM, highlighting the wider assay window of spy molecule 19. The CPMG delays used for spy molecules 6, 11 and 19 were, respectively, 634, 447 and 345 ms (see section 6 for details regarding the choice of CPMG delay).
Supplementary Figure 3. Determination of the affinities of VHL binders using spy molecule 6. (a) Structure, dissociation constant and R² contrast of spy molecule 6. The C₂ was obtained with spy molecule at 50 µM and VBC at 1 µM. (b) Displacement of spy molecule 6 in presence of different concentrations of five VHL binders (molecules 3, 10, 12, 18 and 22). Data obtained from ¹⁹F CPMG experiments using a CPMG delay of 634 ms and 40 scans.

Supplementary Figure 4. Determination of the affinities of VHL binders using spy molecule 11. (a) Structure, dissociation constant and R² contrast of spy molecule 11. The C₂ was obtained with spy molecule at 50 µM and VBC at 1 µM. (b) Displacement of spy molecule 11 in presence of different concentrations of five VHL binders (molecules 3, 18 and 22). Competitors 10 and 12 were not used in this case because the chemical shifts of their fluorine peaks overlayed with the spy molecule. Data obtained from ¹⁹F CPMG experiments using a CPMG delay of 447 ms and 40 scans.
Supplementary Figure 5. Binders of other sites present in the VBC complex do not displace spy molecule 19. $^{19}$F CPMG NMR peak of spy molecule 19 at different conditions: free in solution (blue), in presence of VBC (red) and in presence of protein and molecules MB756 (green), MB1200 (violet) and MB235 (yellow), binders of other sites present in VBC previously reported.\textsuperscript{(1)} As these compounds bind to sites not targeted by the spy molecule, no displacement was observed, showing that the competition experiment is site specific. Experiments were performed with a CPMG delay of 258 ms and 120 scans.
2. Compound synthesis and characterization

All the compounds, reagents and solvents used, aside from the compounds specifically prepared for this work, were obtained from commercial sources and used without further purification. For compound purification of intermediates, flash column chromatography was performed using a Teledyne Isco CombiFlash Rf or Rf200i, with RediSep Rf Disposable Columns (Normal phase). Where specified, compounds were purified using a Gilson Preparative HPLC System equipped with a Waters X-Bridge C18 column (100 mm x 19 mm; 5 µm particle size) using an eleven minutes gradient (25 mL/min) of: 1) 5% to 95% of acetonitrile : 0.1% formic acid, or 2) 5% to 95% of acetonitrile : 0.1% ammonia.

The NMR characterization was performed either on a Bruker 500 Ultrashield or Bruker Ascend 400 spectrometers. The splitting of the NMR signals are described as follows: singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m) and combinations in case of multiple signal splitting. Chemical shifts are described as parts per million (ppm) and coupling constants (J) were calculated in hertz (Hz). The proton (^1H) and carbon (^13C) spectra were referenced as follows: d6-Chloroform – CDCl3 (δ_H = 7.26 ppm / δ_C = 77.1 ppm) and d5-Methanol – CD3OD (δ_H = 3.34 ppm / δ_C = 49.1 ppm). For compounds where amide rotamers could be observed, just the signal of the major rotamer was listed.

Reactions were monitored using an Agilent Technologies 1200 series analytical HPLC connected to an Agilent Technologies 6130 quadrupole LC/MS containing an Agilent diode array detector and a Waters XBridge column (50 mm × 2.1 mm, 3.5 µm particle size) for separation of the compounds. Samples were eluted with a 3 minutes gradient of 5% to 95% acetonitrile : 0.1% formic acid.

Abbreviations: ACN (acetonitrile), DCM (dichloromethane), DIPEA (N,N-diisopropylethylamine), DMA (dimethylacetamide), DMF (N,N-dimethylformamide), EtOAc (ethyl acetate), Et2O (diethyl ether), HATU (1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate), MgSO4 (Magnesium sulphate), MeOH (methanol), THF (Tetrahydrofuran) and TEA (triethylamine).
For describing the synthesis and characterisation of spy molecules and intermediates, compounds were numbered as shown in the two schemes below. The respective compound numbering in the main text is indicated where applicable (e.g. compound S3a corresponds to spy molecule 1 in the main text, and so on).

Scheme 2.1 Synthesis of spy molecules with a trifluoromethyl modification on an aromatic position.

Scheme 2.2 Synthesis of spy molecules with a trifluoromethyl modification on an aliphatic position.

Compounds S1a, S1c and S8a were purchased from commercial sources and used without further purification. The spectroscopic characterization and yields for intermediates S7b, S8b and S9b can be found elsewhere, as these were previously prepared by our group,[2] while all the remaining compounds were synthesized and characterized as described below. For the NMR characterization of compounds S3a-d and S9a-b, either a chloride or a formate salt was obtained depending on the purification strategy used.
General procedure i. Coupling of aryl bromides with 4-methylthiazole – Synthesis of S1b, S1d and S7b

The aryl bromide (1 equiv.) was dissolved in dimethylacetamide (3 mL per mmol of bromide), followed by the sequential addition of 4-methylthiazole (2 equiv.), potassium acetate (2 equiv.) and palladium (II) acetate (0.02 equiv.). The reaction was stirred for approximately two hours at 150 °C under nitrogen atmosphere. The mixture was extracted with brine and dichloromethane. Combined organic phases were concentrated and DMA was removed in the vacuum pump. The desired product was then purified by flash column chromatography with an increasing elution of ethyl acetate (0-100%) in heptane.

4-(4-methylthiazol-5-yl)-3-(trifluoromethyl)benzonitrile (S1b):

\[
\begin{array}{c}
\text{N} \\
\text{C} \\
\text{F}_3 \\
\text{S} \\
\text{N}
\end{array}
\]

Prepared from 0.3163 g (1.3 mmol) of the respective bromide (S1a), resulting in 0.221 g (0.8 mmol, 65%) of the desired product as a white solid. \( ^1H \) NMR (CDCl\(_3\), 400 MHz) \( \delta \): 8.84 (s, 1H); 8.08 (d, \( J_{\text{H-H}} = 1.4 \) Hz, 1H); 7.89 (dd, \( J_{\text{H-H}} = 7.9, 1.4 \) Hz, 1H); 7.54 (d, \( J_{\text{H-H}} = 7.9 \) Hz, 1H); 2.25 (s, 3H); \( ^{13}C \) NMR (CDCl\(_3\), 100 MHz) \( \delta \): 152.75, 152.15, 136.04 (q, \( ^3J_{\text{C-F}} = 1.7 \) Hz), 135.06, 134.93, 132.37 (q, \( ^2J_{\text{C-F}} = 31.3 \) Hz), 130.36 (q, \( ^3J_{\text{C-F}} = 5.4 \) Hz), 125.06, 122.56 (q, \( ^1J_{\text{C-F}} = 274.6 \) Hz), 117.16, 113.76, 15.72; \( ^{19}F \) NMR (CDCl\(_3\), 470 MHz) \( \delta \): -60.10.

4-(4-methylthiazol-5-yl)-2-(trifluoromethyl)benzonitrile (S1d):

\[
\begin{array}{c}
\text{N} \\
\text{C} \\
\text{F}_3 \\
\text{S} \\
\text{N}
\end{array}
\]

Prepared from 0.8932 g (3.6 mmol) of the respective bromide (S1c), resulting in 0.555 g (2.2 mmol, 61%) of the desired product as a bright yellow solid. \( ^1H \) NMR (CDCl\(_3\), 400 MHz): 8.81 (s, 1H); 7.91 (d, \( J_{\text{H-H}} = 8.08 \) Hz, 1H); 7.86 (d, \( J_{\text{H-H}} = 1.5 \) Hz, 1H); 7.76 (dd, \( J_{\text{H-H}} = 1.5, 8.08 \) Hz, 1H); 2.59 (s, 3H); \( ^{13}C \) NMR (CDCl\(_3\), 125 MHz): 152.49, 151.25, 137.69, 135.29, 133.61 (q, \( ^2J_{\text{C-F}} = 32.8 \) Hz), 132.50, 128.96, 127.27 (q, \( ^3J_{\text{C-F}} = 4.5 \) Hz), 122.28 (q, \( ^1J_{\text{C-F}} = 273.8 \) Hz), 115.32, 109.14, 16.58; \( ^{19}F \) NMR (CDCl\(_3\), 470 MHz) \( \delta \): -60.10.

4-(4-methylthiazol-5-yl)benzonitrile (S7b):

\[
\begin{array}{c}
\text{N} \\
\text{C} \\
\text{S} \\
\text{N}
\end{array}
\]

Previously prepared and characterized.[2]
**General procedure ii. Reduction of nitriles to amines – Synthesis of S2a-d and S8b**

To a stirring solution of the nitrile (1 equiv.) in THF under nitrogen atmosphere, was added a solution of LiAlH₄ (1 equiv. from a 1M solution in THF). After an overnight period, the mixture was cooled down in an ice bath and diluted with diethyl ether. Water was then slowly added (1 μL per mg of LiAlH₄ added), followed by the addition of 15% NaOH (1 μL per mg of LiAlH₄ added) and once more water (3 μL per mg of LiAlH₄ added). The ice bath was removed and the mixture. MgSO₄ was added and after 15 additional minutes stirring the mixture was filtered, then extracted with HCl solution (pH ≈ 2). The organic phase was discarded and the pH of the aqueous phase was raised to 12 by adding NaOH 1 M. This solution was extracted three times with DCM and the organic phase was concentrated and purified (purification strategy specified for each compound).

**4-bromo-3-(trifluoromethyl)phenyl)methanaminium formate (S2a):**

Prepared from 0.264 g (1.06 mmol) of the respective nitrile (S1a), resulting in 0.110 g (0.35 mmol, 35%) of the desired product as a white solid. Compound obtained as a formate salt after purification in reverse phase HPLC using a 5 to 95% gradient of formic acid 0.1% and acetonitrile. ¹H NMR (CD₃OD, 400 MHz) δ: 8.55 (Formic acid), 7.95-7.89 (m, 2H), 7.64-7.60 (m, 1H), 4.19-4.16 (s, 2H); ¹³C NMR (CD₃OD, 100 MHz) δ: 170.08 (Formic acid), 137.05, 136.10, 135.20, 131.73 (q, J_C-F = 31.2 Hz), 129.66 (q, J_C-F = 5.2 Hz), 124.34 (q, J_C-F = 273.3 Hz), 121.31, 43.57; ¹⁹F NMR (CD₃OD, 470 MHz) δ: -64.05 (CF₃).

**4-(4-methylthiazol-5-yl)-3-(trifluoromethyl)phenyl)methanaminium formate (S2b):**

Prepared from 0.221 g (0.82 mmol) of the respective nitrile (S1b), resulting in 0.092 g (0.29 mmol, 36%) of the desired product as a yellow solid. Compound obtained as a formate salt after purification in reverse phase HPLC using a 5 to 95% gradient of formic acid 0.1% and acetonitrile. ¹H NMR (CD₃OD, 500 MHz) δ: 9.04 (s, 1H), 8.02-7.99 (s, 1H), 7.82-7.79 (d, J_H-H = 7.9 Hz, 1H), 7.58-7.55 (d, J_H-H = 7.9 Hz, 1H), 4.26 (s, 2H), 2.22 (s, 3H); ¹⁹F NMR (CD₃OD, 470 MHz) δ: -62.36 (CF₃).

**4-bromo-2-(trifluoromethyl)phenyl)methanaminium formate (S2c):**

Prepared from 0.523 g (2.09 mmol) of the respective nitrile (S1c), resulting in 0.195 g (0.65 mmol, 31%) of the desired product as a white solid. Compound obtained as a formate salt after purification in reverse phase HPLC
using a 5 to 95% gradient of formic acid 0.1% and acetonitrile. $^1$H NMR (CD$_3$OD, 500 MHz) δ: 8.52 (Formic acid), 7.98-7.97 (d, $J_{HH} = 1.6$ Hz, 1H), 7.95-7.92 (dd, $J_{HH} = 1.6$, 8.3 Hz, 1H), 7.65-7.62 (d, $J_{HH} = 8.3$ Hz, 1H), 4.24 (s, 2H); $^{13}$C NMR (CD$_3$OD, 125 MHz) δ: 169.65 (Formic acid), 137.32, 133.94, 133.75, 131.55 (q, $^2 J_{CF} = 31.2$), 130.71 (q, $^3 J_{CF} = 5.8$), 124.76 (q, $^1 J_{CF} = 273.62$), 124.04, 40.71 (q, $^4 J_{CF} = 2.68$); $^{19}$F NMR (CD$_3$OD, 470 MHz) δ: -61.08 (CF$_3$).

(4-(4-methylthiazol-5-yl)-2-(trifluoromethyl)phenyl)methanamine (S2d):

![Chemical structure](image)

Prepared from 0.398 g (1.49 mmol) of the respective nitrile (S1d), resulting in 0.157 g (0.49 mmol, 33%) of the desired product as a yellow solid. Compound obtained as a free amine after purification in reverse phase HPLC using a 5 to 95% gradient of ammonia 0.1% and acetonitrile. $^1$H NMR (CD$_3$OD, 500 MHz) δ: 8.96 (s, 1H), 7.84-7.76 (m, 3H), 4.05 (s, 2H), 2.53 (s, 3H); $^{13}$C NMR (CD$_3$OD, 100 MHz) δ: 153.72, 150.26, 142.43, 134.38, 132.24, 131.86, 131.77, 129.35 (q, $^2 J_{CF} = 30.2$ Hz), 127.50 (q, $^3 J_{CF} = 30.2$ Hz), 125.81 (q, $^1 J_{CF} = 273.7$ Hz), 42.83, 15.87; $^{19}$F NMR (CD$_3$OD, 470 MHz) δ: -59.67 (CF$_3$).

(4-(4-methylthiazol-5-yl)phenyl)methanamine (S8b):

![Chemical structure](image)

Previously prepared and characterized.[2]

**General procedure iii. Amide coupling with Boc-L-hydroxyproline and deprotection – Synthesis of S3a-d and S9a-b**

To a solution of amine (1 equiv.) in DMF, Boc-L-hydroxyproline (1 equiv.) was added and the mixture was stirred at room temperature. DIPEA (2 equiv.) was added dropwise and the mixture was stirred for 5 minutes at room temperature. HATU (1.1 equiv.) was added and the mixture was stirred at room temperature for 30-90 minutes (LCMS monitoring). Water was added and the mixture was extracted with ethyl acetate. The combined organic phases were washed with brine, dried over MgSO$_4$ and evaporated under reduced pressure to give the corresponding crude, which was purified by flash column chromatography with an increasing gradient of DCM and 20% MeOH in DCM to yield the desired product. The Boc protected compound was dissolved in DCM, followed by the dropwise addition of a 4M HCl solution in dioxane (at least 3 equiv.). Often an insoluble precipitate starts to be formed. A few drops of MeOH were added to make the solution homogeneous, being kept stirring for approximately one hour. The DCM and the HCl were removed by flushing nitrogen into the solution and residual solvents were evaporated under reduced pressure. To remove traces of impurities, compounds S3a, S3b, S3d and S9a were furtherly purified by preparative HPLC, being obtained as a formate salt, while the remaining compounds were obtained as a chloride salt.
(2S,4R)-2-((4-bromo-3-(trifluoromethyl)benzyl)carbamoyl)-4-hydroxyprrolidin-1-ium formate (S3a - spy molecule 1):

Prepared from 198 mg (0.78 mmol) of amine S2a, resulting in 248 mg (0.60 mmol, 78%) of the desired product as a white solid. Compound obtained as a formate salt after purification in reverse phase HPLC using a 5 to 95% gradient of formic acid 0.1% and acetonitrile. HRMS (ESI) [M+H]+ (m/z): Calculated for C13H14BrF3N2O2: 367.0264; Observed: 367.0299; 1H NMR (CD3OD, 400 MHz) δ: 8.50 (Formic acid), 7.80 (d, JH-H = 8.2 Hz, 1H), 7.74 (d, JH-H = 1.7 Hz, 1H), 7.49 (dd, JH-H = 1.7, 8.2 Hz, 1H), 4.58 (m, 1H), 4.49 (s, 2H), 4.42 (dd, JH-H = 7.6, 10.3 Hz, 1H), 3.38 (dd, JH-H = 3.7, 12.1 Hz, 1H), 3.26 (d, JH-H = 12.1 Hz, 1H), 2.44 (dd, JH-H = 1.5, 7.6, 13.4 Hz, 1H), 2.04 (ddd, JH-H = 4.2, 10.3, 13.4 Hz, 1H); 13C NMR (CD3OD, 100 MHz) δ: 171.98, 170.29 (Formic acid), 140.99, 137.34, 134.74, 131.96 (q, JCF = 31.3 Hz), 129.06 (q, JCF = 5.6 Hz), 120.22, 72.47, 60.96, 56.09, 44.19, 40.96; 19F NMR (CD3OD, 470 MHz) δ: -64.01.

(2S,4R)-2-((4-(4-methylthiazol-5-yl)-3-(trifluoromethyl)benzyl)carbamoyl)-4-hydroxyprrolidin-1-ium formate (S3b - spy molecule 5):

Prepared from 179 mg (0.56 mmol) of amine S2b, resulting in 203 mg (0.47 mmol, 84%) of the desired product as a white solid. Compound obtained as a formate salt after purification in reverse phase HPLC using a 5 to 95% gradient of formic acid 0.1% and acetonitrile. MS [M+H]+ (m/z): 386.1; 1H NMR (CD3OD, 400 MHz) δ: 9.02 (s, 1H), 8.51 (Formic acid), 7.81 (s, 1H), 7.66 (d, JH-H = 7.9 Hz, 1H), 7.46 (d, JH-H = 7.9 Hz, 1H), 4.58 (s, 2H), 4.55 (m, 1H), 4.35 (dd, JH-H = 7.7, 9.9 Hz, 1H), 3.30 (m, 1H), 3.20 (dt, JH-H = 1.4, 12.0 Hz, 1H), 2.41 (ddt, JH-H = 1.5, 7.6, 13.3 Hz, 1H), 2.20 (s, 3H), 2.03 (ddd, JH-H = 4.3, 9.9, 13.4 Hz, 1H); 13C NMR (CD3OD, 100 MHz) δ: 172.51, 169.65 (Formic acid), 154.51, 152.18, 142.12, 135.37, 132.41, 131.77 (q, JCF = 30.1 Hz), 130.17, 128.59, 126.72 (q, JCF = 5.6 Hz), 125.19 (q, JCF = 273.7 Hz), 72.11, 60.40, 55.55, 43.62, 40.41, 15.33; 19F NMR (CD3OD, 470 MHz) δ: -64.01.

(2S,4R)-2-((4-bromo-2-(trifluoromethyl)benzyl)carbamoyl)-4-hydroxyprrolidin-1-ium chloride (S3c - spy molecule 9):
Prepared from 11 mg (0.043 mmol) of amine S2c, resulting in 14 mg (0.035 mmol, 80%) of the desired product as a white solid. HRMS (ESI) [M+H]+ (m/z): Calculated for C_{13}H_{14}BrF_{3}N_{2}O_{2}: 367.0264; Observed: 367.0280; \(^1\)H NMR (CD_{3}OD, 500 MHz) δ: 7.88-7.85 (d, J_{H-H} = 1.8 Hz, 1H), 7.82-7.79 (dd, J_{H-H} = 1.8, 8.4 Hz, 1H), 7.47-7.44(d, J_{H-H} = 8.4 Hz, 1H), 4.61-4.53 (m, 2H), 4.41-3.48 (m, 1H), 4.00-3.76 (t, J_{H-H} = 8.3 Hz, 1H), 3.06-3.01 (dd, J_{H-H} = 4.0, 12.0 Hz, 1H), 2.96-2.91 (dt, J_{H-H} = 1.8, 12.0 Hz, 1H), 2.23-2.17 (ddt, J_{H-H} = 1.8, 8.0, 13.4 Hz, 1H), 1.91-1.85 (ddd, J_{H-H} = 5.0, 8.7, 13.4 Hz, 1H); \(^{13}\)C NMR (CD_{3}OD, 125 MHz) δ: 177.58, 137.72, 136.72, 132.33, 130.63 (q, \(^{2}\)J_{C-F} = 31.5 Hz), 130.09 (q, \(^{3}\)J_{C-F} = 5.8 Hz), 125.02 (q, \(^{1}\)J_{C-F} = 273.6 Hz), 122.07, 73.67, 60.82, 56.15, 40.99, 40.19 (q, \(^{4}\)J_{C-F} = 2.9 Hz); \(^{19}\)F NMR (CD_{3}OD, 470 MHz) δ: -61.86 (CF_{3}).

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(2S,4R)-2-((4-(methylthiazol-5-yl)-2-(trifluoromethyl)benzyl)carbamoyl)-4-hydroxyprrolidin-1-ium chloride (S3d - spy molecule 13):

Prepared from 164 mg (0.60 mmol) of amine S2d, resulting in 239 mg (0.55 mmol, 92%) of the desired product as a white solid. Compound obtained as a formate salt after purification in reverse phase HPLC using a 5 to 95% gradient of formic acid 0.1% and acetonitrile. HRMS (ESI) [M+H]+ (m/z): Calculated for C_{17}H_{13}F_{3}N_{2}O_{2}S: 386.1145; Observed: 386.1168; \(^1\)H NMR (CD_{3}OD, 500 MHz) δ: 8.98 (s, 1H), 8.55 (Formic acid), 7.80 (s, 1H), 7.77 (dd, J_{H-H} = 1.4, 8.1 Hz, 1H), 7.66 (d, J_{H-H} = 8.1 Hz, 1H), 4.69 (m, 2H), 4.48 (m, 1H), 4.18 (t, J_{H-H} = 8.7 Hz, 1H), 3.18 (dd, J_{H-H} = 3.5, 11.7 Hz, 1H), 3.07 (d, J_{H-H} = 12.1 Hz, 1H), 2.53 (s, 3H), 2.32 (ddt, J_{H-H} = 1.6, 7.8, 13.5 Hz, 1H), 1.98 (ddd, J_{H-H} = 4.7, 9.3, 13.5 Hz, 1H); \(^{13}\)C NMR (CD_{3}OD, 125 MHz) δ: 174.57, 170.18 (Formic acid), 153.88, 150.39, 138.01, 134.30, 132.82, 131.65, 131.37, 129.60 (q, \(^{2}\)J_{C-F} = 30.9 Hz), 127.78 (q, \(^{3}\)J_{C-F} = 5.9 Hz), 125.64 (q, \(^{1}\)J_{C-F} = 273.4 Hz), 72.77, 60.56, 55.82, 40.64, 15.87; \(^{19}\)F NMR (CD_{3}OD, 470 MHz) δ: -60.11.

(2S,4R)-2-((4-bromobenzyl)carbamoyl)-4-hydroxyprrolidin-1-ium chloride (S9a):

Prepared from 182 mg (1.00 mmol) of amine S8a, resulting in 294 mg (0.47 mmol, 84%) of the desired product as a white solid. Compound obtained as a formate salt after purification in reverse phase HPLC using a 5 to 95% gradient of formic acid 0.1% and acetonitrile. \(^1\)H NMR (CD_{3}OD, 500 MHz) δ: 7.51-7.47 (m, 2H), 7.26-7.23 (m, 2H), 4.52-4.49 (m, 1H), 4.41-3.8 (s, 2H), 4.27-4.23 (dd, J_{H-H} = 7.9, 9.4 Hz, 1H), 3.27-3.23 (dd, J_{H-H} = 3.8, 12.1
(2S,4R)-4-hydroxy-2-((4-(4-methylthiazol-5-yl)benzyl)carbamoyl)pyrrolidin-1-ium chloride (S9b):

Previously prepared and characterized.\(^2\)

General procedure iv. Amine acetylation – Synthesis of S4a-d and S6a-d

The amine (1 equiv.) was dissolved in DMF, followed by the addition of triethylamine (2.0 equiv.) and 1-acetylimidazole (1.0 equiv.). The mixture was kept stirring overnight, followed by extraction with ethyl acetate and brine. Combined organic phases were dried with MgSO\(_4\), concentrated and purified in the acidic Gilson HPLC system, yielding the desired product.

(2S,4R)-1-acetyl-N-(4-bromo-3-(trifluoromethyl)benzyl)-4-hydroxypyrrolidine-2-carboxamide (S4a - spy molecule 2):

Prepared from 50.3 mg (0.125 mmol) of hydrochloride salt of amine S3a, resulting in 47.7 mg (0.117 mmol, 93%) of the desired product as a white solid. HRMS (ESI) [M+H]\(^+\) (m/z): Calculated for C\(_{15}\)H\(_{17}\)BrF\(_3\)N\(_2\)O\(_3\): 409.0369; Observed: 409.0387; \(^1\)H NMR (CD\(_3\)OD, 400 MHz) \(\delta\): 7.79 (d, \(J_{H-H} = 8.3\) Hz, 1H), 7.73 (d, \(J_{H-H} = 1.6\) Hz, 1H), 7.49 (dd, \(J_{H-H} = 1.6, 8.3\) Hz, 1H), 4.48 (m, 4H), 3.80 (dd, \(J_{H-H} = 4.2, 11.0\) Hz, 1H), 3.58 (dd, \(J_{H-H} = 1.8, 11.0\) Hz, 1H), 2.27 (ddd, \(J_{H-H} = 1.6, 3.0, 8.0, 13.1\) Hz, 1H), 2.12 (s, 3H), 2.07 (ddd, \(J_{H-H} = 4.6, 8.3, 13.1\) Hz, 1H); \(^13\)C NMR (CD\(_3\)OD, 100 MHz) \(\delta\): 175.88, 173.49, 141.40, 137.19, 134.38, 131.77 (q, \(J_{C-F} = 31.3\) Hz), 128.72 (q, \(J_{C-F} = 5.5\) Hz), 125.32 (q, \(J_{C-F} = 273.1\) Hz), 119.77, 71.70, 61.21, 58.21, 43.87, 40.35, 23.18; \(^19\)F NMR (CD\(_3\)OD, 470 MHz) \(\delta\): -63.86 (CF\(_3\)).

(2S,4R)-1-acetyl-4-hydroxy-N-(4-(4-methylthiazol-5-yl)-3-(trifluoromethyl)benzyl)pyrrolidine-2-carboxamide (S4b - spy molecule 6):

Prepared from 26 mg (0.060 mmol) of formate salt of amine S3b, resulting in 20 mg (0.047 mmol, 78%) of the desired product as a white solid. HRMS (ESI) [M+H]\(^+\) (m/z): Calculated for C\(_{19}\)H\(_{20}\)F\(_3\)N\(_3\)O\(_3\)S: 428.1250;
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Observed: 428.1272; $^1$H NMR (CD$_3$OD, 500 MHz) δ: 9.04-9.02 (s, 1H), 7.83-7.81 (s, 1H), 7.74-7.66 (d, $J_{H-H}$ = 7.9 Hz, 1H), 7.49-7.44 (d, $J_{H-H}$ = 7.9 Hz, 1H), 4.66-4.44 (m, 4H), 3.83-3.79 (dd, $J_{H-H}$ = 4.3, 11.1 Hz, 1H), 3.61-3.57 (dd, $J_{H-H}$ = 1.7, 11.1 Hz, 1H), 2.48-2.27 (m, 1H), 2.22-2.20 (s, 3H), 2.20-2.07 (m, 1H), 2.14-1.96 (s, 3H); $^{13}$C NMR (CD$_3$OD, 125 MHz) δ: 175.20, 172.75, 154.45, 152.11, 142.44, 135.25, 132.04, 131.57 (q, $^2J_{C-F}$ = 29.8 Hz), 129.70 (q, $^3J_{C-F}$ = 1.9 Hz), 128.81, 126.42 (q, $^3J_{C-F}$ = 5.3 Hz), 125.25 (q, $^1J_{C-F}$ = 273.1 Hz), 70.96, 60.48, 57.48, 43.39, 39.63, 22.44, 15.34; $^{19}$F NMR (CD$_3$OD, 470 MHz) δ: -60.75 (CF$_3$).

(2S,4R)-1-acetyl-N-(4-bromo-2-(trifluoromethyl)benzyl)-4-hydroxypyrrolidine-2-carboxamide (S4c - spy molecule 10):

Prepared from 29 mg (0.072 mmol) of amine S3c, resulting in 22 mg (0.054 mmol, 75%) of the desired product as a white solid. HRMS (ESI) [M+H]$^+$ (m/z): Calculated for C$_{15}$H$_{16}$BrF$_3$N$_2$O$_3$: 409.0369; Observed: 409.0377; $^1$H NMR (CD$_3$OD, 500 MHz) δ: 7.89-7.80 (m, 2H), 7.66-7.47 (d, $J_{H-H}$ = 8.4 Hz, 1H), 4.66-4.43 (m, 4H), 3.83-3.79 (dd, $J_{H-H}$ = 4.1, 11.0 Hz, 1H), 3.61-3.57 (dd, $J_{H-H}$ = 1.7, 11.1 Hz, 1H), 2.45-2.26 (dddd, $J_{H-H}$ = 1.7, 2.8, 7.8, 13.2 Hz, 1H), 2.21-2.07 (dddd, $J_{H-H}$ = 4.6, 8.5, 13.2 Hz, 1H), 2.15-1.97 (s, 3H); $^{13}$C NMR (CD$_3$OD, 125 MHz) δ: 175.33, 172.81, 137.72, 136.75, 132.12, 130.26 (q, $^2J_{C-F}$ = 31.3 Hz), 129.81 (q, $^3J_{C-F}$ = 6.1 Hz), 125.05 (q, $^1J_{C-F}$ = 273.5 Hz), 121.81, 70.98, 60.49, 57.52, 40.29 (q, $^4J_{C-F}$ = 3.4 Hz), 39.57, 22.44; $^{19}$F NMR (CD$_3$OD, 470 MHz) δ: -62.14 (CF$_3$).

(2S,4R)-1-acetyl-4-hydroxy-N-(4-(4-methylthiazol-5-yl)-2-(trifluoromethyl)benzyl)pyrrolidine-2-carboxamide (S4d - spy molecule 14):

Prepared from 39 mg (0.090 mmol) of amine S3d, resulting in 28 mg (0.066 mmol, 72%) of the desired product as a white solid. HRMS (ESI) [M+H]$^+$ (m/z): Calculated for C$_{19}$H$_{20}$F$_3$N$_3$O$_3$: 428.1250; Observed: 428.1265; $^1$H NMR (CD$_3$OD, 500 MHz) δ: 8.98 (s, 1H), 7.86-7.76 (m, 3H), 4.75-4.51 (m, 4H), 3.84-3.80 (dd, $J_{H-H}$ = 4.2, 11.1 Hz, 1H), 3.63-3.56 (dt, $J_{H-H}$ = 1.7, 11.1 Hz, 1H), 2.53 (s, 3H), 2.47-2.27 (dddd, $J_{H-H}$ = 1.6, 2.9, 7.9, 13.2 Hz, 1H), 2.24-2.10 (m, 1H), 2.15-2.00 (s, 3H); $^{13}$C NMR (CD$_3$OD, 125 MHz) δ: 175.35, 172.81, 153.75, 150.23, 138.38, 134.32, 132.30, 131.83, 130.80, 129.08 (q, $^2J_{C-F}$ = 30.9 Hz), 127.43 (q, $^3J_{C-F}$ = 5.9 Hz), 125.70 (q, $^1J_{C-F}$ = 273.6 Hz), 70.98, 60.52, 57.53, 40.50 (q, $^4J_{C-F}$ = 3.2 Hz), 39.61, 22.47, 15.90; $^{19}$F NMR (CD$_3$OD, 470 MHz) δ: -60.45 (CF$_3$).

(2S,4R)-1-((S)-2-acetamido-3,3-dimethylbutanoyl)-N-(4-bromo-3-(trifluoromethyl)benzyl)-4-hydroxypyrrolidine-2-carboxamide (S6a - spy molecule 4):
Overall yield of 93% (90 mg, 0.17 mmol), starting from amine S3a (77 mg, 0.18 mmol). Yield includes also step (vi). HRMS (ESI) [M+H]+ (m/z): Calculated for C₁₉H₂₀F₃N₆O₅S: 428.1250; Observed: 428.1265; ¹H NMR (CD₃OD, 500 MHz) δ: 7.82-7.58 (m, 3H), 4.63 (s, 1H), 4.60-4.45 (m, 3H), 4.34 (d, J₃₅-H = 15.5 Hz, 1H), 3.92 (d, J₃₅-H = 11.0 Hz, 1H), 3.81 (dd, J₃₅-H = 3.8, 11.0 Hz, 1H), 2.42-2.19 (m, 1H), 2.16-1.99 (m, 4H), 1.04 (s, 9H); ¹³C NMR (CD₃OD, 125 MHz) δ: 174.75, 173.22, 172.39, 154.46, 152.09, 142.49, 135.13, 132.28, 131.57 (q, J₂₁-CF = 30.9 Hz), 128.15 (q, J₂₂-CF = 5.4 Hz), 124.56 (q, J₂₃-CF = 272.5 Hz), 119.03, 71.17, 60.83, 59.25, 58.06, 43.21, 39.00, 36.53, 27.03, 22.39; ¹⁹F NMR (CD₃OD, 470 MHz) δ: -62.30 (CF₃).

(2S,4R)-1-((S)-2-acetamido-3,3-dimethylbutanoyl)-4-hydroxy-N-(4-(4-methylthiazol-5-yl)-3-(trifluoromethyl)benzyl)pyrrolidine-2-carboxamide (S6b - spy molecule 8):

Overall yield of 84% (66 mg, 0.12 mmol), starting from amine S3b (63 mg, 0.15 mmol). Yield includes also step (vi). HRMS (ESI) [M+H]+ (m/z): Calculated for C₂₅H₂₅F₃N₆O₅S: 541.2091; Observed: 541.2105; ¹H NMR (CD₃OD, 500 MHz) δ: 9.03 (s, 1H), 7.87-7.84 (m, 3H), 7.78-7.69 (d, J₅₆-H = 7.9 Hz, 1H), 7.48-7.40 (d, J₅₆-H = 7.9 Hz, 1H), 4.82-4.43 (m, 5H), 3.96-3.92 (d, J₅₆-H = 11.0 Hz, 1H), 3.85-3.81 (dd, J₅₆-H = 3.9, 11.0 Hz, 1H), 2.42-2.23 (m, 1H), 2.23-2.19 (s, 3H), 2.18-2.06 (ddd, J₅₆-H = 4.5, 9.2, 13.1 Hz, 1H), 2.05-1.99 (s, 3H), 1.08-1.02 (s, 9H); ¹³C NMR (CD₃OD, 125 MHz) δ: 174.83, 173.22, 172.39, 154.46, 152.09, 142.49, 135.13, 132.28, 131.57 (q, J₂₁-CF = 29.7 Hz), 129.69 (q, J₂₂-CF = 1.9 Hz), 128.81, 126.59 (q, J₂₃-CF = 5.3 Hz), 125.24 (q, J₂₄-CF = 273.3 Hz), 71.19, 60.87, 59.26, 58.08, 43.51, 39.05, 36.54, 27.06, 22.39, 15.35; ¹⁹F NMR (CD₃OD, 470 MHz) δ: -60.65 (CF₃).

(2S,4R)-1-((S)-2-acetamido-3,3-dimethylbutanoyl)-N-(4-bromo-3-(trifluoromethyl)benzyl)-4-hydroxypyrrolidine-2-carboxamide (S6c - spy molecule 12):

Prepared from 17 mg (0.042 mmol) of amine S3c, resulting in 18 mg (0.034 mmol, 82%) of the desired product as a white solid. Yield includes also step (vi). HRMS (ESI) [M+H]+ (m/z): C₁₉H₁₉BrF₃N₆O₅: 522.1210; Observed: 522.1180; ¹H NMR (CD₃OD, 500 MHz) δ: 7.87-7.83 (d, J₅₆-H = 1.1 Hz, 1H), 7.77-7.72 (m, 2H), 4.64 (s, 1H),
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4.71-4.41 (m, 4H), 3.96-3.93 (d, J_H-H = 11.1 Hz, 1H), 3.84-3.80 (dd, J_H-H = 3.9, 11.1 Hz, 1H), 2.39-2.22 (m, 1H), 2.14-2.07 (ddd, J_H-H = 4.4, 9.4, 13.1 Hz, 1H), 2.05-2.00 (s, 3H), 1.09-1.02 (s, 9H); \(^{13}\)C NMR (CD\(_{3}\)OD, 125 MHz) \(\delta\): 175.02, 173.24, 172.47, 137.74, 136.54, 132.45, 130.32 (q, \(^{2}\)J_C-F = 31.3 Hz), 129.80 (q, \(^{3}\)J_C-F = 6.1 Hz), 125.05 (q, \(^{4}\)J_C-F = 273.7 Hz), 121.79, 71.23, 60.90, 59.30, 58.11, 40.40 (q, \(^{5}\)J_C-F = 3.3 Hz), 38.94, 36.58, 27.04, 22.39; \(^{19}\)F NMR (CD\(_{3}\)OD, 470 MHz) \(\delta\): -62.12 (CF\(_3\)).

**(2S,4R)-1-((S)-2-acetamido-3,3-dimethylbutanoyl)-4-hydroxy-N-(4-(4-methylthiazol-5-yl)-3-(trifluoromethyl)benzyl)pyrrolidine-2-carboxamide (S6d - spy molecule 16):**

![Image of molecular structure](image)

Prepared from 53 mg (0.123 mmol) of amine S3d, resulting in 51 mg (0.094 mmol, 77%) of the desired product as a white solid. Yield includes also step (vi). HRMS (ESI) [M+H]\(^{+}\) (m/z): C\(_{25}\)H\(_{31}\)F\(_{11}\)N\(_{3}\)O\(_{7}\): 541.2091; Observed: 541.2091; \(^{1}\)H NMR (CD\(_{3}\)OD, 500 MHz) \(\delta\): 8.98 (s, 1H), 7.97-7.93 (d, J_H-H = 8.1 Hz, 1H), 7.80-7.76 (d, J_H-H = 1.3 Hz, 1H), 7.76-7.69 (ddd, J_H-H = 1.3, 8.1 Hz, 1H), 4.81-4.75 (d, J_H-H = 16.2 Hz, 1H), 4.67-4.51 (m, 4H), 3.97-3.93 (dd, J_H-H = 11.0 Hz, 1H), 3.86-3.81 (dd, J_H-H = 3.9, 11.0 Hz, 1H), 2.54-2.51 (s, 3H), 2.31-2.24 (m, 1H), 2.17-2.10 (ddd, J_H-H = 4.5, 9.2, 13.1 Hz, 1H), 2.06-2.02 (s, 3H), 1.09-1.03 (s, 9H); \(^{13}\)C NMR (CD\(_{3}\)OD, 125 MHz) \(\delta\): 175.06, 173.25, 172.49, 153.78, 150.21, 138.42, 134.11, 132.30, 131.83, 131.14, 129.17 (q, \(^{2}\)J_C-F = 30.9 Hz), 127.47 (q, \(^{3}\)J_C-F = 5.9 Hz), 125.70 (q, \(^{4}\)J_C-F = 273.4 Hz), 71.25, 60.93, 59.33, 58.13, 40.64 (q, \(^{5}\)J_C-F = 3.3 Hz), 38.97, 36.61, 27.06, 22.39, 15.89; \(^{19}\)F NMR (CD\(_{3}\)OD, 470 MHz) \(\delta\): -61.96.

**General procedure v. Amide coupling with 3,3-dimethylbutyric acid – Synthesis of S5a-d**

To a solution of amine (1 equiv.) in DMF, 3,3-dimethylbutyric acid (1 equiv.) was added and the mixture was stirred at room temperature. DIPEA (2 equiv.) was added dropwise and the mixture was stirred for 5 minutes at room temperature. HATU (1.1 equiv.) was added and the mixture was stirred at room temperature for 60 minutes (LCMS monitoring). Water was added and the mixture was extracted with ethyl acetate. The combined organic phases were washed with brine, dried over MgSO\(_4\) and evaporated under reduced pressure to give the corresponding crude, which was purified in the acidic Gilson preparative HPLC.

**(2S,4R)-N-(4-bromo-3-(trifluoromethyl)benzyl)-1-(3,3-dimethylbutanoyl)-4-hydroxypyrrolidine-2-carboxamide (S5a - spy molecule 3):**

![Image of molecular structure](image)

Prepared from 50.6 mg (0.125 mmol) of hydrochloride salt of the amine S3a resulting in 49.8 mg (0.107 mmol, 86%) of the desired product as a white solid. HRMS (ESI) [M+H]\(^{+}\) (m/z): Calculated for C\(_{19}\)H\(_{24}\)BrF\(_3\)N\(_{2}\)O\(_{3}\):
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465.0995; Observed: 465.1013; ¹H NMR (CD₃OD, 400 MHz) δ: 7.82-7.75 (d, J_H-H = 8.2 Hz, 1H), 7.76-7.73 (m, 1H), 7.56-7.47 (dd, J_H-H = 1.6, 8.2 Hz, 1H), 4.58-4.36 (m, 4H), 3.80-3.75 (dd, J_H-H = 4.1, 11.0 Hz, 1H), 3.74-3.63 (dt, J_H-H = 1.7, 11.0 Hz, 1H), 2.37-1.81 (m, 4H), 1.10-0.97 (s, 9H); ¹³C NMR (CD₃OD, 100 MHz) δ: 175.29, 173.80, 140.67, 136.36, 133.75, 131.03 (q, ²J_C-F = 31.8 Hz), 128.08 (q, ³J_C-F = 5.5 Hz), 124.56 (q, ⁴J_C-F = 272.7 Hz), 119.01, 71.10, 60.54, 57.82, 47.76, 43.17, 39.24, 32.74, 30.47; ¹⁹F NMR (CD₃OD, 470 MHz) δ: -63.81 (CF₃).

(2S,4R)-1-(3,3-dimethylbutanoyl)-4-hydroxy-N-(4-(4-methylthiazol-5-yl)-3-(trifluoromethyl)benzyl)pyrrolidine-2-carboxamide (S5b - spy molecule 7):

![Structure 1](image1.png)

Prepared with 0.023 g (0.053 mmol) of amine S3b, resulting in 0.020 mg (0.042 mmol, 80%) of product as a white solid. HRMS (ESI) [M+H]+ (m/z): Calculated for C₂₃H₂₅F₃N₃O₅S: 484.1876; Observed: 484.1891; ¹H NMR (CD₃OD, 500 MHz) δ: 9.04-9.02 (s, 1H), 7.87-7.84 (m, 1H), 7.75-7.68 (m, 1H), 7.48-7.41 (d, J_H-H = 7.8 Hz, 1H), 4.65-4.43 (m, 4H), 3.82-3.78 (dd, J_H-H = 4.2, 11.1 Hz, 1H), 3.75-3.65 (dt, J_H-H = 2.0, 11.1 Hz, 1H), 2.38-2.24 (m, 3H), 2.22-2.20 (s, 3H), 2.19-2.06 (ddd, J_H-H = 4.4, 8.4, 13.3 Hz, 1H), 1.10-0.97 (s, 9H); ¹³C NMR (CD₃OD, 125 MHz) δ: 175.37, 173.82, 154.44, 152.09, 142.49, 135.18, 132.21, 131.59 (q, ²J_C-F = 30.5 Hz), 129.69 (q, ³J_C-F = 1.9 Hz), 128.83, 126.55 (q, ⁴J_C-F = 5.5 Hz), 125.25 (q, ⁵J_C-F = 273.1 Hz), 71.13, 60.58, 57.85, 47.77, 43.65, 39.28, 32.77, 30.48, 15.89; ¹⁹F NMR (CD₃OD, 470 MHz) δ: -60.68 (CF₃).

(2S,4R)-N-(4-bromo-2-(trifluoromethyl)benzyl)-1-(3,3-dimethylbutanoyl)-4-hydroxypyrrrolidine-2-carboxamide (S5c - spy molecule 11):

![Structure 2](image2.png)

Prepared from 17 mg (0.042 mmol) of amine S3c, resulting in 15 mg (0.032 mmol, 76%) of the desired product as a white solid. HRMS (ESI) [M+H]+ (m/z): Calculated for C₁₉H₂₃BrF₃N₂O₅S: 465.0995; Observed: 465.0997; ¹H NMR (CD₃OD, 500 MHz) δ: 7.90-7.83 (d, J_H-H = 1.8 Hz, 1H), 7.83-7.60 (dd, J_H-H = 1.8, 8.3 Hz, 1H), 7.72-7.50 (d, J_H-H = 8.3 Hz, 1H), 4.66-4.42 (m, 4H), 3.81-3.77 (dd, J_H-H = 4.1, 11.1 Hz, 1H), 3.74-3.66 (dd, J_H-H = 1.9, 11.1 Hz, 1H), 2.40-2.16 (m, 3H), 2.14-2.07 (ddd, J_H-H = 4.6, 8.5, 13.2 Hz, 1H), 1.12-1.02 (s, 9H); ¹³C NMR (CD₃OD, 125 MHz) δ: 175.51, 173.92, 137.74 (q, ²J_C-F = 1.2 Hz), 136.65, 132.32, 130.32 (q, ³J_C-F = 31.3 Hz), 129.80 (q, ⁴J_C-F = 6.1 Hz), 125.05 (q, ⁵J_C-F = 273.8 Hz), 121.79, 71.14, 60.58, 57.91, 47.82, 40.36 (q, ⁶J_C-F = 3.3 Hz), 39.21, 32.87, 30.50; ¹⁹F NMR (CD₃OD, 470 MHz) δ: -62.11 (CF₃).
Prepared from 24 mg (0.056 mmol) of amine S3d, resulting in 22 mg (0.045 mmol, 82%) of the desired product as a white solid.

HRMS (ESI) [M+H]+ (m/z): Calculated for C23H28F3N3O3S: 484.1876; Observed: 484.1894;

1H NMR (CD3OD, 500 MHz) δ: 9.00-8.98 (s, 1H), 7.91-7.87 (d, JH-H = 7.9 Hz, 1H), 4.76-4.51 (m, 4H), 3.83-3.78 (dd, JH-H = 4.1, 11.1 Hz, 1H), 3.74-3.67 (dt, JH-H = 1.5, 11.1 Hz, 1H), 2.53 (s, 3H), 2.41-2.11 (m, 4H), 1.12-1.02 (s, 9H);

13C NMR (CD3OD, 125 MHz) δ: 175.55, 173.95, 154.03, 149.74, 138.59, 134.26, 132.13, 132.03, 131.06, 129.16 (q, JCF = 30.9 Hz), 127.48 (q, JCF = 5.8 Hz), 125.69 (q, JCF = 273.5 Hz), 71.16, 60.61, 57.94, 47.84, 40.59 (q, JCF = 3.1 Hz), 39.25, 32.89, 30.51, 15.68; 19F NMR (CD3OD, 470 MHz) δ: -61.95.

**General procedure vi. Amide coupling with Boc-L-tert-leucine and deprotection - Synthesis of intermediates of compounds S6a-d and S12a-b**

Same as “general procedure iii”, just replacing the Boc-L-hydroxyproline with Boc-L-tert-leucine. All the crude intermediates prepared at this step were directly used in the next steps without further purification and characterization after deprotection of the Boc group.

**General procedure vii. Amine trifluoroacetylation – Synthesis of S10a-b and S12a-b**

To a solution of the amine (1 equiv.) in dry MeOH (1 ml per mmol of amine) was added triethylamine (2 equiv.). Ethyl trifluoroacetate (1.25 equiv.) was added and the reaction was stirred at room temperature for approximately 24 hours (LCMS monitoring). The solvent was evaporated under reduced pressure and the crude mixture was extracted with ethyl acetate and 1.0 M HCl solution. Combined organic phases were dried over MgSO4, concentrated and purified in the acidic Gilson preparative HPLC system, yielding the desired product.
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MHz) δ: 173.19, 157.56 (q, 2J_C,F = 37.11 Hz), 139.11, 132.69, 130.44, 121.98, 117.73 (q, 1J_C,F = 286.8 Hz), 71.08, 61.89, 57.21 (q, 4J_C,F = 3.0 Hz), 43.53, 38.57; 19F NMR (CD3OD, 470 MHz) δ: -72.35 (CF3).

(2S,4R)-4-hydroxy-N-(4-(4-methylthiazol-5-yl)benzyl)-1-(2,2,2-trifluoroacetyl)pyrrolidine-2-carboxamide (S10b - spy molecule 18):

Prepared from 28 mg (0.079 mmol) of amine S9b, resulting in 19 mg (0.046 mmol, 58%) of the desired product as a white solid. HRMS (ESI) [M+H]+ (m/z): Calculated for C18H19F3N3O3S: 414.1094; Observed: 414.1117; 1H NMR (CD3OD, 500 MHz) δ: 8.91 (s, 1H), 7.50-7.41 (m, 4H), 4.69-4.64 (d, J_H,H = 8.5 Hz, 1H), 4.59-4.40 (m, 3H), 3.89-3.85 (dd, J_H,H = 3.6, 11.5 Hz, 1H), 3.83-3.79 (dd, J_H,H = 1.4, 11.5 Hz, 1H), 2.54-2.50 (s, 3H), 2.37-2.31 (ddt, J_H,H = 1.8, 7.8, 13.3 Hz, 1H), 2.14-2.07 (ddd, J_H,H = 4.3, 9.3, 13.3 Hz, 1H); 13C NMR (CD3OD, MHz) δ: 173.23, 157.58 (q, 2J_C,F = 36.8 Hz), 153.00, 149.20, 140.12, 133.48, 131.79, 130.59, 129.03, 117.75 (q, 1J_C,F = 286.8 Hz), 71.10, 61.93, 57.23 (q, 4J_C,F = 3.0 Hz), 43.82, 38.61, 15.89; 19F NMR (CD3OD, 470 MHz) δ: -73.74 (CF3).

(2S,4R)-N-(4-hromobenzyl)-1-((S)-3,3-dimethyl-2-(2,2,2-trifluoroacetamido)butanoyl)-4-hydroxy-N-(4-(4-methylthiazol-5-yl)benzyl)pyrrolidine-2-carboxamide (S12a - spy molecule 21):

Prepared from 24 mg (0.070 mmol) of amine S9a, resulting in 23 mg (0.045 mmol, 65%) of the desired product as a white solid. HRMS (ESI) [M+H]+ (m/z): Calculated for C29H25BrF3N3O4: 508.1053; Observed: 508.1046; 1H NMR (CD3OD, 500 MHz) δ: 8.58 (s, NH), 7.53-7.29 (m, 4H), 4.77-4.75 (s, 1H), 4.61-4.26 (m, 4H), 3.88-3.81 (m, 2H), 2.42-2.22 (m, 1H), 2.12-2.06 (ddd, J_H,H = 4.4, 9.4, 13.1 Hz, 1H), 1.10-1.04 (s, 9H); 13C NMR (CD3OD, 125 MHz) δ: 174.38, 170.74, 158.81 (q, 2J_C,F = 37.6 Hz), 139.34, 132.57, 130.46, 121.82, 117.58 (q, 1J_C,F = 286.3 Hz), 71.17, 60.95, 59.62, 58.30, 43.50, 39.03, 37.38, 26.93; 19F NMR (CD3OD, 470 MHz) δ: -76.65 (CF3).

(2S,4R)-1-((S)-3,3-dimethyl-2-(2,2,2-trifluoroacetamido)butanoyl)-4-hydroxy-N-(4-(4-methylthiazol-5-yl)benzyl)pyrrolidine-2-carboxamide (S12b - spy molecule 22):
Prepared from 60 mg (0.17 mmol) of amine S9b, resulting in 63 mg (0.12 mmol, 71%) of the desired product as a white solid. HRMS (ESI) [M+H]+ (m/z): Calculated for C_{24}H_{30}F_3N_4Os: 527.1934; Observed: 527.1901; 1H NMR (CD_{2}OD, 500 MHz) δ: 8.91-8.90 (s, 1H), 7.51-7.43 (m, 4H), 4.77 (s, 1H), 4.81-4.36 (m, 4H), 3.89-3.62 (m, 2H), 2.52-2.49 (s, 3H), 2.45-2.24 (m, 1H), 2.18-2.08 (ddd, J_{H-H} = 4.4, 9.3, 13.1 Hz, 1H), 1.11-1.05 (s, 9H); 13C NMR (CD_{2}OD, 100 MHz) δ: 174.38, 170.77, 152.94, 149.15, 140.34, 133.50, 131.64, 130.47, 129.04, 117.58 (q, J_{C-F} = 38.4 Hz), 152.94, 149.15, 140.34, 133.50, 131.64, 130.47, 129.04, 117.58 (q, J_{C-F} = 38.4 Hz), 71.18, 60.98, 59.64, 58.31, 43.81, 39.05, 37.40, 26.96, 15.89; 19F NMR (CD_{2}OD, 470 MHz) δ: -75.18.

**General procedure viii. Amide coupling with 3,3,3-fluoropropanoic acid – Synthesis of S11a-b**

To a solution of amine (1 equiv.) in DMF, 3,3,3-Trifluoropropanoic acid (1 equiv.) was added and the mixture was stirred at room temperature. HATU (1.1 equiv.) was added and the mixture was stirred at room temperature for 30-90 minutes (TLC monitoring). Water was added and the mixture was extracted with ethyl acetate. The combined organic phases were washed with brine, dried over MgSO_{4} and evaporated under reduced pressure to give the corresponding crude, which was purified by flash column chromatography with an increasing gradient of DCM and 20% MeOH in DCM to yield the desired product.

**(2S,4R)-N-(4-bromobenzyl)-4-hydroxy-1-(3,3,3-trifluoropropanoyl)pyrrolidine-2-carboxamide (S11a - spy molecule 19):**

![Image of 2S,4R)-N-(4-bromobenzyl)-4-hydroxy-1-(3,3,3-trifluoropropanoyl)pyrrolidine-2-carboxamide](image)

Prepared from 37 mg (0.107 mmol) of amine S9a, resulting in 33 mg (0.081 mmol, 75%) of the desired product as a white solid. HRMS (ESI) [M+H]+ (m/z): Calculated for C_{13}H_{17}BrF_{3}N_{2}O_{3}: 409.0369; Observed: 409.0389; 1H NMR (CD_{2}OD, 500 MHz) δ: 7.52-7.47 (m, 2H), 7.29-7.25 (m, 2H), 4.59-4.33 (m, 4H), 3.81-3.77 (dd, J_{H-H} = 4.3, 11.0 Hz, 1H), 3.65-3.58 (m, 1H), 3.58-2.93 (m, 2H), 2.45-2.24 (m, 1H), 2.20-2.05 (ddd, J_{H-H} = 4.7, 8.2, 13.2 Hz, 1H); 13C NMR (CD_{2}OD, 125 MHz) δ: 174.37, 165.27 (q, J_{C-F} = 3.5 Hz), 139.20, 132.66, 130.43, 125.90 (q, J_{C-F} = 75.3 Hz), 121.89, 70.83, 60.70, 56.98, 43.48, 39.75 (q, J_{C-F} = 28.9 Hz), 39.39; 19F NMR (CD_{2}OD, 470 MHz) δ: -64.04 (CF_{3}).

**(2S,4R)-4-hydroxy-N-(4-(4-methylthiazol-5-yl)benzyl)-1-(3,3,3-trifluoropropanoyl)pyrrolidine-2-carboxamide (S11b - spy molecule 20):**

![Image of 2S,4R)-4-hydroxy-N-(4-(4-methylthiazol-5-yl)benzyl)-1-(3,3,3-trifluoropropanoyl)pyrrolidine-2-carboxamide](image)

Prepared from 22 mg (0.062 mmol) of amine S9b, resulting in 22 mg (0.051 mmol, 83%) of the desired product as a white solid. HRMS (ESI) [M+H]+ (m/z): Calculated for C_{19}H_{28}F_{3}N_{4}O_{5}: 428.1250; Observed: 428.1262; 1H
NMR (CD$_3$OD, 500 MHz) $\delta$: 8.93-8.89 (s, 1H), 7.50-7.44 (m, 4H), 4.65-4.44 (m, 4H), 3.83-3.79 (dd, $J_{\text{H-H}} = 4.3, 10.9$ Hz, 1H), 3.78-3.58 (dd, $J_{\text{H-H}} = 2.0, 10.9$ Hz, 1H), 3.58-2.93 (m, 2H), 2.52-2.50 (s, 3H), 2.47-2.27 (m, 1H), 2.24-2.09 (dd, $J_{\text{H-H}} = 4.7, 8.2, 13.2$ Hz, 1H); $^{13}$C NMR (CD$_3$OD, 125 MHz) $\delta$: 174.41, 165.29 (q, $^{3}J_{\text{C-F}} = 3.3$ Hz), 152.98, 149.17, 140.23, 133.51, 131.70, 130.56, 129.03, 125.92 (q, $^{1}J_{\text{C-F}} = 275.3$ Hz), 70.85, 60.74, 57.00, 43.78, 39.77 (q, $^{2}J_{\text{C-F}} = 29.0$ Hz), 39.43, 15.88; $^{19}$F NMR (CD$_3$OD, 470 MHz) $\delta$: -64.04 (CF$_3$).
3. **Protein expression, purification and biotinylation**

The VHL E3 ligase is a multi-protein complex composed of five proteins: VHL protein (pVHL), elongin B (eloB), elongin C (eloC), Cullin-2 (Cul2) and Ring-box protein 1 (Rbx1).\[^3\] Since the compounds developed in this work bind solely to the VHL protein, the VBC complex (equimolar complex of pVHL\(_{54-213}\), eloB\(_{1-104}\) and eloC\(_{17-112}\)) was used in all experiments, as it can be readily expressed in *E. coli* with high yields,\[^4\] while the full E3 ligase would require baculovirus-insect cells expression system.\[^5\] The expression and purification of VBC was performed as described previously by our group\[^2\] and employed directly in all NMR experiments.

For the surface plasmon resonance (SPR) experiments, a VBC complex containing an AviTag™ in the N-terminus of eloB (AviVBC) was purified using the same procedure described for VBC. The modified eloB/eloC expression plasmid was previously developed in-house by Dr. Michael Roy and kindly shared. The AviVBC complex was site-specifically biotinylated in the AviTag using the GST-BirA method previously described by Fairhead and Howarth.\[^6\]
4. Surface plasmon resonance experiments

The SPR experiments were performed with a Biacore T200 instrument (GE Healthcare). All measurements were performed at 20 °C with buffer containing 10 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM TCEP, 0.005% (v/v) Tween® 20 and 2% (v/v) dimethyl sulfoxide (DMSO). Biotinylated AviVBC (~0.5 μM) was immobilised at 22 °C onto a Series S sensor chip SA (GE Heathcare) to levels of approximately 3500-4000 response units (RU).

Solutions of each spy molecule were prepared in buffer at concentrations based on previous structure-activity relationship studies of VHL ligands (K_D expected in the nanomolar range for structures like spy molecules 16 and 22, or in the milimolar range for spy molecules similar to 1 and 9). From this first screen, the binding affinities were roughly estimated using the Biacore T200 evaluation software (GE Healthcare), then measurements were repeated using concentrations above and below the K_D obtained in the first round to generate better curves for fitting the data accurately. Contact and dissociation times varied across the different compounds tested, but in general fast binding kinetics were observed for all compounds, fully reaching steady-state or being completely dissociated from the surface in less than sixty seconds.

Data analysis was performed using the steady state responses of the double-referenced sensorgrams (raw data subtracted from blank and reference surface injections) obtained for each concentration tested. These responses were plotted against the respective concentrations and the data fitted to a 1:1 binding model using the Biacore T200 evaluation software and the following equation:

\[ R_{eq} = \frac{C \times R_{MAX}}{K_D + C} + offset \]

Where \( R_{eq} \) is the steady-state response at a given concentration C. Deviations in \( R_{eq} \) were corrected by adding an ‘offset’ term to the equation. \( K_D \) is the dissociation constant to be determined and \( R_{MAX} \) is the maximum response expected for a given compound according to the equation below:

\[ R_{MAX} = n \times R_{Protein} \left( \frac{MW_{Compound}}{MW_{Protein}} \right) \]

Where n is the stoichiometry of the interaction (in this case, n = 1) and \( R_{Protein} \) is the immobilization level of protein. \( MW_{Compound} \) and \( MW_{Protein} \) are the molecular weights of compound and protein, respectively. Sensorgrams and fitting parameters for all spy molecules can be found in section 7.
5. **Measurement of the transverse relaxation rates by $^{19}$F CPMG NMR**

All the NMR experiments were performed in a 500 MHz Bruker AVANCE NMR spectrometer equipped with a CPQCI-F cryoprobe. To measure the transverse relaxation rates ($R_2$), a solution of each spy molecule at 100 μM was prepared in 50 mM potassium phosphate monobasic (KH₂PO₄), pH 7.5, 100 mM NaCl, 1 mM TCEP, 2% (v/v) DMSO, 20% D₂O and 10 μM trifluoroacetic acid (TFA). For each solution, $^{19}$F CPMG experiments (decoupled) were performed varying the total CPMG filter (50, 100, 200, 400, 800, 1200, 1600 and 3200 ms). Due to the fast relaxation of molecules 21 and 22, the experiments were repeated with shorter CPMG filters (50, 100, 150, 200, 300, 400, 700 and 1000 ms). The fluorine peaks were integrated and plotted against the respective CPMG filters. The $R_2$ relaxation rates were obtained from fitting the data as an exponential decay (GraphPad Prism 6) according to the equation below: [7]

\[
I(t) = I(0) \times e^{-R_2 t}
\]

Where $I(t)$ is the $^{19}$F signal intensity or integral, $t$ is the total CPMG filter in seconds, $I(0)$ is the signal intensity when $t = 0$. To obtain the $R_2$ upon addition of protein, all the experiments above were repeated in presence of VBC at 1 μM. Experiments were performed as triplicates and the $R_2$ contrasts ($C_2$) were obtained according to the equation below: [8]

\[
C_2 = \frac{R_2^{\text{observed}} - R_2^{\text{Free}}}{R_2^{\text{observed}}}
\]

Where $R_2^{\text{Free}}$ is the $R_2$ obtained for the spy molecule free in solution and $R_2^{\text{observed}}$ is the $R_2$ obtained with the spy molecule in presence of VBC. The raw data, fitting, $R_2$ and $C_2$ values for each spy molecule can be found in section 8.1. For the $R_2$ measurements using molecule 19 at varied concentrations of spy molecule and protein, see section 8.2. For the $R_2$ measurements using spy molecules 6 and 11 (used to generate figures S2, S3 and S4) see section 8.3.
6. Competition experiments by $^{19}$F NMR

As full measurements of $R_2$ would be very time consuming, the competition experiments were performed using a single $^{19}$F CPMG experiment (decoupled) per sample with a fixed CPMG delay. To determine the best CPMG delay for a given spy molecule and assay condition, the procedure described in Figure S5 was developed based on the transverse relaxation rate equations.\(^[7]\) By knowing the relaxation rates of the spy molecule free in solution and in presence of protein, the CPMG delay where the difference between the NMR peaks is maximum is hereon referred as $d_{\text{max}}$. The $d_{\text{max}}$ for all the conditions tested for each spy molecule can be found in section 8, together with the respective values of $R_2$ and $C_2$.

$$ I_F(t) = I_0 e^{-R_2^F t} $$
$$ I_B(t) = I_0 e^{-R_2^B t} $$
$$ I_D(t) = I_F(t) - I_B(t) = I_0 (e^{-R_2^F t} - e^{-R_2^B t}) $$

When $t = d_{\text{max}}$, the maximum of the difference curve is described as:

$$ \frac{d}{dt} I_D(t) = 0 $$
$$ \frac{d}{dt} I_0 (e^{-R_2^F t} - e^{-R_2^B t}) = 0 $$

$$ R_2^B e^{-R_2^B t} - R_2^F e^{-R_2^F t} = 0 \quad \Leftrightarrow \quad t = d_{\text{max}} = \frac{\ln \left( \frac{R_2^F}{R_2^B} \right)}{R_2^B - R_2^F} $$

**Method for selecting the best CPMG delay for competition experiments.** With the transverse relaxation rates of the spy molecule free in solution ($R_2^F$, resulting in the blue plot) and bound to protein ($R_2^B$, resulting in the red plot), the difference between the two curves is described by $I(t)^D$ (cyan curve). To obtain $d_{\text{max}}$, the delay where the difference curve reaches its maximum, the first derivative of $I(t)^D$ was obtained and equalled to 0, subsequently isolating $t$ (CPMG filter).

After the $d_{\text{max}}$ for each condition was established, the competition experiments with spy molecules 6 (Figure S3), 11 (Figure S4) and 19 (Figure 5 and S5) were performed with solutions containing 50 mM potassium phosphate monobasic (KH$_2$PO$_4$), pH 7.5, 100 mM NaCl, 1 mM TCEP, 2% (v/v) DMSO, 20% D$_2$O and 10 µM trifluoroacetic acid (TFA). Each assay consisted of two controls:
1) Spy molecule free in solution
2) Spy molecule in presence of protein

Samples containing different concentrations of competitors were prepared in presence of spy molecule and protein and \(^{19}\text{F}\) CPMG spectra at the respective \(d_{\text{max}}\) were collected. The displacement of the spy molecule was obtained from the equation below:

\[
\text{Displacement} = \frac{I_C - I_P}{I_C - I_P} \times 100\%
\]

Where \(I_F\) is the integral of the fluorine peak of the spy molecule free in solution, \(I_P\) is the integral of the fluorine peak of the spy molecule in presence of protein and \(I_C\) is the integral of the fluorine peak of the spy molecule in presence of protein and a competitor at a given concentration.

To determine the dissociation constant of a competitor, the displacement of the spy molecule was plotted against the concentration of the competitors, and then fitted to a “log\(_{10}\) [Inhibitor] versus Normalised response” model using GraphPad Prism 6.0, resulting in the plots observed in Figures 5, S3 and S4. By knowing the concentrations of spy molecule, protein and \(K_D\) of the spy molecule (\(K_S\)), the IC\(_{50}\) values obtained from the fitting were converted into the \(K_D\) of competitor (\(K_i\)) using the Nikolovska-Coleska relationship:\(^9\)

\[
K_i = \frac{[C]_{50}}{K_S + [P]_{0} + 1}
\]

Where \([C]_{50}\) and \([S]_{50}\) are, respectively, the free concentrations of competitor and spy molecule at 50% inhibition (concentration of competitor equals the IC\(_{50}\)), and \([P]_0\) is the free concentration of protein in presence of just the spy molecule. As the total concentrations of protein (\(P_t\)) and spy molecule (\(S_t\)) are known, these values can be obtained from the equations below:\(^9\)

\[
P_0 = \frac{[P]_t - K_S - [S]_t - \sqrt{(K_S + [S]_t - [P]_t)^2 + 4[P]_t K_S}}{2}
\]

\[
[S]_{50} = [S]_t - \frac{([P]_t - [P]_0)}{2}
\]

\[
[C]_{50} = IC_{50} - [P]_0 + \frac{([P]_t - [P]_0)}{2} \times \left(1 + \frac{K_S}{[S]_{50}}\right)
\]
7. Surface plasmon resonance – Sensorgrams and data fitting

Spy molecule 1 (Compound S3a)

Blank and reference surface subtracted responses according to the concentration of spy molecule. **Theoretical $R_{\text{MAX}} = 25.4$**

| [C] (µM) | log₁₀[C] | Response (RU) |
|----------|-----------|---------------|
| 14.0625  | -4.85     | 2.6           |
| 28.125   | -4.55     | 2.9           |
| 56.25    | -4.25     | 3.3           |
| 112.5    | -3.95     | 3.5           |
| 225      | -3.65     | 4             |
| 450      | -3.35     | 4.8           |

Data could not be fitted. Responses increase with concentration, but too far from the theoretical $R_{\text{MAX}}$. $K_D >>> 1.0$ mM.
Blank and reference surface subtracted responses according to the concentration of spy molecule. **Theoretical R\text{MAX} = 29.7**

| [C] (µM) | log\text{10}[C] | Response (RU) |
|----------|-----------------|---------------|
| 12.5     | 4.90            | 0.1           |
| 25.0     | 4.60            | 0.8           |
| 50.0     | 4.30            | 1.5           |
| 100.0    | 4.00            | 3.0           |
| 200.0    | 3.70            | 5.5           |
| 400.0    | 3.40            | 10.6          |
| 800.0    | 3.10            | 16.3          |

Data fitting using the Biacore T200 evaluation software. As responses were lower than the theoretical R\text{MAX}, fitting was performed with a fixed R\text{MAX}.

| K\text{D} (µM) | SE (K\text{D}) | R\text{MAX} (RU) | SE(R\text{MAX}) | offset (RU) | SE(offset) | Ch\text{²} (RU\text{²}) |
|----------------|----------------|-------------------|-----------------|-------------|------------|--------------------------|
| 657.3          | 49.0           | 29.7              | -               | -0.62       | 0.34       | 0.262                    |
Supporting Information

Spy molecule 3 (Compound S5a)

Blank and reference surface subtracted responses according to the concentration of spy molecule. Theoretical \( R_{\text{MAX}} = 34.0 \)

| [C] (µM) | \( \log_{10}[C] \) | Response (RU) |
|----------|-----------------|--------------|
| 12.5     | 4.90            | 1.6          |
| 25.0     | 4.60            | 3.0          |
| 50.0     | 4.30            | 5.3          |
| 100.0    | 4.00            | 8.8          |
| 200.0    | 3.70            | 13.0         |
| 400.0    | 3.40            | 18.1         |
| 800.0    | 3.10            | 21.9         |

Data fitting using the Biacore T200 evaluation software. As responses were lower than the theoretical \( R_{\text{MAX}} \), fitting was performed with a fixed \( R_{\text{MAX}} \).

| \( K_D \) (µM) | SE (\( K_D \)) | \( R_{\text{MAX}} \) (RU) | SE(\( R_{\text{MAX}} \)) | offset (RU) | SE(offset) | \( \chi^2 \) (RU²) |
|----------------|---------------|----------------|-----------------|------------|-----------|--------------|
| 407.2          | 54.0          | 34.0           | -               | 1.13       | 0.76      | 0.961        |
Spy molecule 4 (Compound S6a)

Blank and reference surface subtracted responses according to the concentration of spy molecule.

Theoretical $R_{\text{MAX}} = 35.3$

| [C] (µM) | log$_{10}$[C] | Response (RU) |
|----------|----------------|---------------|
| 0.023    | -7.64          | 2.9           |
| 0.069    | -7.16          | 3.4           |
| 0.206    | -6.69          | 4.6           |
| 0.617    | -6.21          | 7.2           |
| 1.852    | -5.73          | 12.2          |
| 5.556    | -5.26          | 20.5          |
| 16.667   | -4.78          | 27.9          |
| 50.000   | -4.30          | 32.3          |

Data fitting using the Biacore T200 evaluation software:

| $K_D$ (µM) | SE ($K_D$) | $R_{\text{MAX}}$ (RU) | SE($R_{\text{MAX}}$) | offset (RU) | SE(offset) | $\text{Chi}^2$ (RU²) |
|------------|------------|------------------------|----------------------|-------------|------------|---------------------|
| 4.388      | 0.120      | 32.2                   | 0.22                 | 3           | 0.11       | 0.0344              |
**Spy molecule 5 (Compound S3b)**

Blank and reference surface subtracted responses according to the concentration of spy molecule:

| [C] (µM) | log₁₀[C] | Response (RU) |
|----------|----------|---------------|
| 14.0625  | -4.85    | 2.7           |
| 28.125   | -4.55    | 2.8           |
| 56.25    | -4.25    | 3.0           |
| 112.5    | -3.95    | 4.0           |
| 225      | -3.65    | 5.0           |
| 450      | -3.35    | 6.8           |

Data could not be fitted. Responses increase with concentration, but too far from the theoretical $R_{\text{MAX}}$. $K_D >>> 1.0$ mM.
Spy molecule 6 (Compound S4b)

Blank and reference surface subtracted responses according to the concentration of spy molecule:

**Theoretical R\text{\text{MAX}} = 28.5**

| [C] (µM) | log₁₀[C] | Response (RU) |
|----------|----------|---------------|
| 14.0625  | -4.85    | 3.0           |
| 28.125   | -4.55    | 4.0           |
| 56.25    | -4.25    | 4.6           |
| 225      | -3.65    | 9.5           |
| 450      | -3.35    | 11.8          |
| 900      | -3.05    | 18.4          |

Data fitting using the Biacore T200 evaluation software. As responses were much lower than the theoretical R\text{\text{MAX}}, fitting was performed with a fixed R\text{\text{MAX}}:

| K\text{\text{D}} (µM) | SE (K\text{\text{D}}) | R\text{\text{MAX}} (RU) | SE(R\text{\text{MAX}}) | offset (RU) | SE(offset) | Chi² (RU²) |
|-----------------------|----------------------|-------------------------|------------------------|--------------|------------|------------|
| 877.7                 | 98.0                 | 28.5                    | -                      | 2.9          | 0.49       | 0.526      |
Spy molecule 7 (Compound S5b)

Blank and reference surface subtracted responses according to the concentration of spy molecule.

**Theoretical $R_{MAX} = 32.0$**

| [C] (µM) | $\log_{10}[C]$ | Response (RU) |
|----------|----------------|---------------|
| 3.125    | -5.51          | 7.2           |
| 6.250    | -5.20          | 7.8           |
| 12.500   | -4.90          | 10.5          |
| 25.000   | -4.60          | 15.4          |
| 50.000   | -4.30          | 18.8          |
| 100.000  | -4.00          | 23.5          |
| 200.000  | -3.70          | 29.8          |

Data fitting using the Biacore T200 evaluation software:

| $K_D$ (µM) | SE ($K_D$) | $R_{MAX}$ (RU) | SE($R_{MAX}$) | offset (RU) | SE(offset) | $\chi^2$ (RU$^2$) |
|------------|------------|----------------|---------------|-------------|------------|------------------|
| 67.12      | 14.00      | 31.8           | 2             | 5.6         | 0.79       | 0.704            |
Spy molecule 8 (Compound S6b)

Blank and reference surface subtracted responses according to the concentration of spy molecule.

Theoretical $R_{\text{MAX}} = 35.5$

| [C] (µM) | $\log_{10}[C]$ | Response (RU) |
|----------|-----------------|---------------|
| 0.781    | -6.11           | 12.6          |
| 1.563    | -5.81           | 17.8          |
| 3.125    | -5.51           | 24.0          |
| 6.250    | -5.20           | 27.4          |
| 12.500   | -4.90           | 32.0          |
| 25.000   | -4.60           | 34.3          |
| 50.000   | -4.30           | 38.0          |

Data fitting using the Biacore T200 evaluation software:

| $K_D$ (µM) | SE ($K_D$) | $R_{\text{MAX}}$ (RU) | SE($R_{\text{MAX}}$) | offset (RU) | SE(offset) | $\chi^2$ (RU²) |
|------------|------------|------------------------|-----------------------|-------------|------------|---------------|
| 2.991      | 0.750      | 32.1                   | 2.1                   | 6.4         | 2.4        | 1.16          |
Spy molecule 9 (Compound S3c)

Blank and reference surface subtracted responses according to the concentration of spy molecule.

Theoretical $R_{\text{MAX}} = 24.0$

| [C] (µM) | $\log_{10}[C]$ | Response (RU) |
|----------|----------------|---------------|
| 14.063   | -4.85          | 2.5           |
| 28.125   | -4.55          | 2.6           |
| 56.250   | -4.25          | 3.1           |
| 112.500  | -3.95          | 3.0           |
| 225.000  | -3.65          | 3.8           |
| 450.000  | -3.35          | 4.5           |
| 900.000  | -3.05          | 7.2           |

Data could not be fitted. Responses increase with concentration, but too far from the theoretical $R_{\text{MAX}}$. $K_D \gg 1.0 \text{ mM}$. 
Blank and reference surface subtracted responses according to the concentration of spy molecule.

Theoretical $R_{\text{MAX}} = 26.6$

| [C] (µM) | $\log_{10}([C])$ | Response (RU) |
|----------|------------------|---------------|
| 14.063   | -4.85            | 3.2           |
| 28.125   | -4.55            | 3.3           |
| 56.250   | -4.25            | 4.3           |
| 112.500  | -3.95            | 5.3           |
| 225.000  | -3.65            | 6.3           |
| 450.000  | -3.35            | 8.9           |
| 900.000  | -3.05            | 13.9          |

Data fitting using the Biacore T200 evaluation software. As responses were lower than the theoretical $R_{\text{MAX}}$, fitting was performed with a fixed $R_{\text{MAX}}$.

| $K_D$ (µM) | SE ($K_D$) | $R_{\text{MAX}}$ (RU) | SE($R_{\text{MAX}}$) | offset (RU) | SE(offset) | Chi² (RU²) |
|------------|------------|------------------------|----------------------|--------------|------------|------------|
| 1352       | 94         | 26.6                   | -                    | 2.9          | 0.24       | 0.159      |
Spy molecule 11 (Compound S5c)

Blank and reference surface subtracted responses according to the concentration of spy molecule.

Theoretical $R_{\text{MAX}} = 29.8$

| [C] (µM) | $\log_{10}[C]$ | Response (RU) |
|----------|-----------------|---------------|
| 6.250    | -5.20           | 5.5           |
| 12.500   | -4.90           | 6.2           |
| 25.000   | -4.60           | 8.2           |
| 50.000   | -4.30           | 11.9          |
| 100.000  | -4.00           | 14.4          |
| 200.000  | -3.70           | 18.0          |
| 400.000  | -3.40           | 21.5          |
| 800.000  | -3.10           | 24.0          |

Data fitting using the Biacore T200 evaluation software:

| $K_D$ (µM) | SE ($K_D$) | $R_{\text{MAX}}$ (RU) | SE($R_{\text{MAX}}$) | Offset (RU) | SE(Offset) | Chi$^2$ (RU$^2$) |
|------------|------------|------------------------|-----------------------|-------------|------------|-----------------|
| 109.7      | 8.9        | 23.5                   | 0.43                  | 4           | 0.31       | 0.11            |
Spy molecule 12 (Compound S6c)

Blank and reference surface subtracted responses according to the concentration of spy molecule.

Theoretical $R_{\text{MAX}} = 33.2$

| [C] (µM) | log$_{10}$[C] | Response (RU) |
|----------|----------------|---------------|
| 0.078    | -7.11          | 6.2           |
| 0.156    | -6.81          | 8.4           |
| 0.313    | -6.51          | 10.7          |
| 0.625    | -6.20          | 14.9          |
| 1.250    | -5.90          | 19.2          |
| 2.500    | -5.60          | 24.0          |
| 5.000    | -5.30          | 26.8          |
| 10.000   | -5.00          | 30.1          |

Data fitting using the Biacore T200 evaluation software:

| $K_D$ (µM) | SE ($K_D$) | $R_{\text{MAX}}$ (RU) | SE($R_{\text{MAX}}$) | offset (RU) | SE(offset) | $\chi^2$ (RU$^2$) |
|------------|------------|------------------------|----------------------|-------------|------------|-------------------|
| 1.142      | 0.120      | 26.8                   | 0.61                 | 4.9         | 0.51       | 0.255             |
Spy molecule 13 (Compound S3d)

Blank and reference surface subtracted responses according to the concentration of spy molecule.

Theoretical $R_{\text{MAX}} = 24.3$

| [C] (µM) | $\log_{10}[C]$ | Response (RU) |
|----------|----------------|---------------|
| 14.063   | -4.85          | 3.8           |
| 28.125   | -4.55          | 4.9           |
| 56.250   | -4.25          | 6.4           |
| 112.500  | -3.95          | 8.4           |
| 225.000  | -3.65          | 11.1          |
| 450.000  | -3.35          | 12.8          |
| 900.000  | -3.05          | 22.2          |

Data fitting using the Biacore T200 evaluation software: The fitted $K_D$ presented a very large error and high $R_{\text{MAX}}$. The results not significant even using different fitting methods (fixing $R_{\text{MAX}}$ or the offset). Large difference in response between concentrations of 450 and 900 µM might indicate unspecific / promiscuous binding to the protein surface. Similar result obtained from different repeats of different stocks of the compound.
Spy molecule 14 (Compound S4d)

Blank and reference surface subtracted responses according to the concentration of spy molecule.

Theoretical $R_{MAX} = 26.8$

| [C] (µM) | $\log_{10}[C]$ | Response (RU) |
|----------|----------------|---------------|
| 6.250    | -5.20          | 5.5           |
| 12.500   | -4.90          | 6.7           |
| 25.000   | -4.60          | 10.1          |
| 50.000   | -4.30          | 14.2          |
| 100.000  | -4.00          | 19.8          |
| 200.000  | -3.70          | 25.1          |
| 400.000  | -3.40          | 31.0          |
| 800.000  | -3.10          | 40.3          |

Data fitting using the Biacore T200 evaluation software: The only acceptable fitting resulted in a very large $R_{MAX}$. (1.6 times larger than the theoretical). Fitting with fixed theoretical $R_{MAX}$ presented a large error in the $K_D$. Compound might bind to the VHL-HIF site, but also bind unspecifically / promiscuously to the protein surface.
Blank and reference surface subtracted responses according to the concentration of spy molecule.

\[ \text{Theoretical } R_{\text{MAX}} = 30.0 \]

| [C] (µM) | \( \log_{10}[C] \) | Response (RU) |
|----------|----------------|--------------|
| 1.563    | -5.81          | 6.3          |
| 3.125    | -5.51          | 8.5          |
| 6.250    | -5.20          | 10.4         |
| 12.500   | -4.90          | 15.7         |
| 25.000   | -4.60          | 19.0         |
| 50.000   | -4.30          | 23.3         |
| 100.000  | -4.00          | 28.7         |
| 200.000  | -3.70          | 33.7         |

Data fitting using the Biacore T200 evaluation software:

| \( K_D \) (µM) | SE (\( K_D \)) | \( R_{\text{MAX}} \) (RU) | SE(\( R_{\text{MAX}} \)) | offset (RU) | SE(offset) | \( \text{Chi}^2 \) (RU²) |
|----------------|---------------|-----------------|----------------|-------------|------------|-------------------|
| 35.16          | 7.00          | 31.8            | 1.5            | 5.7         | 0.92        | 1.13              |
Spy molecule 16 (Compound S6d)

Blank and reference surface subtracted responses according to the concentration of spy molecule.

Theoretical R\textsubscript{MAX} = 33.3

| [C] (µM) | \(\log_{10}[C]\) | Response (RU) |
|----------|-----------------|-------------|
| 0.039    | -7.41           | 10.4        |
| 0.078    | -7.11           | 13.6        |
| 0.156    | -6.81           | 18.2        |
| 0.313    | -6.51           | 23.1        |
| 0.625    | -6.20           | 27.2        |
| 1.250    | -5.90           | 30.8        |
| 2.500    | -5.60           | 33.2        |
| 5.000    | -5.30           | 35.8        |

Data fitting using the Biacore T200 evaluation software:

| \(K_D\) (µM) | SE (\(K_D\)) | \(R_{\text{MAX}}\) (RU) | SE(\(R_{\text{MAX}}\)) | offset (RU) | SE(offset) | \(\text{Chi}^2\) (RU²) |
|--------------|--------------|--------------------------|--------------------------|-------------|------------|-------------------------|
| 0.2675       | 0.0300       | 29.3                     | 0.74                     | 6.9         | 0.82       | 0.319                   |
Spy molecule 17 (Compound S10a)

Blank and reference surface subtracted responses according to the concentration of spy molecule.

**Theoretical R\textsubscript{MAX} = 24.1**

| [C] (µM) | log\textsubscript{10}[C] | Response (RU) |
|----------|-----------------|---------------|
| 7.031    | -5.15           | 3.9           |
| 14.063   | -4.85           | 4.6           |
| 28.125   | -4.55           | 5.2           |
| 56.250   | -4.25           | 7.7           |
| 112.500  | -3.95           | 8.7           |
| 225.000  | -3.65           | 10.3          |
| 450.000  | -3.35           | 13.2          |
| 900.000  | -3.05           | 19.6          |

Data fitting using the Biacore T200 evaluation software. As responses were lower than the theoretical R\textsubscript{MAX}, fitting was performed with a fixed R\textsubscript{MAX}.

| K\textsubscript{D} (µM) | SE (K\textsubscript{D}) | R\textsubscript{max} (RU) | SE(R\textsubscript{max}) | offset (RU) | SE(offset) | Ch\textsuperscript{2} (RU\textsuperscript{2}) |
|-------------------------|------------------------|---------------------------|--------------------------|-------------|------------|-----------------------------------|
| 645.0                   | 100.0                  | 24.1                      | -                        | 4.4         | 0.58       | 0.985                             |
Spy molecule 18 (Compound S10b)
Blank and reference surface subtracted responses according to the concentration of spy molecule.

Theoretical $R_{\text{MAX}} = 24.7$

| [C] (µM) | $\log_{10}[C]$ | Response (RU) |
|----------|----------------|---------------|
| 1.172    | -5.93          | 7.1           |
| 2.344    | -5.63          | 7.9           |
| 4.688    | -5.33          | 11.3          |
| 9.375    | -5.03          | 13.5          |
| 18.750   | -4.73          | 18.3          |
| 37.500   | -4.43          | 22.6          |
| 75.000   | -4.12          | 26.1          |
| 150.000  | -3.82          | 30.5          |

Data fitting using the Biacore T200 evaluation software:

| $K_D$ (µM) | SE ($K_D$) | $R_{\text{MAX}}$ (RU) | SE($R_{\text{MAX}}$) | Offset (RU) | SE(offset) | Chi² (RU²) |
|------------|------------|-----------------------|----------------------|-------------|------------|------------|
| 24.76      | 3.40       | 27.8                  | 0.92                 | 6.00        | 0.58       | 0.43       |
Spy molecule 19 (Compound S11a)

Blank and reference surface subtracted responses according to the concentration of spy molecule.

Theoretical $R_{\text{MAX}} = 24.7$

| [C] (µM) | $\log_{10}[C]$ | Response (RU) |
|----------|----------------|---------------|
| 6.250    | -5.20          | 5.3           |
| 12.500   | -4.90          | 6.6           |
| 25.000   | -4.60          | 8.8           |
| 50.000   | -4.30          | 11.6          |
| 100.000  | -4.00          | 15.3          |
| 200.000  | -3.70          | 19.4          |
| 400.000  | -3.40          | 21.9          |
| 800.000  | -3.10          | 27.2          |

Data fitting using the Biacore T200 evaluation software:

| $K_D$ (µM) | SE ($K_D$) | $R_{\text{MAX}}$ (RU) | SE($R_{\text{MAX}}$) | offset (RU) | SE(offset) | $\text{Chi}^2$(RU²) |
|------------|------------|------------------------|-----------------------|-------------|------------|---------------------|
| 144.8      | 29.0       | 25.4                   | 1.3                   | 4.7         | 0.73       | 0.727               |
Spy molecule 20 (Compound S11b)

Blank and reference surface subtracted responses according to the concentration of spy molecule.

Theoretical $R_{\text{MAX}} = 25.4$

| [C] (µM) | $\log_{10}$[C] | Response (RU) |
|----------|----------------|--------------|
| 0.977    | -6.01          | 6.4          |
| 1.953    | -5.71          | 9.1          |
| 3.906    | -5.41          | 11.7         |
| 7.813    | -5.11          | 16.1         |
| 15.625   | -4.81          | 20.0         |
| 31.250   | -4.51          | 23.6         |
| 62.500   | -4.20          | 27.0         |
| 125.000  | -3.90          | 30.5         |

Data fitting using the Biacore T200 evaluation software:

| $K_D$ (µM) | SE ($K_D$) | $R_{\text{MAX}}$ (RU) | SE($R_{\text{MAX}}$) | offset (RU) | SE(offset) | $\chi^2$ (RU²) |
|------------|------------|------------------------|-----------------------|--------------|------------|----------------|
| 12.41      | 1.90       | 27.1                   | 0.86                  | 5.00         | 0.77       | 0.53           |
Spy molecule 21 (Compound S12a)

Blank and reference surface subtracted responses according to the concentration of spy molecule.

Theoretical $R_{\text{MAX}} = 30.5$

| [C] (µM) | $\log_{10}[C]$ | Response (RU) |
|----------|----------------|--------------|
| 0.023    | -7.63          | 5.9          |
| 0.047    | -7.33          | 7.9          |
| 0.094    | -7.03          | 10.8         |
| 0.188    | -6.73          | 13.7         |
| 0.375    | -6.43          | 16.9         |
| 0.750    | -6.12          | 20.5         |
| 1.500    | -5.82          | 24.6         |
| 3.000    | -5.52          | 28.3         |

Data fitting using the Biacore T200 evaluation software:

| $K_D$ (µM) | SE ($K_D$) | $R_{\text{MAX}}$ (RU) | SE($R_{\text{MAX}}$) | offset (RU) | SE(offset) | Chi² (RU²) |
|------------|------------|------------------------|-----------------------|-------------|------------|------------|
| 0.4468     | 0.0850     | 25.4                   | 1.1                   | 5.5         | 0.76       | 0.692      |
Spy molecule 22 (Compound S12b)

Blank and reference surface subtracted responses according to the concentration of spy molecule.

Theoretical $R_{\text{MAX}} = 36.6$

| [C] (µM) | $\log_{10}[C]$ | Response (RU) |
|----------|----------------|---------------|
| 0.0039   | -8.41          | 6.1           |
| 0.0078   | -8.11          | 7.1           |
| 0.0156   | -7.81          | 9.9           |
| 0.0313   | -7.51          | 13.5          |
| 0.0625   | -7.20          | 18.9          |
| 0.1250   | -6.90          | 25.0          |
| 0.2500   | -6.60          | 30.0          |
| 0.5000   | -6.30          | 35.4          |

Data fitting using the Biacore T200 evaluation software:

| $K_D$ (µM) | SE ($K_D$) | $R_{\text{MAX}}$ (RU) | SE($R_{\text{MAX}}$) | Offset (RU) | SE(offset) | Chi² (RU²) |
|------------|------------|------------------------|-----------------------|-------------|------------|------------|
| 0.09693    | 0.00680    | 36.9                   | 0.65                  | 4.6         | 0.35       | 0.178      |
8. $^{19}$F CPMG signal intensity versus CPMG filter

8.1 Spy molecules at 100 μM in absence or in presence of VBC 1 μM

\[
\text{Contrast} = R_2 \text{ contrast} - C_2 \%
\]

|                | Spy molecule 100 μM | Spy molecule 100 μM + VBC 1 μM |
|----------------|---------------------|-------------------------------|
| $R_2$ (s$^{-1}$) | 1.069 ± 0.028       | 1.115 ± 0.020                 |
| R-square       | 0.995               | 0.998                         |
| $d_{\text{max}}$ (s) | 0.916          | 0.826                         |
**SUPPORTING INFORMATION**

**Spy molecule 100 μM**
- $R_2$ (s$^{-1}$): 1.326 ± 0.025
- R-square: 0.998

**Spy molecule 100 μM + VBC 1 μM**
- $R_2$ (s$^{-1}$): 1.847 ± 0.033
- R-square: 0.998

| Contrast      | $R_2$ contrast – $C_2$ (%) | $d_{max}$ (s) |
|---------------|-----------------------------|---------------|
| Spy molecule 100 μM | 28.2 ± 2.3                 | 0.636         |

**Spy molecule 100 μM**
- $R_2$ (s$^{-1}$): 1.175 ± 0.027
- R-square: 0.996

**Spy molecule 100 μM + VBC 1 μM**
- $R_2$ (s$^{-1}$): 1.924 ± 0.037
- R-square: 0.998

| Contrast      | $R_2$ contrast – $C_2$ (%) | $d_{max}$ (s) |
|---------------|-----------------------------|---------------|
| Spy molecule 100 μM | 38.9 ± 2.5                 | 0.658         |
Spy molecule 100 μM

- $R_2 (s^{-1})$: 0.915 ± 0.012
- R-square: 0.999

Spy molecule 100 μM + VBC 1 μM

- $R_2 (s^{-1})$: 0.936 ± 0.011
- R-square: 0.999

**Contrast**

- $R_2$ contrast – $C_2$ (%): 2.2 ± 1.8
- $d_{max}$ (s): 1.080

Spy molecule 100 μM

- $R_2 (s^{-1})$: 1.096 ± 0.020
- R-square: 0.998

Spy molecule 100 μM + VBC 1 μM

- $R_2 (s^{-1})$: 1.760 ± 0.039
- R-square: 0.997

**Contrast**

- $R_2$ contrast – $C_2$ (%): 37.7 ± 2.6
- $d_{max}$ (s): 0.713
Spy molecule 100 μM

R₂ (s⁻¹) 1.368 ± 0.033
R-square 0.998

Spy molecule 100 μM + VBC 1 μM

R₂ (s⁻¹) 3.614 ± 0.088
R-square 0.999

Contrast

R₂ contrast – C₂ (%) 62.1 ± 3.0
Dₘₐₓ (s) 0.433

Spy molecule 100 μM

R₂ (s⁻¹) 1.349 ± 0.027
R-square 0.997

Spy molecule 100 μM + VBC 1 μM

R₂ (s⁻¹) 1.639 ± 0.022
R-square 0.999

Contrast

R₂ contrast – C₂ (%) 17.7 ± 2.2
Dₘₐₓ (s) 0.671
Spy molecule 100 μM

R₂ (s⁻¹) 1.268 ± 0.026
R-square 0.997

Spy molecule 100 μM + VBC 1 μM

R₂ (s⁻¹) 1.279 ± 0.022
R-square 0.998

Contrast

R₂ contrast – C₂ (%) 0.9 ± 2.7

dₘₐₓ (s) 0.785

Spy molecule 100 μM

R₂ (s⁻¹) 1.249 ± 0.018
R-square 0.999

Spy molecule 100 μM + VBC 1 μM

R₂ (s⁻¹) 2.596 ± 0.046
R-square 0.999

Contrast

R₂ contrast – C₂ (%) 51.9 ± 2.1

dₘₐₓ (s) 0.543
Spy molecule 100 μM

- \( R_2 (s^{-1}) \): 1.427 ± 0.033
- R-square: 0.997

Spy molecule 100 μM + VBC 1 μM

- \( R_2 (s^{-1}) \): 2.740 ± 0.096
- R-square: 0.995

Contrast

- R₂ contrast – C₂ (%): 47.9 ± 4.1
- \( d_{\text{max}} \) (s): 0.497

Spy molecule 100 μM

- \( R_2 (s^{-1}) \): 1.512 ± 0.029
- R-square: 0.998

Spy molecule 100 μM + VBC 1 μM

- \( R_2 (s^{-1}) \): 1.637 ± 0.028
- R-square: 0.998

Contrast

- R₂ contrast – C₂ (%): 7.6 ± 2.4
- \( d_{\text{max}} \) (s): 0.635
Spy molecule 100 μM
\[ R_2 (s^{-1}) \] 1.558 ± 0.028
\[ \text{R-square} \] 0.998

Spy molecule 100 μM + VBC 1 μM
\[ R_2 (s^{-1}) \] 1.609 ± 0.017
\[ \text{R-square} \] 0.999

Contrast
\[ R_2 \text{ contrast} – C_2 \% \] 3.2 ± 2.0
\[ d_{max} \text{ (s)} \] 0.632

Spy molecule 100 μM
\[ R_2 (s^{-1}) \] 1.579 ± 0.022
\[ \text{R-square} \] 0.999

Spy molecule 100 μM + VBC 1 μM
\[ R_2 (s^{-1}) \] 3.425 ± 0.073
\[ \text{R-square} \] 0.998

Contrast
\[ R_2 \text{ contrast} – C_2 \% \] 53.9 ± 2.5
\[ d_{max} \text{ (s)} \] 0.419
Spy molecule 100 μM

| Parameter          | Value         |
|--------------------|---------------|
| $R_2$ (s$^{-1}$)   | 1.545 ± 0.035 |
| R-square           | 0.997         |

Spy molecule 100 μM + VBC 1 μM

| Parameter          | Value         |
|--------------------|---------------|
| $R_2$ (s$^{-1}$)   | 2.634 ± 0.058 |
| R-square           | 0.998         |

Contrast

| Parameter          | Value         |
|--------------------|---------------|
| $R_2$ contrast – $C_2$ (%) | 41.3 ± 2.7   |
| $d_{max}$ (s)      | 0.490         |

Spy molecule 100 μM

| Parameter          | Value         |
|--------------------|---------------|
| $R_2$ (s$^{-1}$)   | 1.805 ± 0.032 |
| R-square           | 0.998         |

Spy molecule 100 μM + VBC 1 μM

| Parameter          | Value         |
|--------------------|---------------|
| $R_2$ (s$^{-1}$)   | 1.828 ± 0.031 |
| R-square           | 0.999         |

Contrast

| Parameter          | Value         |
|--------------------|---------------|
| $R_2$ contrast – $C_2$ (%) | 1.3 ± 2.4   |
| $d_{max}$ (s)      | 0.551         |
Spy molecule 100 μM
\[ R_2 \text{ (s}^{-1}) \quad 1.213 \pm 0.028 \]
R-square \[ 0.996 \]

Spy molecule 100 μM + VBC 1 μM
\[ R_2 \text{ (s}^{-1}) \quad 3.189 \pm 0.066 \]
R-square \[ 0.998 \]

Contrast
\[ R_2 \text{ contrast – } C_2 \% \quad 62.0 \pm 2.6 \]
\[ d_{\text{max}} \text{ (s)} \quad 0.489 \]

Spy molecule 100 μM
\[ R_2 \text{ (s}^{-1}) \quad 1.167 \pm 0.014 \]
R-square \[ 0.999 \]

Spy molecule 100 μM + VBC 1 μM
\[ R_2 \text{ (s}^{-1}) \quad 4.858 \pm 0.123 \]
R-square \[ 0.998 \]

Contrast
\[ R_2 \text{ contrast – } C_2 \% \quad 76.0 \pm 3.2 \]
\[ d_{\text{max}} \text{ (s)} \quad 0.386 \]
Spy molecule 100 μM

\[ R_2 (s^{-1}) \] = 1.526 ± 0.041

R-square = 0.996

Spy molecule 100 μM + VBC 1 μM

\[ R_2 (s^{-1}) \] = 5.096 ± 0.186

R-square = 0.995

Contrast

\[ R_2 \text{ contrast} - C_2 \text{ (%)} \] = 70.1 ± 4.5

\[ d_{\text{max}} (s) \] = 0.338

Spy molecule 100 μM

\[ R_2 (s^{-1}) \] = 1.609 ± 0.042

R-square = 0.996

Spy molecule 100 μM + VBC 1 μM

\[ R_2 (s^{-1}) \] = 3.116 ± 0.153

R-square = 0.990

Contrast

\[ R_2 \text{ contrast} - C_2 \text{ (%)} \] = 48.4 ± 5.6

\[ d_{\text{max}} (s) \] = 0.439
Spy molecule 100 μM

\[ R_2 (s^{-1}) = 3.959 \pm 0.262 \]
\[ \text{R-square} = 0.970 \]

Spy molecule 100 μM + VBC 1 μM

\[ R_2 (s^{-1}) = 4.109 \pm 0.247 \]
\[ \text{R-square} = 0.976 \]

Contrast

\[ R_2 \text{ contrast} - C_2 (%) = 3.7 \pm 8.8 \]
\[ d_{\text{max}} (s) = 0.248 \]

Spy molecule 100 μM

\[ R_2 (s^{-1}) = 3.987 \pm 0.241 \]
\[ \text{R-square} = 0.975 \]

Spy molecule 100 μM + VBC 1 μM

\[ R_2 (s^{-1}) = 4.018 \pm 0.245 \]
\[ \text{R-square} = 0.974 \]

Contrast

\[ R_2 \text{ contrast} - C_2 (%) = 0.8 \pm 8.6 \]
\[ d_{\text{max}} (s) = 0.250 \]
8.2 Spy molecule 19 and VBC at different concentrations

### Spy molecule 19 at 50 µM

| Parameter | [VBC] µM | 0.000 | 0.125 | 0.250 | 0.500 | 1.000 |
|-----------|----------|-------|-------|-------|-------|-------|
| $R_2$ (s⁻¹) | 1.475 ± 0.059 | 1.969 ± 0.071 | 2.316 ± 0.168 | 3.857 ± 0.121 | 6.424 ± 0.262 |
| R-square | 0.986 | 0.991 | 0.967 | 0.995 | 0.993 |
| $R_2$ contrast – $C_2$ (%) | - | 22.5 ± 4.2 | 34.1 ± 7.9 | 60.4 ± 3.8 | 76.2 ± 5.2 |
| $d_{max}$ (s) | - | 0.575 | 0.528 | 0.398 | 0.293 |

### Spy molecule 19 at 25 µM

| Parameter | [VBC] µM | 0.000 | 0.125 | 0.250 | 0.500 | 1.000 |
|-----------|----------|-------|-------|-------|-------|-------|
| $R_2$ (s⁻¹) | 1.582 ± 0.049 | 2.107 ± 0.095 | 2.811 ± 0.156 | 4.008 ± 0.200 | 6.934 ± 0.348 |
| R-square | 0.992 | 0.986 | 0.984 | 0.989 | 0.989 |
| $R_2$ contrast – $C_2$ (%) | - | 27.6 ± 5.1 | 45.7 ± 6.3 | 61.9 ± 5.9 | 78.0 ± 6.4 |
| $d_{max}$ (s) | - | 0.555 | 0.475 | 0.389 | 0.280 |
**Supporting Information**

**Spy molecule 19 at 10 \( \mu \text{M} \)**

| Parameter                        | \([\text{VBC}] \, \mu\text{M}\) |
|----------------------------------|-----------------------------------|
|                                  | 0.000    | 0.125    | 0.250    | 0.500    | 1.000    |
| \(R_z\) (s\(^{-1}\))            | 1.464 ± 0.096 | 2.346 ± 0.193 | 2.807 ± 0.131 | 4.288 ± 0.304 | 7.974 ± 0.727 |
| R-square                         | 0.962     | 0.961     | 0.988     | 0.979     | 0.967     |
| \(R_2\) contrast – \(C_2\) (%)   | -         | 35.0 ± 8.9 | 45.6 ± 5.3 | 64.4 ± 8.5 | 80.9 ± 11.7 |
| \(d_{\text{max}}\) (s)           | -         | 0.524     | 0.476     | 0.374     | 0.256     |
8.3 Spy molecules 6 and 11 at 50 μM in absence or in presence of VBC 1 μM

**Spy molecule 100 μM**

| Parameter          | Value         |
|--------------------|---------------|
| $R_2$ (s$^{-1}$)   | $1.255 \pm 0.043$ |
| R-square           | 0.989         |

**Spy molecule 100 μM + VBC 1 μM**

| Parameter          | Value         |
|--------------------|---------------|
| $R_2$ (s$^{-1}$)   | $1.951 \pm 0.062$ |
| R-square           | 0.994         |

**Contrast**

| Parameter          | Value         |
|--------------------|---------------|
| $R_2$ contrast – $C_2$ (%) | $35.7 \pm 4.0$ |
| $d_{max}$ (s)      | 0.634         |

**Spy molecule 100 μM**

| Parameter          | Value         |
|--------------------|---------------|
| $R_2$ (s$^{-1}$)   | $1.534 \pm 0.071$ |
| R-square           | 0.984         |

**Spy molecule 100 μM + VBC 1 μM**

| Parameter          | Value         |
|--------------------|---------------|
| $R_2$ (s$^{-1}$)   | $3.122 \pm 0.145$ |
| R-square           | 0.990         |

**Contrast**

| Parameter          | Value         |
|--------------------|---------------|
| $R_2$ contrast – $C_2$ (%) | $50.9 \pm 5.7$ |
| $d_{max}$ (s)      | 0.447         |
9. NMR spectra of synthesized compounds

**Compound S1b**

NMR spectra of synthesized compounds.

---

**Current Data Parameters**

- **NAME**: 9GC-10-0654
- **EXPROD**: 12
- **FNAME**: 1

**F2 - Acquisition Parameters**

- **Data**: 20150313
- **Time**: 10.14
- **INTER**: 1.00
- **Program**: 5 mm Pake 2.89
- **Pulse**: 1.00 ms
- **FIDLE**: 1.00 ms
- **SOLVENT**: DMSO
- **NS**: 16
- **DS**:
  - **SN**: 3,501.23 Hz
  - **TUBERS**: 3.50123 Hz
  - **AQ**: 3.50139 Hz
  - **DN**: 41.02 Hz
  - **DG**: 11.7 Hz
  - **DF**:
  - **TD**: 0.100 Hz

**------------- CHANNEL F1 -------------**

- **Sample**: 400.1204 Hz
- **TD**: 10.0 Hz
- **PLMS**: 20.0000 Hz

**F1 - Processing parameters**

- **SP**: 400.1204 Hz
- **MTR**: 18
- **HIM**: 0
- **DB**: 0.11 Hz
- **PC**: 1.00
Compound S1b

Current Data Parameters
NAME  ALC-GC-R044
EXPNO  14
PROCNO  1

F2 - Acquisition Parameters
Date_  20160511
Time_  19.04
INSTRUM_ spect
PROBND_ 5 mm PADDY1 JC
PULPROG_ zgpg30
T0_ 119.44
SOLVENT_ CDCl3
NS_ 512
DS_ 4
SWH_ 25000.000 Hz
F1WINS_ 0.2120066 Hz
AQ_ 2.3000000 sec
RG_ 136.14
DW_ 25.000 usec
DE_ 7.92 usec
TE_ 298.2 K
DI_ 1.0000000 sec
D1_ 0.0300000 sec
TD0_ 1

----------- CHANNEL f1 -----------
SFO1_ 100.623851 MHz
NUC1_ 13C
F1_ 10.00 usec
PLM1_ 36.0000000 W

----------- CHANNEL f2 -----------
SFO2_ 400.1316065 MHz
NUC2_ 1H
CDEPRG[2]_ waltz64
FCEPD2_ 90.00 usec
PLM2_ 20.0000000 W
PLM12_ 0.2460001 W
PLM13_ 0.2000000 W

F2 - Processing parameters
SI_ 131072
SP_ 100.6127525 MHz
MOE_ EM
USB_ 0
LA_ 1.00 Hz
GB_ 0
PC_ 1.40
SUPPORTING INFORMATION

Compound S2a

Current Data Parameters
NAME          GC-S2A-F1
EXPNO         10
PROCNO        1

F2 - Acquisition Parameters
Date          20181126
Time          9.55
INSTROM       spect
PROBMD        5 mm PABBO DB/
PULPROG       zgfhiquq.2
TO            131072
SOLVENT       MeOD
NS            16
DS            4
SWH           113636.367 Hz
PIDRXS        0.866977 Hz
AQ            0.0767168 sec
KG            322
DW            4.400 usec
DE            6.50 usec
TE            303.4 K
D1            1.00000000 sec
D11           0.03000000 sec
D12           0.00002000 sec
TD0           1

-------- CHANNEL f1 --------
SP01         470.5453180 MHz
NUC1         19F
FR           14.50 usec
PLW1         45.00000000 W

-------- CHANNEL f2 --------
SP02         500.1320056 MHz
NUC2         1H
CPRESS[2]    waltz16
PDCP2        80.00 usec
PLW2         20.850000038 W
PLW12        0.359169999 W

F2 - Processing parameters
SI            65536
SP            470.5923772 MHz
MOQ          RH
SSB           0
LA            0.30 Hz
PC            1.00
Compound S2b

Current Data Parameters
NAME GC-32B-2
EXPNO 11
PROCNO 1

F2 - Acquisition Parameters
Date_ 20181126
Time 10.29
INSTRUM spect
PROBMD 5 mm PABBO DB/
PULPROG zgfhig2n.2
TO 111072
SOLVENT MeOD
NS 16
DS 4
SWH 113636.36 Hz
F1DRXN 8.866977 Hz
AQ 0.5767168 sec
DG 322
DW 4400 usec
DE 650 usec
TE 303.4 K
D1 1.00000000 sec
D11 0.03000000 sec
D12 0.00002000 sec
TD0 1

======== CHANNEL f1 ========
SP01 5453180 MHz
NUC1 19F
F1 14.50 usec
PLW1 45.00000000 W

======== CHANNEL f2 ========
SP02 59001320055 MHz
NUC2 1H
CPFPAG[2] waltz16
PCPF2 80.00 usec
PLK2 20.85000038 W
PLW1 39169999 W

F2 - Processing parameters
SI 65536
SP 470.5923772 MHz
MOW 127
SSB 0
LS 0.03 Hz
GB 0
PC 1.00
Compound S2c

Current Data Parameters
NAME GC-S2c
EXPNO 11
PROCNO 1

F2 - Acquisition Parameters
Date_ 20181126
Time 11:50
INSTROM spect
PROBMD 5 mm CPQC1 1H/
PULPROG zgpg10
TO 55400
SOLVENT MeOD
NS 2048
DS 4
SWH 29761.904 Hz
FIDRMS 0.425575 Hz
AQ 1.0987200 sec
RG 190.78
DW 16.800 usec
DE 18.00 usec
TE 303.1 K
D1 2.00000000 sec
D11 0.03000000 sec
TD0 1

======== CHANNEL f1 ========
SFO1 125.7703637 MHz
NUC1 13C
P1 12.00 usec
PLM1 133.00000000 W

======== CHANNEL f2 ========
SFO2 500.1320005 MHz
NUC2 1H
CPDPRG1 1H
F1FD2 100.00 usec
PLM2 5.19999981 W
PLM12 0.06406900 W
PLM13 0.06406900 W

F2 - Processing parameters
SI 131072
SP 125.7575591 MHz
WOW EM
SUB 0
LA 0
GB 0
PEC 1.40
Compound S2c

Current Data Parameters
NAME: GC-S2C-GILSON
EXPNO: 11
PROCNO: 1

F2 - Acquisition Parameters
Date_._ 20181126
Time: 9.49
INSTRUM: spect
PROBMD: 5 mm PABBO DB/
PULPROG: zgfhigun.2
TO: 131.072
SOLVENT: MeOD
NS: 16
DS: 4
SWH: 113636.367 Hz
FIDRMS: 8.866977 Hz
AQ: 0.5767168 sec
RG: 322
DW: 4.400 usec
DE: 6.50 usec
TE: 303.4 K
DI: 1.0000000 sec
D11: 0.0300000 sec
D12: 0.0000000 sec
TD0: 1

======== CHANNEL f1 ========
SP01: 470.5453160 MHz
NUC1: 19F
F1: 14.50 usec
PLW1: 45.00000000 W

======== CHANNEL f2 ========
SP02: 500.1320065 MHz
NUC2: 1H
CPFINC[2] waltz16
CPD2: 80.00 usec
PLK2: 20.05000000 W
PLK12: 0.35916999 W

F2 - Processing parameters
SI: 65536
SP: 470.5923772 MHz
MOW: EM
SSB: 0
LA: 0.30 Hz
GB: 0
PC: 1.00
SUPPORTING INFORMATION

Compound S2d

Current Data Parameters
NAME ALC-DC-S2d
EXPNO 30
PROCNO 1

F2 – Acquisition Parameters
DATE 20181121
TIMA 5.25
INSTNM
INSTNR 5 mm PADC
PULPROG udept
TD 17996
SOLVENT Methanol
NS 5000
DS
SW 25000.000 Hz
FDRES 1.388188 Hz
AQ 0.3992290 sec
NS 396.14
DW 20.000 sec
DP 30.000 sec
ET 394.6 Hz
D1 3.0000000 sec
D12 0.0000000 sec
D2 200.0000000 sec
D2G 1

CHANNEL F1
SF01 100.6238346 MHz
MC1 1560
F1 10.0000000
F12 2000.000000
F14 500.000000
FF01 36.00000000 W
GREN051 Cpl6Chmp.4
SPO2 0.500
SDF051 0 Hz
SDFM01 5.50000007 W
SDFM01 0.500
SDFP05 0 Hz
SDFE 5.50000007 W

CHANNEL C2
SF02 400.1316006 MHz
MC02 16
CF2PG12 weiz84
FD01 90.0000000
F1Q01 40.00000000 W
F1M02 0.24441008 W

F2 – Processing parameters
SJ 28214
SF 100.6126170 MHz
SMR 0
LM 0
GN 2.00 Hz
FC 1.40

77
Compound S2d

Current Data Parameters
NAME GC-B049
EXPNO 2
PROCNO 1

F2 - Acquisition Parameters
Date_ 20160519
Time 13.52
INSTRUM spect
PROBMD 5 mm QNP 1H/13
FURLEQ zgfhigqr.2
TO 111072
SOLVENT MeOD
NS 16
DS 4
SWH 113636.367 Hz
P1DRX5 0.866977 Hz
AQ 0.0767168 sec
KG 263
DW 4.400 usec
DE 6.500 usec
TE 298.2 K
D1 1.00000000 sec
D11 0.03000000 sec
D12 0.00000000 sec
TD0 1

====== CHANNEL f1 ======
SP1 470.5433180 MHz
NUC1 19F
P1 13.500 usec
PLW1 25.00000000 W

====== CHANNEL f2 ======
SP2 500.1320055 MHz
NUC2 1H
C12F14C12 1.00000000 W
PCD2 80.000 usec
PLW2 25.00000000 W
PLW12 0.39063001 W

F2 - Processing parameters
SI 65536
SP 470.5923772 MHz
MOD EM
SSB 0
LA 0.30 Hz
GB 0
PC 1.00
Spy molecule 1 (Compound S3a)
Spy molecule 1 (Compound S3a)
Spy molecule 5 (Compound S3b)
Spy molecule 5 (Compound S3b)

Current Data Parameters
NAME: ALC-SC-0033
EXPN: 2
PROCNO: 1
F2 - Acquisition Parameters
DATA: 20160316
Mode: 5.10
INTGRM: Read
POLORD: 5 mm FID
PULPROG: adeq1
TD: 17946
SOLVENT: MeOD
N1: 5800
DS: 25000.000 Hz
PDRES: 1.289108 Hz
AQ: 0.3992900 sec
N0: 394.14
SW: 201.0000000000000 sec
TE: 290.2 sec
D1: 0.0000000000000 sec
D11: 0.0000000000000 sec
D20: 200.0000000000000 sec
DS: 1

---------- CHANNEL F1 ----------
SF01 100.623836 MHz
MC1 1000.0000000 sec
F1 10.00 sec
F13 2001.00 sec
F19 500.00 sec
FLM1 36.0000000 MHz
GSHIFT1 Cmpchamp.4
SPD2 2.500000
SPDP2 0.000 Hz
SPDP3 5.50040007 W
SPDN3 0.000 MHz
SPD2 2.50040007 W

---------- CHANNEL C2 ----------
SF02 400.1351605 MHz
MC2 1800.0000000 sec
CFDGR12 weitz04
PLD2 90.0000000 sec
PLA2 20.0000000000000 MHz
FLM12 0.24691000 MHz

F2 - Processing parameters
S2: 20121.0
SF: 100.6236170 MHz
SMM: 0.000 Hz
LS: 0
GR: 0.000 Hz
PC: 1.60
Spy molecule 5 (Compound S3b)

Current Data Parameters
NAME AC-GC-R038
EXPNO 1
PROCNO 1

F2 - Acquisition Parameters
Date_ 20160316
Time 14.35
INSTRUM spect
PROBMD 5 mm QNP 1H/13
PULPROG zgfhigqn.2
TD 11072
SOLVENT MeOD
NS 64
DS 4
SWH 113636.367 Hz
FIDWAX 8.866977 Hz
AQ 0.5767168 sec
KQ 812
DW 4.400 usec
DE 6.50 usec
TE 295.0 K
D1 1.00000000 sec
D11 0.03000000 sec
D12 0.00002000 sec
T0 1

=-=-=-=-=-=- CHANNEL f1 -=-=-=-=-=-=
SPO1 470.5453180 MHz
NUC1 19F
P1 13.50 usec
PLW1 25.00000000 W

=-=-=-=-=-=- CHANNEL f2 -=-=-=-=-=-=
SPO2 500.1320005 MHz
NUC2 1H
CF3FG[2] waltz16
PCPD2 80.00 usec
PLW2 25.00000000 W
PLW12 0.39063001 W

F2 - Processing parameters
SI 65536
SP 470.5923772 MHz
MD 470 E2
SSB 0
LA 0.30 Hz
GB 0
PC 1.00
Spy molecule 9 (Compound S3c)
Spy molecule 13 (Compound S3d)
Spy molecule 13 (Compound S3d)
Spy molecule 13 (Compound S3d)

Current Data Parameters
NAME  GC-RS53
EXPN0  4
PROCNO  1

F2 - Acquisition Parameters
Date_  20160530
Time  20.31
INSTRUM  spect
PROBMD  5 mm QNP 1H/13
FUPLP0RG  zgfhigqnc.2
TO  131072
SOLVENT  MeOD
NS  16
DS  4
SWH  113636.367 Hz
FIDDLY5  0.866977 Hz
AQ  0.5767168 sec
WG  362
DW  4.400 usec
DE  6.50 usec
TE  298.2 K
D1  1.0000000 sec
D11  0.0300000 sec
D12  0.0000200 sec
TD0  1

======== CHANNEL f1 ========
SP01  470.5453180 MHz
NUC1  19F
FI  13.50 usec
PLW1  25.00000000 W

======== CHANNEL f2 ========
SP02  500.1320005 MHz
NUC2  1H
CP8Pw[2  waltz16
CPD2  80.00 usec
PLW2  25.00000000 W
PLW12  0.39063001 W

F2 - Processing parameters
SI  65536
SP  470.5923772 MHz
MOD  1EM
SUB  0
LA  0.30 Hz
GB  0
PA  1.00
Spy molecule 2 (Compound S4a)
Spy molecule 2 (Compound S4a)
Spy molecule 2 (Compound S4a)
Spy molecule 6 (Compound S4b)
Spy molecule 6 (Compound S4b)
Spy molecule 10 (Compound S4c)
Spy molecule 10 (Compound S4c)
Spy molecule 14 (Compound S4d)
Spy molecule 14 (Compound S4d)

Current Data Parameters
NAME GC-R594
EXPNO 12
PROCNO 1

F2 - Acquisition Parameters
Date_ 20160530
Time 23:11
INSTRUM spect
PROBMD 5 mm QNP 18/13
PULPROG zgfix160.2
TO 131072
SOLVENT MeOD
N5 16
D5 4
SWH 113636.36 Hz
F1D4MX 5.8666777 Hz
AQ 0.9767168 sec
KG 362
DW 4.400 usec
DE 6.50 usec
TE 298.2 K
D1 1.0000000 sec
D11 0.03000000 sec
D12 0.00000000 sec
TD0 1

======== CHANNEL f1 ========
SFO1 470.5453160 MHz
NUC1 19F
F1 13.50 usec
PLW1 25.00000000 W

======== CHANNEL f2 ========
SFO2 590.1320005 MHz
NUC2 1H
CPPEF2[2] waltz16
PCPD2 80.00 usec
PLW2 25.00000000 W
PLW12 0.39063001 W

F2 - Processing parameters
SI 65536
SP 470.5923772 MHz
NOW 0.30 Hz
GB 0
PC 1.00
Spy molecule 7 (Compound S5b)
Spy molecule 7 (Compound S5b)
SUPPORTING INFORMATION

Spy molecule 11 (S5c)
Spy molecule 11 (Compound S5c)
Spy molecule 11 (Compound S5c)

Current Data Parameters
NAME: GCF11
EXPNO: 120
PROCNO: 1

F2 - Acquisition Parameters
Date: 20181114
Time: 11:14
INSTRUM: spect
PROBMD: 5 mm CPQC1 1H/
PULPROG: zgfhfqnq.2
TO: 131072
SOLVENT: MeOD
NS: 256
DS: 4
SWH: 113636.367 Hz
FIDNEX: 8.866977 Hz
AQ: 0.0767168 sec
KG: 190.78
DW: 4.000 usec
DE: 18.00 usec
TE: 293.1 K
D1: 1.00000000 sec
D11: 0.03000000 sec
D12: 0.00020000 sec
TD0: 1

======== CHANNEL f1 ========
SP01: 470.5453160 MHz
NUC1: 19F
F1: 12.00 usec
PLW1: 5.19999981 W

======== CHANNEL f2 ========
SP02: 500.1320005 MHz
NUC2: 1H
CP2FPG[2]: waltz16
PCPD2: 100.00 usec
PLW2: 5.19999981 W
PLW12: 0.06406900 W

F2 - Processing parameters
SI: 256244
SP: 470.5923772 MHz
MD: 128
SSB: 0
LB: 1.50 Hz
GB: 1.00
Spy molecule 15 (Compound S5d)
Spy molecule 4 (Compound S6a)
Spy molecule 4 (Compound S6a)
Spy molecule 4 (Compound S6a)
Spy molecule 8 (Compound S6b)
Spy molecule 8 (Compound S6b)
Spy molecule 8 (Compound S6b)

Bruker Data Parameters

Current Data Parameters
NAME  GCP08
EXPNO  70
PROCNO  1

F2 - Acquisition Parameters
Date_  20181114
Time  9.57
INSTRUM  spect
PROBMD  5 mm CPQC1 1H/
PULPROG  zgfhigqn.2
TO  111.072
SOLVENT  MeOD
NS  256
DS  4
SWH  13636.167 Hz
FIDNKS  8.866977 Hz
AQ  0.9767168 sec
KG  190.78
DW  4.400 usec
DE  18.00 usec
TE  293.2 K
D1  1.00000000 sec
D11  0.03000000 sec
D12  0.00002000 sec
D00  1

====== CHANNEL f1 ======
SP01  470.1453180 MHz
NUC1  19F
F1  12.00 usec
PLW1  5.19999981 W

====== CHANNEL f2 ======
SP02  500.1320005 MHz
NUC2  1H
CPFPAG[2  waltz16
PFPD2  100.00 usec
PLW2  5.19999991 W
PLW12  0.06406900 W

F2 - Processing parameters
ST  262144
SP  470.1923772 MHz
MOD  EM
SSB  0
LR  1.50 Hz
GB  0
PC  1.00
Spy molecule 12 (Compound S6c)
Spy molecule 12 (Compound S6c)

Current Data Parameters
NAME: GCF12
EXPNO: 84
PROCNO: 1

F2 - Acquisition Parameters
Date_: 20181117
Time: 1.17
INSTRTUM: spect
PROPBD: 5 mm CFCQI 1H/
PULPROG: zgpa90
TD: 65400
SOLVENT: MeOD
NS: 3000
DS: 4
SMH: 29761.994 Hz
FIDRES: 0.455075 Hz
AQ: 1.0987220 sec
RG: 190.78
DW: 16.800 usec
DE: 18.000 usec
TE: 293.1 K
D1: 2.00000000 sec
D11: 0.03000000 sec
TD0: 1

-------- CHANNEL f1 --------
SF01: 125.7703637 MHz
NUC1: 13C
P1: 22.000 usec
PLM1: 133.00000000 MHz

-------- CHANNEL f2 --------
SF02: 500.1320005 MHz
NUC2: 1H
CPDPRG[2]: 1H
CPDPD2: 100.000 usec
PLK2: 5.33999981 W
PLM2: 0.06406900 W
PLM3: 0.06406900 W

F2 - Processing parameters
SI: 131072
SF: 125.7575997 MHz
MDW: EM
SSB: 0
LB: 1.00 Hz
GB: 0
PC: 1.40
Spy molecule 12 (Compound S6c)

Current Data Parameters
NAME GCF12
EXPNO 80
PROCNO 1

F2 - Acquisition Parameters
Date_ 20181114
Time 10.16
INSTRUM spect
PROBMD 5 mm CPQC1 1H/
PULPROG zgfhigqn.2
TO 111072
SOLVENT MeOD
NS 256
DS 4
SWH 113636.367 Hz
FIDRES 8.866977 Hz
AQ 0.5767168 sec
KG 190.78
DW 4.400 usec
DE 18.00 usec
TE 293.1 K
D1 1.0000000 sec
D11 0.0300000 sec
D12 0.00002000 sec
T00 1

====== CHANNEL f1 ======
SP01 470.5453180 MHz
NUC1 19F
P1 12.00 usec
PLW1 5.19999981 W

====== CHANNEL f2 ======
SP02 500.1320005 MHz
NUC2 1H
CPFAG[2] waltz16
PCPD2 120.00 usec
PLK2 5.19999981 W
PLW12 0.06406900 W

F2 - Processing parameters
SI 262144
SP 470.5923772 MHz
MOW EM
SSB 0
LA 1.50 Hz
GB 0
DC 1.00
Spy molecule 16 (Compound S6d)
Spy molecule 16 (Compound S6d)

Current Data Parameters
NAME       GC-R558
EXPNO      27
PROCNO     1

F2 - Acquisition Parameters
Date_      20160531
Time       2.19
INSTROK    spect
PROCMD      5 mm QNP 1H/13
FUPROG     zgfhigq0.2
TO         131.072
SOLVENT    MeOD
NS         16
DS         4
SWH        113636.367 Hz
FIDFRQ     2.8669777 Hz
AQ         0.9767168 sec
WG         322
DW         4.400 usec
DE         6.50 usec
TE         298.2 K
DI         1.0000000 sec
D11        0.0300000 sec
D12        0.00002000 sec
TD0        1

=-=-=-=-= CHANNEL f1 ==-=-=-=-
SP01       470.543160 MHz
NUC1       19F
P1         13.50 usec
PLW1       25.00000000 W

=-=-=-=-= CHANNEL f2 ==-=-=-=-
SP02       590.1320005 MHz
NUC2       1H
CPFPBK[2]  waltz16
PCPD2      80.00 usec
PLW2       25.00000000 W
PLW12      0.39063001 W

F2 - Processing parameters
SI         65536
SP         470.5923772 MHz
MOD        EM
-SSB       0
LB         0.30 Hz
GB         0
FC         1.00
Spy molecule 17 (Compound S10a)
Spy molecule 17 (Compound S10a)
Spy molecule 18 (Compound S10b)

**Current Data Parameters**
- **NAME**: 2018/2214
- **TIME**: 6.52
- **INSTRUM**: Spec60
- **PROCADD**: 5 mm CP/D/C 147
- **DIPLUG**: 2930
- **SOLVENT**: MEOH
- **DI**: 440
- **SN**: 10000.000 Hz
- **DF**: 6.000000 Hz
- **DF**: 190.000 sec
- **DF**: 10.000000 sec
- **DF**: 293.2 K
- **DI**: 3.00000000 sec

**-------- CHANNEL 1 --------**
- **SN**: 500.133045 Hz
- **DF**: 41.000000 sec
- **FD**: 1.9999981 Hz

**F1 - Processing parameters**
- **SS**: 10000.000 Hz
- **MDW**: 10000.000 Hz
- **DD**: 0
- **LB**: 0.30 Hz
- **PG**: 1.00
Spy molecule 18 (Compound S10b)

**Current Data Parameters**
- NAME: GCP20
- EXPNO: 148
- PROCNO: 1

**F2 - Acquisition Parameters**
- Date: 20181116
- Time: 1.25
- INSTRUM: spect
- PROBD: 5 mm CPQCI 1H/
- PULP: zpgp30
- TD: 65400
- SOLVENT: MeOD
- NS: 4000
- DS: 4
- SWH: 29761.904 Hz
- FIDRES: 0.455075 Hz
- AQ: 1.0987200 sec
- RG: 190.78
- DM: 16.800 usec
- DE: 18.00 usec
- Jk: 293.1 K
- D1: 2.0000000 sec
- D11: 0.03000000 sec
- TRO: 1

**CHANNEL F1**
- SF01: 125.7703637 MHz
- NUC1: 13C
- P1: 12.00 usec
- PLW1: 133.00000000 W

**CHANNEL F2**
- SF02: 500.1320000 MHz
- NUC2: 1H
- CPDP: [2] wellt26
- PCPD2: 100.00 usec
- PLM1: 5.19999981 W
- PLM12: 0.0665600 W
- PLM13: 0.066560 W

**F2 - Processing parameters**
- SI: 131072
- SF: 125.7575991 MHz
- CW: 2M
- SSB: 0
- LB: 1.00 Hz
- PC: 1.40
Spy molecule 18 (Compound S10b)

Spy molecule 18 (S10b)

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Current Data Parameters
NAME GCP20
EXPNO 146
PROCNO 1

F2 - Acquisition Parameters
Date_ 20181114
Time 8.48
INSTRUM spect
PROBMD 5 mm CPQC1 1H/
PULPROG zgfhigqn.2
TO 111072
SOLVENT MeOD
NS 256
DS 4
SWH 113636.367 Hz
FD fwhm 8.866977 Hz
AQ 0.9767168 sec
KG 190.78
DW 1.400 usec
DE 18.00 usec
TE 293.2 K
D1 1.0000000 sec
D12 0.0000000 sec
D10 1

====== CHANNEL f1 ======
SP01 470.1453180 MHz
NUC1 19F
P1 12.00 usec
PLW1 5.19999981 W

===== CHANNEL f2 ======
SP02 500.1320005 MHz
NUC2 1H
CPPEAK2 waltz16
CPD2 100.00 usec
PLW2 5.19999981 W
PLW12 0.05406900 W

F2 - Processing parameters
SI 262144
FP 470.1923772 MHz
MOD EM
SBB 0
LB 1.50 Hz
GB 0
PC 1.00
Spy molecule 19 (Compound S11a)
Spy molecule 19 (Compound S11a)

Current Data Parameters
NAME  GCF18
EXPNO  165
PROCNO  1

F2 - Acquisition Parameters
Date_  20181114
Time  4.32
INSTRUM  spect
PROBHD  5 mm CPCI 1H/13C
FULPROG  zgpg30
TD  65400
SOLVENT  Me2CO
NS  2048
DS  4
SWB  29761.904 Hz
FIDRES  0.455075 Hz
AQ  1.0987200 sec
RG  190.78
DW  16.5000000 sec
DE  18.0000000 sec
TE  293.2 K
D1  2.000000000 sec
D11  0.030000000 sec
TD6  1

----- CHANNEL f1 ------
SFC1  125.7703637 MHz
NUC1  13C
P1  12.0000000 sec
PLM1  133.0000000000 W

----- CHANNEL f2 ------
SFC2  500.13200005 MHz
NUC2  1H
CFDPRG[2  waltz16
PCPD2  108.0000000 sec
PLM2  5.19999991 W
PLM12  0.06406900 W
PLM13  0.06406900 W

F2 - Processing parameters
SI  133.972
SF  125.7575999 MHz
MOD  EM
SSB  0
LB  1.00 Hz
GR  0
FC  1.40
Spy molecule 20 (Compound S11b)

Current Data Parameters
NAME:  GCF21
EXPNO:  174
PROCNO:  1

F2 - Acquisition Parameters
Date:  20181114
Time:  6.40
INSTNUM:  spect
PROBMD:  5 mm CPQC1 LR/
PULPROG:  zpg10
TO:  65400
SOLVENT:  MeOD
NS:  2048
DS:  4
SWH:  29761.904 Hz
FIDRMS:  0.425575 Hz
AQ:  1.0987200 sec
RG:  390.78
DW:  16.800 usec
DE:  18.00 usec
TE:  293.2 K
D1:  2.00000000 sec
D11:  0.03000000 sec
TD0:  1

-------- CHANNEL f1 --------
SP01:  125.7703637 MHz
NC1:  13C
F1:  12.00 usec
PLM1:  133.00000000 W

-------- CHANNEL f2 --------
SP02:  500.1320065 MHz
NC2:  1H
CPDPRG[1]:  waltz16
CPDDE:  100.00 usec
PLM2:  5.19999981 W
PLM12:  0.06409600 W
PLM13:  0.06409600 W

F2 - Processing parameters
SI:  131072
SP:  125.7575992 MHz
MOW:  0
EBB:  0
LA:  1.00 Hz
PF:  1.40
Spy molecule 20 (Compound S11b)
Spy molecule 21 (Compound S12a)
Spy molecule 21 (Compound S12a)
Spy molecule 21 (Compound S12a)
Spy molecule 22 (Compound S12b)
10. References

[1] X. Lucas, I. Van Molle, A. Ciulli, J. Med. Chem. 2018, 61, 7387-7393.

[2] C. Galdeano, M. S. Gadd, P. Soares, S. Scaffidi, I. Van Molle, I. Birced, S. Hewitt, D. M. Dias, A. Ciulli, J. Med. Chem. 2014, 57, 8657-8663.

[3] a) T. Kamura, D. M. Koepp, M. N. Conrad, D. Skowyra, R. J. Moreland, O. Iliopoulos, W. S. Lane, W. G. Kaelin, Jr., S. J. Elledge, J. W. Harper, J. W. Conaway, Science 1999, 284, 657-661; b) A. Pause, S. Lee, R. A. Worrell, D. Y. Chen, W. H. Burgess, W. M. Linehan, R. D. Klausner, Proc. Natl. Acad. Sci. U.S.A 1997, 94, 2156-2161.

[4] C. E. Stebbins, W. G. Kaelin, Jr., N. P. Pavletich, Science 1999, 284, 455-461.

[5] T. A. F. Cardote, M. S. Gadd, A. Ciulli, Structure 2017, 25, 901-911 e903.

[6] M. Fairhead, M. Howarth, Methods Mol. Biol. 2015, 1266, 171-184.

[7] C. Dalvit, Prog. Nucl. Magn. Reson. Spectrosc. 2007, 51, 243-1.

[8] R. Buratto, D. Mammoli, E. Chiarparin, G. Williams, G. Bodenhausen, Angew. Chem., Int. Ed. 2014, 53, 11376-11380.

[9] a) R. Z. Cer, U. Mudunuri, R. Stephens, F. J. Lebeda, Nucleic Acids Res. 2009, 37, W441-W445; b) Z. Nikolovska-Coleska, R. Wang, X. Fang, H. Pan, Y. Tomita, P. Li, P. P. Roller, K. Krajewski, N. G. Saito, J. A. Stuckey, S. Wang, Anal. Biochem. 2004, 332, 261-273.