Development of new chemical tools to study the cannabinoid receptor type 2

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Chapter 3

Discovery of a Photoaffinity Probe that Captures the Active Conformation of the Cannabinoid CB2 Receptor

Discovery of a Photoaffinity Probe that Captures the Active Conformation of the Cannabinoid CB_2 Receptor

Introduction

G protein-coupled receptors (GPCRs) constitute a valuable protein family for drug discovery. The cannabinoid receptor type 2 (CB_2R) is a GPCR expressed in cells of the immune system and is considered as a potential drug target due to its anti-inflammatory effects upon activation.[1] However, its low endogenous levels and inducible nature complicate the understanding of its cellular mechanism-of-action, which makes the drug discovery process more challenging. Chemical and biological tools can aid in the cellular characterization of CB_2R.[2] Antibodies are widely used to study GPCR localization and expression, but CB_2R antibodies suffer from poor quality, low selectivity and large batch to batch variability.[3] Therefore, the development of fluorescent chemical probes targeting CB_2R has become an attractive strategy to study its cellular localization, dynamics and occupancy by drugs.[4–6] For example, previously a probe with a silicon-rhodamine fluorophore was reported that was cell permeable and able to selectively label the CB_2 receptor in CB_2R overexpressing cells as well as in primary cultures of human macrophages.[1]

LEI-121 (**1**, Figure 3.1) was previously developed at Leiden University, a photoaffinity probe that is able to monitor endogenous CB_2R expression levels and their occupancy.[7] LEI-121 is a bifunctional CB_2R-selective probe based on the 5-fluoropyridin-2-yl-benzyl-imidazolidine-2,4-dione scaffold.[8] It includes a diazirine group that forms a covalent bond with CB_2R upon photoactivation. The alkyne click handle allows for ligand binding to the receptor prior to incorporation of a fluorescent tag. This This two-step photoaffinity-based protein profiling (ABPP) strategy prevents a decrease of the binding affinity and reduces non-specific binding due to the bulky fluorophore. LEI-121 was able to label CB_2R in gel-based ABPP using recombinant CB_2R, and detected endogenous CB_2R in primary human immune cells using flow cytometry.[7]

LEI-121 is an inverse agonist and reduces the constitutive activity of CB_2Rs.[7] To complete the toolbox and allow targeting of active CB_2Rs, a new probe that behaves as an agonist was developed. Here the structure-based design and synthesis of photo-affinity probes (**2**-**4**, Figure 3.1) is described, which were based on the recently published structure of LEI-102, a close analog of LEI-121, in complex with the CB_2R elucidated by cryogenic electron microscopy (cryo-EM) (Figure 3.2).[9] LEI-102 is a high-affinity CB_2R ligand (pK_i of 8.0 ± 0.1) with over 1000-fold selectivity over CB_1R. LEI-102 activates the CB_2 receptor as a partial agonist (pEC_{50} 6.9 ± 0.2, E_{max} 76 ± 1%).[9]

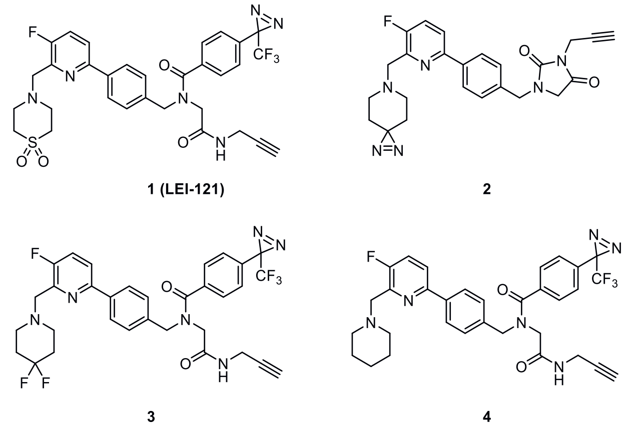


Figure 3.1 The chemical structures of LEI-121 (**1**) and the newly designed two-step photo affinity probes **2-4**.

Results and Discussion

Design

To design novel photoreactive probes, LEI-121 was docked into the recently published CB$_2$R cryo-EM structure (Figure 3.2B). The CB$_2$R-G$_i$ structure was reported in complex with CB$_2$R selective agonist LEI-102 with a resolution 2.9 Å.[9] Considering previous structure activity relationship (SAR) reports[8], the thiomorpholine 1,1-dioxide (**1**) of LEI-121 was substituted for a diaziridine-piperidine (**2**), a difluoropiperidine (**3**) or a piperidine (**4**) to investigate their effect on the potency and functionality of the probe.[8] Furthermore, compound **2** was designed to more closely resemble the structure of LEI-102, in an attempt to create an agonist probe. The hypothesis is that the positioning of the propargylamide in LEI-121 is responsible for its inverse agonistic activity by occupying in a subpocket, which may stabilize the inactive receptor conformation. This resembles the change in functionality, we have previously reported with a lipophilic spacer on this scaffold.[10] In compound **2** the propargyl moiety was introduced on the imidazolidine moiety to act as a ligation handle and the diazirine was moved to the piperidine. Of note, compound **2** had a similar binding pose as LEI-102 in the CB$_2$R structure (Figure 3.2C).

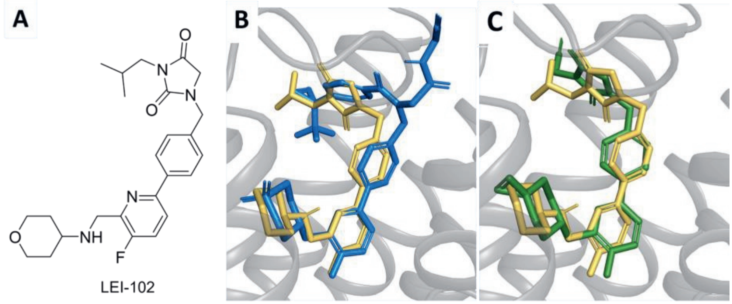
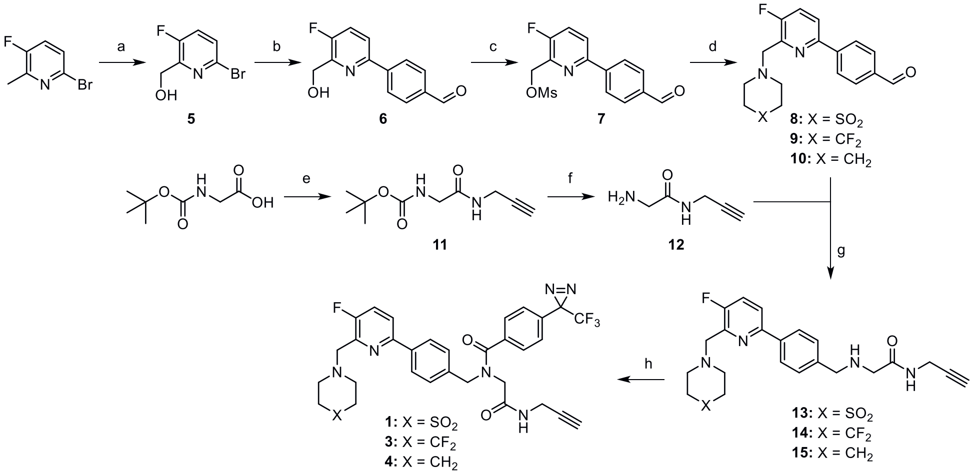


Figure 3.2 The chemical structure of LEI-102 (A), and the docked poses of LEI-121 (blue, B) and **2** (green, C) overlayed with the cryo-EM structure of LEI-102 (yellow). Docking was performed in the cryo-EM structure of CB$_2$R containing LEI-102 (PDB: 8GUT).

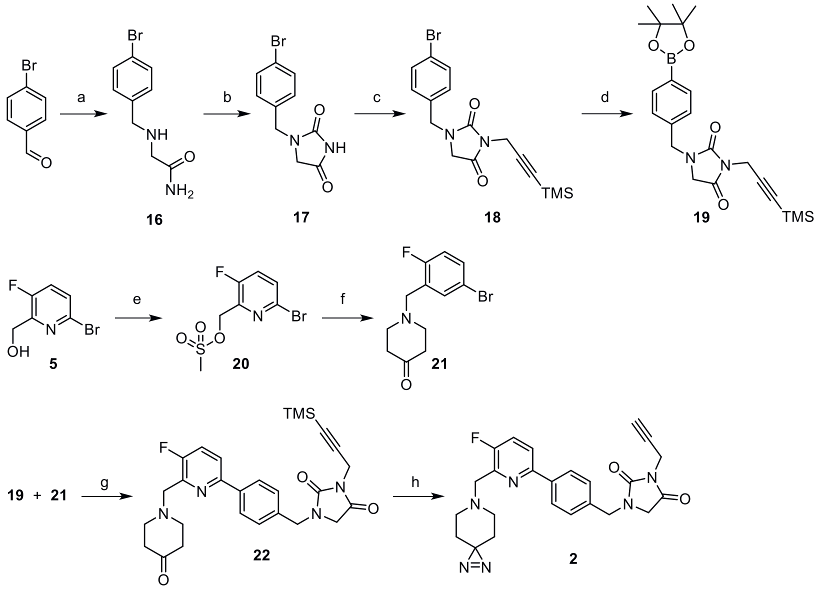
Synthesis

The synthesis of probes **1**,**3** and **4** started with oxidation of 6-bromo-3-fluoro-2-methylpyridine with *m*-CPBA followed by a Boekelheide rearrangement to yield **5**. Conjugation of **5** and (4-formylphenyl) boronic acid through a Suzuki coupling led to pyridyl benzaldehyde **6** (Scheme 3.1). Subsequent mesylation (**6** → **7**) and nucleophilic substitution with the desired piperidines gave the thiomorpholine 1,1-dioxide **8**, 4,4-difluoropiperidine **9** and piperidine **10** intermediates. The alkyne click handle was introduced via reductive amination of 2-amino-*N*-propargyl acetamide (**12**), obtained through HBTU mediated condensation of Boc-Gly-OH with propargylamine followed by Boc deprotection, and intermediates **8**-**10** to gain **13**-**15**. Finally, conjugation of 4-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)benzoic acid to **13**-**15** afforded the probes **1**,**3** and **4**.



Scheme 3.1 The Synthesis of compounds **1**, **3** and **4**. Reagents and conditions: a) Step 1: *m*-CPBA (1.8 eq), 0 °C-RT, DCM (0.2 M), 4 days; Step 2: TFAA (2.2 eq), 0 °C-55 °C, 3 h; Step 3: K_2CO_3 (2.3 eq), THF:MeOH (20:1), 17 h, 35% (three steps); b) (4-formylphenyl) boronic acid (1.5 eq), $Pd(PPh_3)_4$ (0.1 eq), K_2CO_3 (6 eq), toluene : EtOH (0.4 M, 4 : 1 v/v), 80 °C, 96 h, 80%; c) DiPEA (2 eq), MsCl (1 eq), DCM (0.2 M), 0 °C, 1 h, 75%; d) **8**: Thiomorpholine 1,1-dioxide / **9**: 4,4 difluoropiperidine/ **10**: piperidine (1.2 eq), K_2CO_3 (3.6 eq), ACN (0.2 M), 60 °C, 3 h, 70-80%; e) propargylamine (1 eq), *N*-methylmorpholine (1.1 eq), HOBt (1.1 eq), EDC.HCl (1.1 eq), DCM (0.2 M), RT, 6 h, 74%; f) HCl (4 M, 1.6 eq) in dioxane, RT, 2.5 h, 46%; g) acetic acid (1 eq), $NaBH(OAc)_3$ (3.6 eq), THF (0.1 M), RT, 16 h, 43-54%; h) 4-(3-(trifluoromethyl)-3H-diazirin-3-yl)benzoic acid (1 eq), HBTU (1.5 eq), DiPEA (3 eq), DCM (0.1 M), RT, 1 h, 28-31%.

The construction of probe **2** was similar to the synthesis of LEI-102.[9] To start, reductive amination of 4-bromobenzaldehyde and 2-aminoacetamide led to compound **16**. After cyclization the formed imidazolidinedione **17** was alkylated with 3-bromo-1-(trimethylsilyl)-1-propyne (**18**) and subsequent borylation gave building block **19**. Simultaneously, previously synthesized (6-bromo-3-fluoropyridin-2-yl)methanol (**5**) was mesylated (**20**) followed by substituted of the mesyl with piperidin-4-one to gain ketone **21**. Suzuki coupling of **19** and **21** gave key intermediate **22**. The reaction conditions used for the conversion of the ketone in **22** to a diazirine led to simultaneous demasking of the alkyne function to give the photoaffinity probe **2**.



Scheme 3.2 The synthesis of the hydantoin probe **2**. Reagents and conditions: a) Step 1: 2-aminoacetamide hydrochloride (1.0 eq), NaOH (1.1 eq), MeOH:H_2O (0.3 M, 5:1 (v/v)), RT, 18 h; Step 2: $NaBH_4$ (2.1 eq), 18 h, 91% (two steps); b) CDI (2.1 eq), DMAP (2.1 eq), ACN (0.1 M), 60 °C, 70 h, 37%; c) 3-bromo-1-(trimethylsilyl)-1-propyne (1.2 eq), K_2CO_3 (1 eq), DMF (0.3 M), 50 °C, 16 h, 61%; d) bis(pinacol)diborane (1.5 eq), KOAc (4 eq), Pd(dppf)Cl_2 (0.5 eq), 1,4 dioxane (0.1 M), 80 °C, 16 h, used as crude; e) Et_3N (2.3 eq), MsCl (1.7 eq), THF (0.2 M), 0 °C-RT, 1 h, 75%; f) 4-piperidone hydrochloride (1.2 eq), K_2CO_3 (3 eq), ACN (0.2 M), 70 °C, 48 h, 78%; g) K_2CO_3 (1.8 eq), $Pd(PPh_3)_4$ (0.1 eq), toluene:EtOH (0.1 M, 4:1 (v/v)), 50 °C, 16 h, 29%; h) Step 1: NH_3 (g), MeOH (0.2 M), 0 °C, 5 h; Step 2: NH_2SO_3H (1.5 eq), MeOH (0.2 M), 16 h, RT, Step 3: I_2, MeOH, 0 °C 15 min., 27% (three steps).

Molecular Pharmacology

Next probes **1**-**4** were tested in a [^{3}H]CP-55,940 radioligand displacement assay to determine their affinity (pK_i or displacement %) for the CB_2R and CB_1R. Compounds with less than 50% displacement on CB_1R at 1 µM were considered inactive. Additionally, their potency (EC_{50}) and maximal efficacy (E_{max}) were measured in a [^{35}S]-GTPγS functional assay. The results are summarized in Table 3.1.

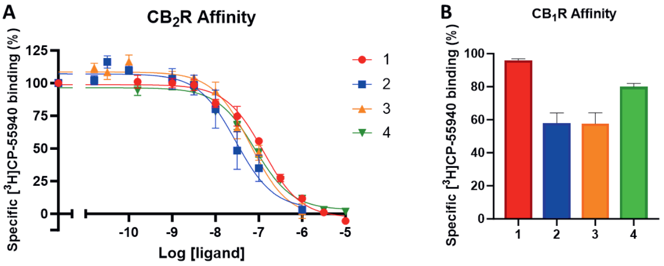


Figure 3.3 The affinity (pK_i or displacement) of compounds **1**-**4** on CB_2R (A) and CB_1R (B). Binding was normalized to binding of [^{3}H]CP-55,940 at 10 µM. Percentage displacement was determined with 1 µM compound. Data are presented as the mean ± SEM from three independent experiment performed in triplicate (CB_1R two experiments in duplicate).

Table 3.1 The Affinity and Potency of the two-step probes **1-4** on CB$_1$R and CB$_2$R.

Probe	**CB$_2$R**			**CB$_1$R**		
	pK$_i$ ± SEM	**pEC$_{50}$ ± SEM**	**E$_{max}$ (% ± SEM)**	**Displacement at 1 µM (%)**	**pEC$_{50}$ ± SEM**	**E$_{max}$ (% ± SEM)**
LEI-102	8.6 ± 0.3	6.9 ± 0.2	76 ± 1	< 50	n.d.	n.d.
LEI-121[9]	7.2 ± 0.4	6.6 ± 0.2	-50 ± 7	<50	n.d.	n.d.
1 (LEI-121)	7.24 ± 0.06	7.06 ± 0.19	-35 ± 3	4 ± 1	6.08 ± 0.21	-26 ± 4
2	7.89 ± 0.13	8.56 ± 0.56	58 ± 4	42 ± 6	6.97 ± 0.13	35 ± 3
3	7.59 ± 0.09	7.75 ± 0.19	-26 ± 2	42 ± 7	7.00 ± 0.23	-16 ± 2
4	7.42 ± 0.05	7.10 ± 0.33	-28 ± 2	20 ± 2	5.98 ± 0.16	-19 ± 3

Binding affinities (pK$_i$ or displacement %) and potency (pEC$_{50}$) were determined with a [^{3}H]CP-55,940 displacement assay and [^{35}S]GTPγS functional assay respectively on CBR overexpressing CHO membranes. Potency values (pEC$_{50}$) were obtained for compounds with displacement ≥ 35% using a [^{35}S]GTPγS assay. Efficacy (E$_{max}$) was normalized to the effect of 10 µM CP-55,940. Data are presented as the mean ± SEM from three independent experiments performed in triplicate.

All probes showed improved affinity for CB$_2$R compared to LEI-121 (Figure 3.3, Table 3.1), while retaining selectivity over CB$_1$R. This was accompanied by increased activity on hCB$_2$R by probes **2**-**4** compared to LEI-121 (Figure 3.4, Table 3.1). Probe **2** had the highest affinity (pK$_i$ 7.89 ± 0.13) and activity (pEC$_{50}$ 8.56 ± 0.56), which was 15-fold increased compared to LEI-102.

Compounds **3** and **4** behaved as inverse agonists with an E$_{max}$ ≈ -30%, comparable to LEI-121 (**1**).[8] In contrast, probe **2** acted as a partial agonist (E$_{max}$ 58% ± 4) (Figure 3.4). While probe **2** at 1 µM showed less than 50% radioligand displacement at the CB$_1$R, the compound did show some activity in the G protein activation assay. Of note, probe **2** was 77-fold selective over CB$_1$R and elicited 40-fold more potent receptor activation for CB$_2$R compared to CB$_1$R.

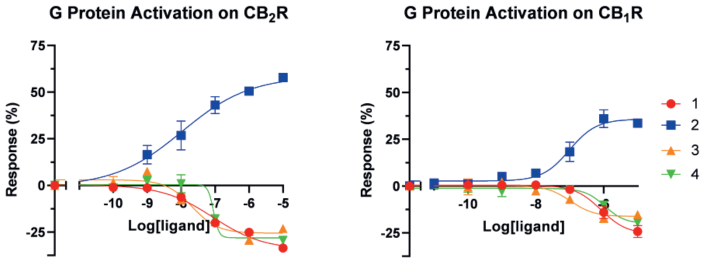


Figure 3.4 G protein activation of CB$_2$R and CB$_1$R by compounds **1**-**4** were measured with [^{35}S]GTPγS functional assays on CBR overexpressing CHO membranes. The results showed the switch of **2** from inverse agonist to partial agonist. Data points are presented as the mean ± SEM of at least three independent experiments performed in triplicate.

CB_2R membranes	+	-	+	+	+	+	+	+	+
Denatured Protein	-	-	+	-	-	-	-	-	-
UV	+	+	+	+	-	+	+	+	+
Click azide	+	+	+	-	+	+	+	+	+
Probe (20 uM)	-	**2**	**2**	**2**	**2**	**2**	**2**	**1**	**1**
CP55,940 (200 uM)	-	-	-	-	-	-	+	-	+

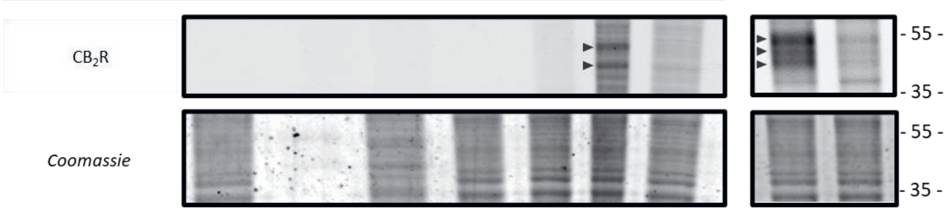


Figure 3.5 Probes **1** and **2** were able to label CB_2R (45-55 kDa) in CB_2R-overexpressing CHO membranes. Denaturation of the protein prior to probe incubation, or omitting the UV irradiation step prevented labelling of CB_2R by the probes. Additionally, no signal was detected in the absence of either click azide or probe. Labelling was outcompeted by CP-55,940. The gels are representative of two independent experiments.

Since probe **2** was the only compound that behaved as a partial agonist, its ability to visualize CB_2R was analysed by two-step pAfBPP. Probe **1** was used as a positive control. To this end, membrane preparations of hCB_2R-overexpressing CHO cells were incubated with probe **1** or **2**. Cross-linking was effected by UV-irradiation (λ = 350 nm, 5 min) using a CaproBox, a device used for controlled irradiation of biological samples with simultaneous cooling at 4 °C, to counteract the heat induced by the irradiation. Next, the membranes were subjected to copper(I)-catalysed click reaction conditions, utilizing $Cy5\text{-}N_3$ as the fluorescent azide to analyse the probe–protein complex by SDS-PAGE and in-gel fluorescence imaging. In this manner, two major bands with an apparent molecular weight of ~47 and ~41 kDa (Figure 3.5) were visualized for both probes, and these were absent in membranes from wild-type CHO cells. Heat-induced denaturation prior to probe incubation also resulted in a loss of fluorescent bands, indicating that the recognition is dependent on an intact three-dimensional protein conformation. The bands were also absent in non-UV treated samples, demonstrating that the probe does not covalently interact with CB_2Rs in the absence of irradiation. Furthermore, omission of the click-mixture showed that labelling was dependent on copper(I)-catalysed azide alkyne click ligation (Figure 3.5). Preincubation with CP-55,940 was able to prevent the CB_2R labelling by probes **1** and **2**. Of note, probe **2** did show two off-targets with a molecular weight around 30 kDa not present for the original LEI-121 and derivatives.[7] The identity of the off-targets is unknown, but one of the off-targets is also competed out by CP-55,940.

The labelling pattern of probes **1** and **2** is slightly different. Probe **2** labels two distinct bands, while labelling with probe **1** yields multiple intense bands. GPCRs including CB_2R can have a wide variety of post translational modifications such as N-glycosylation and phosphorylation.[11,12] Phosphorylation patterns change depending on receptor activity, thus a partial agonist probe may recognize a different receptor population than an inverse agonistic probe.[13]

Conclusion

A structure-based approach exploiting the recently published cryo-EM structure of the CB_2R in complex with LEI-102 was used to develop a new bifunctional photoaffinity probe that stabilized the active conformation of CB_2R. Probe **2** had high binding affinity (pK_i 7.89 ± 0.13), selective of CB_1R and behaved as a partial agonist (EC_{50} 8.56 ± 0.56 with, E_{max} 58% ± 4). Probe **2** labelled the CB_2R upon photoactivation in a slightly different manner compared to LEI-121 (probe **1**), which is an inverse agonist. This may suggest that the stabilization of a different receptor conformations result in a different labelling pattern, which could be dependent on different post-translational modifications.[10,11] Probe **2** has potential to be used to isolate the CB_2R in the active state using a biotin-reporter and affinity enrichment from primary cells and tissues, which may facilitate the identification of post-translational modifications and potential protein interaction partners of the active receptor. To conclude, a novel photoaffinity probe was developed for the CB_2R that may hold promise to study different receptor conformations in relation to its cellular function.

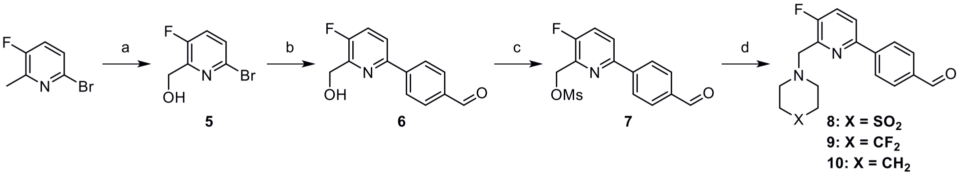
Experimental Section

Chemistry

General Remarks

All reagents and solvents were purchased from commercial sources and were of analytical grade (Sigma-Aldrich, BroadPharm®$^{®}$) and used without further purification. All moisture sensitive reactions were performed under inert atmosphere. Solvents were dried using 4 Å molecular sieves prior to use when anhydrous conditions were required. Water used in reactions was always demineralized. Analytical thin-layer chromatography (TLC) was routinely performed to monitor the progression of a reaction and was conducted on Merck Silica gel 60 F254 plates. Reaction compounds on the TLC plates were visualized by UV irradiation (λ_{254}) and/or spraying with potassium permanganate solution (K_2CO_3 (40 g), $KMnO_4$ (6 g), and H_2O (600 mL)), ninhydrin solution (ninhydrin (1.5 g), n-butanol (100 mL) and acetic acid (3.0 mL)) or molybdenum solution (($(NH_4)_6MO_7O_{24}\cdot 4H_2O$ (25 g/L) and $(NH_4)_4Ce(SO_4)_4\cdot 2H_2O$ (10 g/L) in sulfuric acid (10%)) followed by heating as appropriate. Purification by flash column chromatography was performed using Screening Devices B.V. silica gel 60 (40-63 µm, pore diameter of 60 Å). Solutions were concentrated using a Heidolph laborata W8 4000 efficient rotary evaporator with a Laboport vacuum pump. Analytical purity was determined with liquid chromatography-mass spectrometry (LC-MS) using a Finnigan LCQ Advantage MAX apparatus with electrospray ionization (ESI), equipped with a Phenomenex Gemini 3 µm NX-C18 110Å column (50x4.6mm), measuring absorbance at 254 nm using a Waters 2998 PDA UV detector and the m/z ratio by using an Acquity Single Quad (Q1) detector. Injection was with the Finnigan Surveyor Autosampler Plus and pumped through the column with the Finnigan Surveyor LC pump plus to be analysed with the Finnigan Surveyor PDA plus detector. Samples were analysed using eluent gradient 10% → 90% ACN in MilliQ water (+ 0.1% TFA (v/v)). For purification by mass guided preparative High-Performance Liquid Chromatography (Prep-HPLC) was performed on a Waters AutoPurification HPLC/MS apparatus with a Gemini prep column 5 µm 18C 110 Å (150x21.2mm), Waters 2767 Sample manager, Waters 2545 Binary gradient module, Waters SFO System fluidics organizer, Waters 515 HPLC pump M, Waters 515 HPLC pump L attached to a Waters SQ detector Acquity Ultra performance LC. A five column volume purification protocol was applied with the eluents A: 0.2% aq. TFA, B: ACN, flow 25 mL/min, with a minimum start gradients of 0% to maximum end gradient of 100% of B. All final compounds had a purity > 95%. ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AV 300 (300/75 MHz), AV 400 (400/100 MHz) or AV 500 (500/125 MHz) spectrometer at ambient temperature using $CDCl_3$ or MeOD as solvent. Chemical shifts (δ) are referenced in parts per million (ppm) with tetramethylsilane (TMS) or $CDCl_3$ resonance as the internal standard peak ($CDCl_3$/TMS, δ 0.00 for ^1H (TMS), δ 77.16 for ^{13}C ($CDCl_3$)). The internal standard peaks for MeOD solvent are δ 3.31 (^1H) and 49.00 (^{13}C). Multiplicity is reported as s = singlet, d = doublet, dd = doublet of doublet, dt = doublet of triplet, t = triplet, q = quartet, p = quintet, hept = heptet, m = multiplet. Coupling-constants (*J*) are reported in Hertz (Hz).

Synthesis of Probes 1, 3, and 4.



Scheme 3.3 Synthesis of Intermediates **8**-**10**. Reagents and conditions: a) Step 1: *m*-CPBA (1.8 eq), 0 °C-RT, DCM (0.2 M), 4 days; Step 2: TFAA (2.2 eq), 0 °C-55 °C, 3 h; Step 3: K_2CO_3 (2.3 eq), THF:MeOH (20:1), 17 h, 35% (three steps); b) (4-formylphenyl) boronic acid (1.5 eq), $Pd(PPh_3)_4$ (0.1 eq), K_2CO_3 (6 eq), toluene : EtOH (0.4 M, 4 : 1 v/v), 80 °C, 96 h, 80%; c) DiPEA (2 eq), MsCl (1 eq), DCM (0.2 M), 0 °C, 1 h, 75%; d) **8**: Thiomorpholine 1,1-dioxide / **9**: 4,4 difluoropiperidine/ **10**: piperidine (1.2 eq), K_2CO_3 (3.6 eq), ACN (0.2 M), 60 °C, 3 h, 70-80%.

(6-Bromo-3-fluoropyridin-2-yl)methanol (5): To a stirred and cooled (0 °C) mixture under inert atmosphere of 6-bromo-3-fluoro-2-methylpyridine (10.7 g, 56.3 mmol, 1 eq) in DCM (370 mL) was added portion-wise *m*-CPBA (23.6 g, 70-75%, 100 mmol, 1.8 eq). The reaction mixture was stirred at room temperature (RT) for 4 days. Sat. $NaHCO_3$ (aq) and sat. $Na_2S_2O_3$ (aq) was added (1:1, v/v) and the layers were separated. The aqueous layer was extracted thrice with DCM. The combination of organic layers was dried ($MgSO_4$), filtered, and concentrated under reduced pressure. To the residue was added TFAA (17 mL, 122 mmol, 2.2 eq) at 0 °C. After 15 minutes the temperature was increased to 55 °C for 3 h. The mixture was concentrated under reduced pressure, redissolved in DCM and sat. Na_2CO_3 (aq) was added. The layers were separated and the organic layer was washed with sat. $NaHCO_3$ (aq). The solvent was evaporated and the residue was dissolved in THF:MeOH (20:1, v/v) and K_2CO_3 (18.2 g, 132 mmol, 2.3 eq) was added. After 17 h H_2O was added and the layers were separated. The aqueous layer was extracted thrice with EtOAc. The combination of organic layers was dried ($MgSO_4$), filtered, and the solvent evaporated under reduced pressure. The crude product was purified with flash column chromatography (SiO_2, 10-20% EtOAc in pentane) to yield a white solid (5.79 g, 19.7 mmol, 35%). ^{1}H-NMR (500 MHz, $CDCl_3$) δ 7.42 (ddt, *J* = 8.5, 3.5, 0.7 Hz, 1H), 7.29 (t, *J* = 8.5 Hz, 1H), 4.80 (d, *J* = 3.3 Hz, 2H). ^{13}C-NMR (126 MHz, $CDCl_3$) δ 156.10 (d, *J* = 256.2 Hz), 148.74 (d, *J* = 19.1 Hz), 135.01 (d, *J* = 2.9 Hz), 128.17 (d, *J* = 4.2 Hz), 126.09 (d, *J* = 19.8 Hz), 59.07.

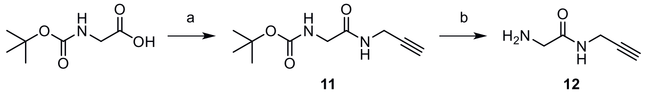
4-(5-Fluoro-6-(hydroxylmethyl) pyridine-2-yl) benzaldehyde (6): Under inert atmosphere of a degassed stirred mixture of **5** (1.90 g, 9.2 mmol, 1 eq), (4-formylphenyl) boronic acid (2.08 g, 13.8 mmol, 1.5 eq) and $Pd(PPh_3)_4$ (1.07 g, 0.9 mmol, 0.1 eq) in toluene:EtOH (22 mL, 4:1 (v/v)) was added aqueous K_2CO_3 (aq) (27.7 mL, 0.2 M, 55.3 mmol, 6 eq) and mixture was heated (80 °C) for 96 h. The mixture was filtered and diluted with H_2O (30 mL) and EtOAc (40 mL). The layers were separated and the aqueous layer extracted thrice with EtOAc. The combination of organic layers was washed with brine, dried ($MgSO_4$), filtered, and the solvent evaporated under reduced pressure. The crude product was purified with flash column chromatography (SiO_2, 30-80% EtOAc in pentane) to yield a yellow solid (1.71 g, 7.4 mmol, 80%). ^{1}H-NMR (500 MHz, $CDCl_3$) δ 10.08 (s, 1H), 8.14 (d, *J* = 8.2 Hz, 2H), 7.99 (d, *J* = 8.2 Hz, 2H), 7.77 (dd, *J* = 8.5, 3.6 Hz, 1H), 7.52 (t, *J* = 8.6 Hz, 1H), 4.87 (d, *J* = 1.8 Hz, 2H), 4.04 (s, 1H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 191.89, 156.22 (d, *J* = 258.2 Hz), 150.62 (d, *J* = 4.7 Hz), 147.21 (d, *J* = 18.6 Hz), 143.23, 136.41, 130.20, 127.29, 123.83 (d, *J* = 18.4 Hz), 121.21 (d, *J* = 4.1 Hz), 59.20.

(3-Fluoro-6-(4-formylphenyl) pyridin-2-yl) methylmethanesulfonate (7): To a cooled (0 °C) and stirred mixture of **6** (1.41 g, 6.1 mmol, 1 eq) in DCM (30 mL) was added DiPEA (2.2 mL, 12.2 mmol, 2 eq) and methanesulfonyl chloride (0.5 mL, 6.1 mmol, 1 eq). After stirring at 0 °C for 1 h the reaction was diluted with H_2O (16 mL) and DCM (10 mL). The layers were separated and the aqueous layer was extracted thrice with DCM. The combination of organic layers was dried ($MgSO_4$), filtered and the solvent evaporated under reduced pressure. The crude product was purified with flash column chromatography (SiO_2, 30-50% EtOAc in pentane) to yield a yellow solid (1.42 g, 4.6 mmol, 75%). ^{1}H-NMR (500 MHz, $CDCl_3$) δ 10.08 (s, 1H), 8.15 (d, *J* = 8.1 Hz, 2H), 7.98 (d, *J* = 8.3 Hz, 2H), 7.89 (dd, *J* = 8.5 Hz, 2.3 Hz, 1H), 7.61 (t, *J* = 8.7 Hz, 1H), 5.49 (d, *J* = 2.1 Hz, 2H), 3.14 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 191.95, 159.43, 156.81, 143.09, 141.44 (d, *J* = 15.2 Hz), 136.72, 130.39, 127.54, 125.01 (d, *J* = 19.4 Hz), 123.60 (d, *J* = 4.5 Hz), 66.70 (d, *J* = 1.9 Hz), 38.32.

4-(6-((1,1-Dioxidothiomorpholino) methyl)-5-fluoropyridin-2-yl) benzaldehyde (8): A stirred mixture of **7** (0.66 g, 2.1 mmol, 1 eq), thiomorpholine 1,1-dioxane (0.35 g, 2.6 mmol, 1.2 eq) and K_2CO_3 (1.07 g, 7.7 mmol, 3.6 eq) in acetonitrile (12 mL) was heated (60 °C) for 3 h. The reaction mixture was diluted at RT with H_2O (15 mL) and DCM (15 mL), the layers were separated and the aqueous layer was extracted thrice with DCM. The combination of organic layers was washed with brine, dried ($MgSO_4$), filtered and the solvent evaporated under reduced pressure. The crude product was purified with flash column chromatography (SiO_2, 30-50% EtOAc in pentane) to yield a yellow solid (0.52 g, 1.5 mmol, 70%). ^{1}H-NMR (500 MHz, $CDCl_3$) δ 10.07 (s, 1H), 8,11 (d, *J* = 8.3 Hz, 2H), 7.99 (d, *J* = 8.4 Hz, 2H), 7.78 (dd, *J* = 8.6, 3.6 Hz, 1H), 7.52 (t, *J* = 8.8 Hz, 1H), 4.03 (d, *J* = 2.4 Hz, 2H), 3.21 (dd, *J* = 7.1, 3.6 Hz, 4H), 3.09 (dd, *J* = 7.5, 3.3 Hz, 4H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 191.96, 158.22 (d, *J* = 260.0 Hz), 151.47 (d, *J* = 4.8 Hz), 144.93 (d, *J* = 15.1 Hz), 136.51, 130.33, 127.42, 124.33 (d, *J* = 20.2 Hz), 121.92 (d, *J* = 4.5 Hz), 56.68 (d, *J* = 2.8 Hz), 51.67, 50.61.

4-(6-((4,4-Difluoropiperidin-1-yl) methyl)-5-fluoropyridin-2-yl) benzaldehyde (9): A stirred mixture of **7** (0.66 g, 2.1 mmol, 1 eq) 4,4-difluoropiperidine hydrochloride (0.41 g, 2.6 mmol, 1.2 eq) and K_2CO_3 (1.07 g, 7.7 mmol, 3.6 eq) in acetonitrile (12 mL) was heated (60 °C) for 3 h. The reaction mixture was diluted at RT with H_2O (15 mL) and DCM (15 mL), the layers were separated and the aqueous layer was extracted thrice with DCM. The combination of organic layers was washed with brine, dried ($MgSO_4$), filtered and the solvent evaporated under reduced pressure. The crude product was purified with flash column chromatography (SiO_2, 70-100% EtOAc in pentane) to yield a yellow solid (0.57 g, 1.7 mmol, 80%). ^{1}H-NMR (500 MHz, $CDCl_3$) δ 10.06 (s, 1H), 8.13 (d, *J* = 8.3 Hz, 2H), 7.97 (d, *J* = 8.5 Hz, 2H), 7.74 (dd, *J* = 8.6, 3.6 Hz, 1H), 7.49 (t, *J* = 8.7 Hz, 1H), 3.90 (d, *J* = 2.5 Hz, 2H), 2.76 (t, *J* = 5.7 Hz, 4H), 2.02 (hept, *J* = 5.8 Hz, 4H). ^{13}C-NMR (126 MHz, $CDCl_3$) δ 192.03, 158.26 (d, *J* = 260.0 Hz), 151.18 (d, *J* = 4.9 Hz), 145.87 (d, *J* = 15.1 Hz), 143.91, 136.37, 130.28, 127.43, 124.02 (d, *J* = 20.3 Hz), 121.98 (t, *J* = 241.5 Hz), 121.50 (d, *J* = 4.4 Hz), 57.09, 49.96 (t, *J* = 5.3 Hz), 34.10 (t, *J* = 22.9 Hz).

4-(5-Fluoro-6-(piperidin-1-ylmethyl) pyridin-2-yl) benzaldehyde (10): A stirred mixture of **7** (0.66 g, 2.1 mmol, 1 eq), piperidine (0.22 g, 2.6 mmol, 1.2 eq) and K_2CO_3 (1.07 g, 7.7 mmol, 3.6 eq) in acetonitrile (12 mL) was heated (60 °C) for 3 h. The reaction mixture was diluted at RT with H_2O (15 mL) and DCM (15 mL), the layers were separated and the aqueous layer was extracted thrice with DCM. The combination of organic layers was washed with brine, dried ($MgSO_4$), filtered and the solvent evaporated under reduced pressure. The crude product was purified with flash column chromatography (SiO_2, 30% EtOAc in pentane) to yield a white solid (0.51 g, 1.7 mmol, 73%). ^{1}H-NMR (500 MHz, $CDCl_3$) δ 10.06 (s, 1H), 8.14 (d, *J* = 8.3 Hz, 2H), 7.96 (d, *J* = 8.4 Hz, 2H), 7.71 (dd, *J* = 8.6, 3.5 Hz, 1H), 7.46 (t, *J* = 8.7 Hz, 1H), 3.83 (d, *J* = 2.6 Hz, 2H), 2.59 (t, *J* = 5.4 Hz, 4H), 1.61 (p, *J* = 5.6 Hz, 4H), 1.46 – 1.37 (m, 2H). ^{13}C-NMR (126 MHz, $CDCl_3$) δ 192.07, 158.35 (d, *J* = 260.0 Hz), 150.95 (d, *J* = 4.8 Hz), 146.50 (d, *J* = 15.2 Hz), 144.19, 136.31, 130.25, 127.48, 123.77 (d, *J* = 20.3 Hz), 121.13 (d, *J* = 4.4 Hz), 58.64 (d, *J* = 3.0 Hz), 54.53, 26.08, 24.25.

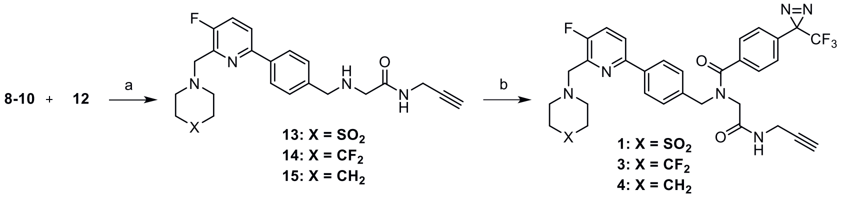


11 **12**

Scheme 3.4 Synthesis of intermediate **12**. Reagents and Conditions: e) propargylamine (1 eq), *N*-methylmorpholine (1.1 eq), HOBt (1.1 eq), EDC.HCl (1.1 eq), DCM (0.2 M), RT, 6 h, 74%; f) HCl (4 M, 1.6 eq) in dioxane, RT, 2.5 h, 46%.

***tert*-Butyl (2-oxo-2-(prop-2-yn-1-ylamino) ethyl) carbamate (11)**: A mixture of *N*-(*tert*-butoxycarbonyl) glycine (5.78 g, 33.0 mmol, 1.1 eq), propargylamine (1.9 mL, 30 mmol, 1 eq), *N*-methylmorpholine (3.6 mL, 33.0 mmol, 1.1 eq), HOBt (5.05 g, 33.0 mmol, 1.1 eq) and EDC.HCl (5.89 g, 33.0 mmol, 1.1 eq) in anhydrous DCM (125 mL) was stirred at RT for 6 h. The mixture was diluted with EtOAc and the combination of organic layers was washed with 0.4 M HCl, H_2O and brine, dried ($MgSO_4$), filtered and the solvent evaporated under reduced pressure. The crude product was purified with recrystallization in EtOAc and hexane to yield a white solid (4.70 g, 22.1 mmol, 74%). ^{1}H-NMR (500 MHz, $CDCl_3$) δ 6.75 (s, 1H), 5.36 (t, *J* = 5.9 Hz, 1H), 4.07 (dd, *J* = 5.4, 2.6 Hz, 2H), 3.84 (d, *J* = 5.7 Hz, 2H), 2.23 (t, *J* = 2.6 Hz, 1H), 1.46 (s, 9H). ^{13}C-NMR (101 MHz, $CDCl_3$) δ 183.38, 169.49, 80.60, 79.22, 71.92, 44.51, 29.27, 28.42.

2-Amino-*N*-(prop-2-yn-1-yl) acetamide. HCl (12): A mixture of 4 M HCl in dioxane (10 mL, 40 mmol, 1.6 eq) and **11** (4.70 g, 24.4 mmol, 1eq) was stirred at RT for 2.5 h. The solvent was evaporated under reduced pressure and the crude product was purified with recrystallization in MeOH to yield a white solid (1.67 g, 11.2 mmol, 46%). ^{1}H-NMR (500 MHz, MeOD) δ 4.05 (d, *J* = 2.6 Hz, 2H), 3.64 (s, 2H), 2.62 (t, *J* = 2.6 Hz, 1H). ^{13}C-NMR (101 MHz, MeOD) δ 171.91, 80.62, 72.72, 41.43, 29.55.



Scheme 3.5 Synthesis of Probes **1**,**3** and **4**. Reagents and Conditions: g) acetic acid (1 eq), $NaBH(OAc)_3$ (3.6 eq), THF (0.1 M), RT, 16 h, 43-54%; h) 4-(3-(trifluoromethyl)-3H-diazirin-3-yl)benzoic acid (1 eq), HBTU (1.5 eq), DiPEA (3 eq), DCM (0.1 M), RT, 1 h, 28-31%.

2-((4-(6-((1,1-Dioxidothiomorpholino) methyl)-5-fluoropyridin-2-yl) benzyl) amino)-*N*-(prop-2-yn-1-yl) acetamide (13): A mixture of **8** (0.24 g, 0.7 mmol, 1 eq), **12** (0.15 g, 1.4 mmol, 2 eq), acetic acid (0.04 mL, 0.7 mmol, 1 eq) and $NaBH(OAc)_3$ (0.53 g, 2.5 mmol, 3.6 eq) in THF (9 mL) was stirred at RT for 16 h. The reaction was diluted with sat. $NaHCO_3$ (aq, 8 mL) and DCM (10 mL). The layers were separated and the aqueous layer extracted thrice with DCM. The combination of organic layers was dried ($MgSO_4$), filtered and the solvent evaporated under reduced pressure. The crude product was purified with flash column chromatography (SiO_2, 5% MeOH (with 1% Et_3N) in DCM) to yield a yellow oil (0.13 g, 0.30 mmol, 43%). ^{1}H-NMR (500 MHz, $CDCl_3$) δ 7.91 (d, *J* = 8.3 Hz, 2H), 7.68 (dd, *J* = 8.6, 3.6 Hz, 1H), 7.47 (t, *J* = 8.8 Hz, 1H), 7.40 (app t, *J* = 8.5 Hz, 3H), 4.07 (dd, *J* = 5.5, 2.6 Hz, 2H), 4.01 (d, *J* = 2.4 Hz, 2H), 3.84 (s, 2H), 3.34 (s, 2H), 3.24 – 3.18 (m, 4H), 3.12 – 3.06 (m, 4H), 2.24 (t, *J* = 2.6 Hz, 1H), 1.84 (s, 1H).^{13}C-NMR (126 MHz, $CDCl_3$) δ 171.21, 157.73 (d, *J* = 257.9 Hz), 152.70 (d, *J* = 4.9 Hz), 144.31 (d, *J* = 14.8 Hz), 140.22, 137.53, 128.75, 127.16, 124.16 (d, *J* = 20.2 Hz), 121.04 (d, *J* = 4.1 Hz), 79.73, 71.60, 56.65 (d, *J* = 2.8 Hz), 53.67, 51.83, 51.72, 50.63, 28.81.

2-((4-(6-((4,4-Difluoropiperidin-1-yl) methyl)-5-fluoropyridin-2-yl) benzyl) amino)-*N*-(prop-2-yn-1-yl) acetamide (14): A mixture of **9** (0.23 g, 0.7 mmol, 1eq), **12** (0.15 g, 1.4 mmol, 2 eq), acetic acid (0.04 mL, 0.7 mmol, 1 eq) and $NaBH(OAc)_3$ (0.53 g, 2.5 mmol, 3.6 eq) in THF (9 mL) was stirred at RT for 16 h. The reaction was diluted with sat. $NaHCO_3$ (aq, 8 mL) and DCM (10 mL). The layers were separated and the aqueous layer extracted thrice with DCM. The combination of organic layers was dried ($MgSO_4$), filtered and the solvent evaporated under reduced pressure. The crude product was purified with flash column chromatography (SiO_2, 5% MeOH (with 1% Et_3N) in DCM) to yield a colourless oil (0.13 g, 0.30 mmol, 45%). ^{1}H-NMR (500 MHz, $CDCl_3$) δ 7.92 (d, *J* = 8.2 Hz, 2H), 7.64 (dd, *J* = 8.6, 3.6 Hz, 1H), 7.43 (t, *J* = 8.8 Hz, 2H), 7.38 (d, *J* = 8.3 Hz, 2H), 4.06 (dd, *J* = 5.5, 2.6 Hz, 2H), 3.88 (d, *J* = 2.5 Hz, 2H), 3.82 (s, 2H), 3.32 (s, 2H), 2.75 (t, *J* = 5.7 Hz, 4H), 2.24 (t, *J* = 2.6 Hz, 1H), 2.01 (hept, *J* = 5.9 Hz, 4H), 1.95 (s, 1H). ^{13}C- NMR (126 MHz, $CDCl_3$) δ 171.27, 157.75 (d, *J* = 257.7 Hz), 152.39 (d, *J* = 4.9 Hz), 145.15 (d, *J* = 14.6 Hz), 139.99, 137.74, 128.63, 127.15, 123.86 (d, *J* = 20.2 Hz), 122.04 (d, *J* = 241.3 Hz), 120.62 (d, *J* = 4.2 Hz), 79.69, 71.56, 57.04, 53.64, 51.78, 49.91 (t, *J* = 5.3 Hz), 34.09 (t, *J* = 22.8 Hz), 28.76.

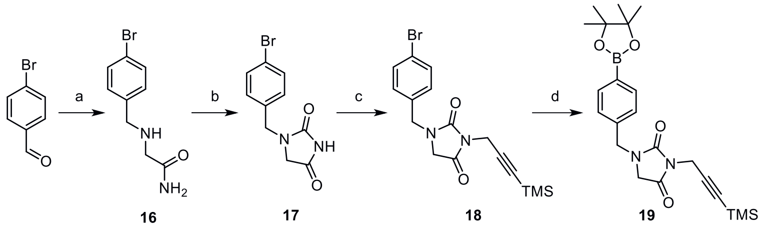
2-((4-(5-Fluoro-6-(piperidin-1-ylmethyl pyridin-2-yl) benzyl) amino)-*N*-(prop-2-yn-1-yl) acetamide (15): A mixture of **10** (0.20 g, 0.7 mmol, 1 eq), **12** (0.15 g, 1.4 mmol, 2 eq), acetic acid (0.04 mL, 0.7 mmol, 1 eq) and $NaBH(OAc)_3$ (0.53 g, 2.5 mmol, 3.6 eq) in THF (9 mL) was stirred at RT for 16 h. The reaction was diluted with sat. $NaHCO_3$ (aq, 8 mL) and DCM (10 mL). The layers were separated and the aqueous layer extracted thrice with DCM. The combination of organic layers was dried ($MgSO_4$), filtered and the solvent evaporated under reduced pressure. The crude product was purified with flash column chromatography (SiO_2, 10% EtOH (with 1% Et_3N) in DCM) to yield a colourless oil (0.14 g, 0.36 mmol, 54%). ^{1}H-NMR (500 MHz, $CDCl_3$) δ 7.93 (d, *J* = 8.2 Hz, 2H), 7.61 (dd, *J* = 8.5, 3.5 Hz, 1H), 7.40 (t, *J* = 8.8 Hz, 2H), 7.37 (d, *J* = 8.3 Hz, 2H), 4.06 (dd, *J* = 5.5, 2.6 Hz, 2H), 3.82 (d, *J* = 2.6 Hz, 2H), 3.81 (s, 2H), 3.32 (s, 2H), 2.59 (s, 4H), 2.23 (t, *J* = 2.6 Hz, 1H), 2.05 (s, 1H), 1.60 (p, *J* = 5.6 Hz, 4H), 1.41 (p, *J* = 6.3 Hz, 2H). ^{13}C-NMR (126 MHz, $CDCl_3$) δ 171.27, 157.83 (d, *J* = 257.7 Hz), 152.14 (d, *J* = 4.8 Hz), 145.62 (d, *J* = 14.1 Hz), 139.83, 137.94, 128.57, 127.18, 123.61 (d, *J* = 20.2 Hz), 120.27 (d, *J* = 4.1 Hz), 79.70, 71.53, 58.55 (d, *J* = 3.2 Hz), 54.40, 53.69, 51.82, 28.75, 26.04, 24.21.

***N*-(4-(6-((1,1-Dioxidothiomorpholino) methyl)-5-fluoropyridin-2-yl) benzyl)-*N*-(2-oxo-2-(prop-2-yn-1-ylamino) ethyl)-4-(3-(trifluoromethyl)-3*H*-diazirin-3-yl) benzamide (1)**: A mixture of HBTU (74.2 mg, 0.20 mmol, 1.5 eq), 4-(3(trifluoromethyl)-3*H*-diazirin-3-yl)) benzonic acid (30 mg, 0.13 mmol, 1 eq) and DiPEA (0.07 mL, 0.40 mmol, 3 eq) in anhydrous DCM (1 mL) was placed under inert atmosphere in a microwave vial. After stirring at RT for 1 h, **13** (63.6 mg, 0.14 mmol, 1.1 eq) dissolved in anhydrous DCM (1 mL) was added dropwise to the reaction mixture. After stirring at RT for 16 h the reaction was diluted with sat. $NaHCO_3$ (aq). The layers were separated and the aqueous layer was extracted thrice with DCM. The combination of organic layers was dried ($MgSO_4$), filtered and the solvent evaporated under reduced pressure. The crude product was purified with flash column chromatography (SiO_2, 70-90% EtOAc in pentane) followed by preparative HPLC and freeze drying to yield a white solid (36.3 mg, 0.06 mmol, 42%). ^{1}H-NMR (400 MHz, MeOD) δ 7.94 (d, *J* = 8.0 Hz, 2H), 7.68 (dd, *J* = 8.6, 3.5 Hz, 1H), 7.56 (d, *J* = 8.0 Hz, 2H), 7.49 (t, *J* = 8.8 Hz, 1H), 7.28 (d, *J* = 8.1 Hz, 2H), 7.22 (d, *J* = 8.1 Hz, 2H), 4.67 (s, 2H), 4.10 (s, 2H), 4.09 – 4.04 (m, 2H), 4.03 (d, *J* = 2.4 Hz, 2H), 3.25 – 3.19 (m, 4H), 3.13 – 3.08 (m, 4H), 2.26 (s, 1H). ^{13}C-NMR (126 MHz, $CDCl_3$) δ 171.93, 168.05, 158.90, 157.86 (d, *J* = 259.7 Hz), 152.29 (d, *J* = 7.5 Hz), 144.39 (d, *J* = 20.4 Hz), 124.29 (d, *J* = 20.1 Hz), 138.18, 136.40, 136.11, 131.56, 127.63, 127.62, 127.54, 126.92, 124.37, 124.21, 123.11, 121.16 (d, *J* = 4.1 Hz), 79.51, 71.99, 56.64, 53.93, 51.74, 50.61, 49.24, 29.38. HRMS $[C_{31}H_{28}F_4N_6O_4S + H]^+$: 657.19016 calculated, 657.18978 found.

***N*-(4-(6-((4,4-Difluoropiperidin-1-yl) methyl)-5-fluoropyridin-2-yl) benzyl)-*N*-(2-oxo-2-(prop-2-yn-1-ylamino) ethyl)-4-(3-(trifluoromethyl)-3*H*-diazirin-3-yl) benzamide (3)**: A mixture of HBTU (74.2 mg, 0.20 mmol, 1.5eq), 4-(3(trifluoromethyl)-3*H*-diazirin-3-yl)) benzonic acid (30 mg, 0.13 mmol, 1 eq) and DiPEA (0.07 mL, 0.40 mmol, 3 eq) in anhydrous DCM (1 mL) was placed under inert atmosphere in a microwave vial. After stirring at RT for 1 h, **14** (61.7 mg, 0.14 mmol, 1.1eq) dissolved in anhydrous DCM (1 mL) was added dropwise to the reaction mixture. After stirring at RT for 16 h the reaction was diluted with sat. $NaHCO_3$ (aq). The layers were separated and the aqueous layer was extracted thrice with DCM. The combination of organic layers was dried ($MgSO_4$), filtered and the solvent evaporated under reduced pressure. The crude product was purified with flash column chromatography (SiO_2, 70-100% EtOAc in pentane) followed by preparative HPLC and freeze drying to yield a white solid (36.6 mg, 0.06 mmol, 44%). ^{1}H-NMR (400 MHz, MeOD) δ 8.03 (t, *J* = 7.5 Hz, 2H), 7.86 (dt, *J* = 8.0, 3.8 Hz, 1H), 7.69

– 7.57 (m, 3H), 7.48 (d, *J* = 7.9 Hz, 1H), 7.38 – 7.28 (m, 3H), 4.82 (s, 1H), 4.62 (s, 1H), 4.14 (s, 1H), 3.97 (dd, *J* = 34.5, 2.5 Hz, 2H), 3.89 (s, 2H), 3.82 (s, 1H), 2.76 (s, 4H), 2.67 – 2.59 (m, 1H), 2.08 – 1.94 (m, 4H). ^{13}C-NMR (126 MHz, $CDCl_3$) δ 172.48, 163.97, 149.08, 148.53, 129.22, 126.92, 124.81, 77.41, 77.16, 76.91, 53.93, 49.61, 49.26, 29.39. HRMS $[C_{32}H_{28}F_6N_6O_2 + H]^+$: 643.22507 calculated, 643.22467 found.

***N*-(4-(5-Fluoro-6-(piperidin-1-ylmethyl) pyridin-2-yl) benzyl)-*N*-(2-oxo-2-(prop-2-yn-1-ylamino) ethyl)-4-(3-(trifluoromethyl)-3*H*-diazirin-3-yl) benzamide (4)**: A mixture of HBTU (74.2 mg, 0.20 mmol, 1.5eq), 4-(3(trifluoromethyl)-3*H*-diazirin-3-yl)) benzonic acid (30 mg, 0.13 mmol, 1 eq) and DiPEA (0.07 mL, 0.40 mmol, 3 eq) in anhydrous DCM (1 mL) was placed under inert atmosphere in a microwave vial. After stirring at RT for 1 h, **15** (56.6 mg, 0.14 mmol, 1.1 eq) dissolved in anhydrous DCM (1 mL) was added dropwise to the reaction mixture. After stirring at RT for 16 h the reaction was diluted with sat. $NaHCO_3$ (aq). The layers were separated and the aqueous layer was extracted thrice with DCM. The combination of organic layers was dried ($MgSO_4$), filtered and the solvent evaporated under reduced pressure. The crude product was purified with flash column chromatography (SiO_2, 90-100% EtOAc in pentane) followed by preparative HPLC and freeze drying to yield a white solid (36.6 mg, 0.06 mmol, 46%). ^{1}H-NMR (500 MHz, $CDCl_3$) δ 7.96 (d, *J* = 7.9 Hz, 2H), 7.61 (dd, *J* = 8.6, 3.5 Hz, 1H), 7.55 (d, *J* = 8.0 Hz, 2H), 7.48 – 7.38 (m, 3H), 7.21 (d, *J* = 8.1 Hz, 2H), 4.65 (s, 2H), 4.16 – 4.00 (m, 4H), 3.82 (d, *J* = 2.7 Hz, 2H), 2.58 (s, 4H), 1.87 (s, 1H), 1.60 (t, *J* = 5.8 Hz, 4H), 1.45 – 1.37 (m, 2H), 1.25 (t, *J* = 7.2 Hz, 1H). ^{13}C-NMR (126 MHz, $CDCl_3$) δ 171.96, 168.09, 162.36, 162.08, 138.81, 137.40, 127.67, 126.92, 125.06 (d, *J* = 20.0 Hz), 122.69, 117.83, 115.51, 73.94, 71.91, 54.50, 53.32, 49.13, 29.34, 22.88, 22.05. HRMS $[C_{32}H_{30}F_4N_6O_2 + H]^+$: 607.24391 calculated, 607.24317 found.



Scheme 3.6 Synthesis of Suzuki Coupling Reagent **19**. Reagents and conditions: a) Step 1: 2-aminoacetamide hydrochloride (1.0 eq), NaOH (1.1 eq), MeOH:H_2O (0.3 M, 5:1 (v/v)), RT, 18 h; Step 2: $NaBH_4$ (2.1 eq), 18 h, 91% (two steps); b) CDI (2.1 eq), DMAP (2.1 eq), ACN (0.1 M), 60 °C, 70 h, 37%; c) 3-bromo-1-(trimethylsilyl)-1-propyne (1.2 eq), K_2CO_3 (1 eq), DMF (0.3 M), 50 °C, 16 h, 61%; d) bis(pinacol)diborane (1.5 eq), KOAc (4 eq), Pd(dppf)Cl_2 (0.5 eq), 1,4 dioxane (0.1 M), 80 °C, 16 h, used as crude.

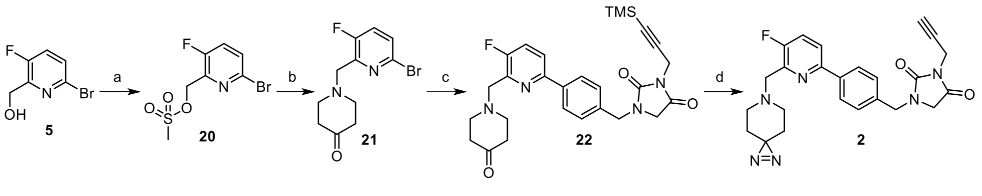
2-((4-Bromobenzyl)amino)acetamide (16): To a stirred mixture of 4-bromobenzaldehyde (9.2 g (49.7 mmol, 1.1 eq) and 2-aminoacetamide hydrochloride (5.06 g, 45.8 mmol, 1.0 eq) in MeOH:H_2O (170 mL, 5:1, v/v) was added NaOH (2.06 g, 51.5 mmol, 1.1 eq). After stirring at RT overnight, $NaBH_4$ (3.6 g, 95.2 mmol, 2.1 eq) was added and the solution was stirred overnight at RT. The solution was acidified to pH 3 with 2 M HCl, then neutralized with sat. $NaHCO_3$ (aq). Methanol was evaporated under reduced pressure and the resulting slurry was filtered to yield a white solid (11.0 g, 45.2 mmol, 91%). ^{1}H-NMR (300 MHz, MeOD) δ 7.69 – 7.59 (m, 2H), 7.47 – 7.38 (m, 2H), 4.22 (s, 2H), 3.81 (s, 2H).

1-(4-Bromobenzyl)imidazolidine-2,4-dione (17): To stirred suspension of **16** (10.0 g, 40.1 mmol, 1.0 eq) in acetonitrile (300 mL) were added CDI (13.86 g, 85.5 mmol, 2.1 eq) and DMAP (10.2 g, 83.5 mmol, 2.1 eq). The mixture was heated (60 °C) under inert atmosphere for 70 h. 1 M HCl (aq, 250 mL) was added and the aqueous layer extracted thrice with EtOAc. The combination of organic layers was washed with H_2O and brine, dried ($MgSO_4$), filtered, and the solvent evaporated under reduced pressure. The crude product was purified with flash column chromatography with dry loading over Celite (SiO_2, 5-10% acetone in DCM) to yield a yellow solid (3.95 g, 14.7 mmol, 37%). ^{1}H-NMR (300 MHz,

$CDCl_3$) δ 7.83 (bs, 1H), 7.56 – 7.45 (m, 2H), 7.20 – 7.10 (m, 2H), 4.49 (s, 2H), 3.79 (s, 2H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ 132.41, 129.95, 77.58, 77.16, 76.74, 50.36, 46.01.

1-(4-Bromobenzyl)-3-(3-(trimethylsilyl) prop-2-yn-1-yl) imidazolidine-2,4-dione (18): To a stirred mixture of **17** (0.52 g, 1.9 mmol, 1 eq) in DMF (6 mL) under inert atmosphere were added K_2CO_3 (0.27 g, 1.9 mmol, 1 eq) and 3-bromo-1-(trimethylsilyl)-1-propyne (0.38 g, 2.3 mmol, 1.2 eq). The reaction mixture was heated (50 °C) for 16 h. and subsequently diluted with 0.4 M K_2CO_3 (aq) and EtOAc. The layers were separated and the aqueous layer was extracted thrice with EtOAc. The combination of organic layers was washed with brine, dried ($MgSO_4$), filtered and the solvent evaporated under reduced pressure. The crude product was purified with flash column chromatography (SiO_2, 40-80% EtOAc in pentane) to yield a yellow oil (0.44 g, 1.2 mmol, 61%). ^1H-NMR (500 MHz, $CDCl_3$) δ 7.48 (d, *J* = 8.3 Hz, 2H), 7.13 (d, *J* = 8.3 Hz, 2H), 4.52 (s, 2H), 4.29 (s, 2H), 3.74 (s, 2H), 0.14 (s, 9H).

1-(4-(4,4,5,5-Tetramethyl-1,3,2-dioxaborolan-2-yl) benzyl)-3-(3-(trimethylsilyl) prop-2-yn-1-yl) imidazolidine-2,4-dione (19): A stirred mixture of **18** (0.23 g, 0.61 mmol, 1 eq), bis(pinacolato)diboron (0.23 g, 0.91 mmol, 1.5 eq), $Pd(dppf)Cl_2$ (0.03 g, 0.03 mmol, 0.5 eq) and KOAc (0.24 g, 2.44 mmol, 4 eq) in 1,4-dioxane (5 mL) under inert atmosphere was heated (80 °C) for 16 h. The reaction mixture was concentrated under reduced pressure. The mixture was diluted with sat. $NaHCO_3$ (aq, 10 mL) and H_2O (15 mL) and the layers were separated. The aqueous layer was extracted thrice with EtOAc. The combination of organic layers was washed with brine, dried ($MgSO_4$), filtered and the solvent evaporated under reduced pressure. The crude product **19** was used directly in the next step to form **22**.



Scheme 3.7 Synthesis of Probe **2**. Reagents and Conditions: a) Et_3N (2.3 eq), MsCl (1.7 eq), THF (0.2 M), 0 °C-RT, 1 h, 75%; b) 4-piperidone hydrochloride (1.2 eq), K_2CO_3 (3 eq), ACN (0.2 M), 70 °C, 48 h, 78%; c) K2CO3 (1.8 eq), Pd(PPh3)4 (0.1 eq), toluene:EtOH (0.1 M, 4:1 (v/v)), 50 °C, 16 h, 29%; d) Step 1: NH3 (g), MeOH (0.2 M), 0 °C, 5 h; Step 2: NH2SO3H (1.5 eq), MeOH (0.2 M), 16 h, RT, Step 3: I2, MeOH, 0 °C 15 min., 27% (three steps).

(6-Bromo-3-fluoropyridin-2-yl)methyl methanesulfonate (20): To a cooled (0 °C) mixture of **5** (1.6 g, 7.8 mmol, 1 eq) and Et_3N (2.5 mL, 17.9 mmol, 2.3 eq) in dry THF (40 mL) was added dropwise MsCl (1.0 mL, 12.9 mmol, 1.7 eq). After stirring at RT for 1 h the solution was concentrated under reduced pressure. DCM and H_2O were added and the layers were separated. The aqueous layer was extracted thrice with DCM. The combination of organic layers was washed with brine, dried ($MgSO_4$), filtered, and the solvent evaporated under reduced pressure to yield a yellow solid (1.65 g, 5.8 mmol, 75%). ^1H-NMR (500 MHz, $CDCl_3$) δ 7.52 (dd, *J* = 8.6, 3.5 Hz, 1H), 7.37 (t, *J* = 8.5 Hz, 1H), 5.33 (d, *J* = 2.1 Hz, 2H), 3.13 (s, 3H). ^{13}C-NMR (126 MHz, $CDCl_3$) δ 157.82 (d, *J* = 261.3 Hz), 142.15 (d, *J* = 16.0 Hz), 130.74 (d, *J* = 4.4 Hz), 127.06 (d, *J* = 20.4 Hz), 65.50 (d, *J* = 1.6 Hz), 38.39.

1-((6-Bromo-3-fluoropyridin-2-yl) methyl) piperidin-4-one (21): A stirred degassed mixture of **20** (0.67 g, 2.4 mmol, 1 eq), 4-piperidone hydrochloride (0.39 g, 2.8 mmol, 1.2 eq) and K_2CO_3 (0.98 g, 7.1 mmol, 3 eq) in acetonitrile (12 mL) was heated (70 °C) for 48 h. The reaction mixture was diluted with H_2O (10 mL) and DCM (5 mL) and the layers were separated. The aqueous layer was extracted thrice with DCM. The combination of organic layers was dried ($MgSO_4$), filtered and the solvent evaporated under reduced pressure. The crude product was purified with flash column chromatography (SiO_2, 30-70% EtOAc in pentane) to yield a yellow solid (0.53 g, 1.9 mmol, 78%).

^{1}H-NMR (500 MHz, $CDCl_3$) δ 7.42 (dd, *J* = 8.5, 3.5 Hz, 1H), 7.30 (t, *J* = 8.5 Hz, 1H), 3.84 (d, *J* = 2.5 Hz, 2H), 2.87 (t, *J* = 6.1 Hz, 4H), 2.46 (t, *J* = 6.2 Hz, 4H). ^{13}C-NMR (126 MHz, $CDCl_3$) δ 208.82, 158.22 (d, *J* = 258.0 Hz), 146.93 (d, *J* = 15.8 Hz), 135.20, 128.69 (d, *J* = 4.4 Hz), 126.31 (d, *J* = 21.6 Hz), 56.40 (d, *J* = 2.8 Hz), 53.02, 41.34.

1-(4-(5-Fluoro-6-((4-oxopiperidin-1-yl) methyl) pyridin-2-yl) benzyl)-3-(3-(trimethylsilyl) prop-2-yn-1-yl) imidazolidine-2,4-dione (22): A stirred mixture of crude **19** (max. 0.61 mmol, 1.5 eq), **21** (0.12 g, 0.41 mmol, 1 eq), K_2CO_3 (0.10 g, 0.73 mmol, 1.8 eq) and $Pd(PPh_3)_4$ (0.07 g, 0.06 mmol, 0.1 eq) in degassed toluene:EtOH (4 mL, 4:1 (v/v)) was heated (50 °C) for 16 h. The mixture was filtered and the residue rinsed with EtOAc. H_2O (10 mL) was added and the layers were separated. The aqueous layer was extracted thrice with EtOAc. The combination of organic layers was washed with brine, dried ($MgSO_4$), filtered and the solvent evaporated under reduced pressure. The crude product was purified with flash column chromatography (SiO_2, 0-10% MeOH in EtOAc) to yield a yellow oil (60.0 mg, 0.12 mmol, 29%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.95 (d, *J* = 8.3 Hz, 2H), 7.66 (dd, *J* = 8.6, 3.6 Hz, 1H), 7.47 (t, *J* = 8.8 Hz, 1H), 7.36 (d, *J* = 8.2 Hz, 2H), 4.63 (s, 2H), 4.32 (s, 2H), 3.98 (d, *J* = 2.4 Hz, 2H), 3.78 (s, 2H), 2.96 (t, *J* = 6.1 Hz, 4H), 2.49 (t, *J* = 6.1 Hz, 4H), 1.23 (s, 9H). ^{13}C-NMR (101 MHz, $CDCl_3$) δ 206.47, 127.72, 126.86, 124.37 (d, *J* = 21.5 Hz), 121.96 (d, *J* = 5.5 Hz), 83.40, 72.26, 56.74, 51.74, 50.66, 43.65, 29.86, 29.56, 0.15.

1-(4-(6-((1,2,6-Triazaspiro [2.5] oct-1-en-6-yl) methyl)-5-fluoropyridin-2-yl) benzyl)-3-(prop-2-yn-1-yl) imidazolidine-2,4-dione (2): In a microwave vial a stirred and cooled (0 °C) solution of **22** (60 mg, 0.12 mmol, 1eq) in anhydrous MeOH (0.5 mL) for 1 h an NH_3 (g) gas flow was established. The reaction was stirred at RT for an additional 5 h. NH_2SO_3H (25 mg, 0.18 mmol, 1.5 eq) dissolved in anhydrous MeOH (0.2 mL) was added dropwise to the reaction mixture. After stirring at RT for 16 h the reaction was filtered over Celite and the solution concentrated under reduced pressure. To a cooled (0 °C) solution of the crude in MeOH (1 mL) was added dropwise sat. I_2 in anhydrous MeOH until a colour change to orange persisted for 15 minutes. The reaction was quenched with sat. $Na_2S_2O_3$ (aq) (2 mL) and diluted with EtOAc. The layers separated and the aqueous layer was extracted thrice with EtOAc. The combination of organic layers was washed with 2 M HCl and brine, dried ($MgSO_4$), filtered and the solvent evaporated under reduced pressure. The crude product was purified with flash column chromatography (SiO_2, 100% EtOAc). The compound was freeze dried to yield a white solid (14.2 mg, 0.03 mmol, 27%). ^{1}H-NMR (500 MHz, $CDCl_3$) δ 7.96 (d, *J* = 8.3 Hz, 2H), 7.65 (dd, *J* = 8.6, 3.5 Hz, 1H), 7.46 (t, *J* = 8.7 Hz, 1H), 7.38 – 7.33 (m, 2H), 4.63 (s, 2H), 4.32 (d, *J* = 2.5 Hz, 2H), 3.95 (d, *J* = 2.5 Hz, 2H), 3.79 (s, 2H), 2.81 (t, *J* = 5.8 Hz, 4H), 2.25 (t, *J* = 2.5 Hz, 1H), 1.33 (t, *J* = 5.7 Hz, 4H). ^{13}C-NMR (126 MHz, $CDCl_3$) δ 168.70, 158.00 (d, *J* = 258.2 Hz), 155.58, 152.02 (d, *J* = 4.9 Hz), 145.61, 138.77, 135.82, 128.79, 127.65, 123.93 (d, *J* = 20.4 Hz), 120.70 (d, *J* = 4.1 Hz), 71.86, 57.84 (d, *J* = 2.9 Hz), 51.99, 49.29, 46.70, 31.37, 28.30. HRMS $[C_{24}H_{23}FN_6O_2 + H]^+$: 447.19393 calculated, 447.19360 found.

Biology

"General Remarks", "Cell Culture", "Membrane Preparation", "[^{3}H]CP-55,940 Heterologous Displacement", "[^{35}S]GTPγS Binding Assay" and "Quantification and Statistical Analysis" have been previously described in **Chapter 2**.

SDS PAGE

CHO-K1 hCB_2R_bgal membranes were diluted to 0.78 ug/uL and homogenized for 15 seconds with an Ultra Turrax homogenizer (IKA-Werke GmbH & Co. KG, Staufen, Germany). Benzonase was added (1:10000 dilution from $2.5 \cdot 10^6$ U/mL). To each sample was added 16 µL membrane followed by 1 µL 360 µM CP-55,940 or DMSO. To the denatured sample was also added 2 µL 10% SDS. After incubation of 30 minutes at RT 1 µL 36 µM probe was added to the samples. After 30 minutes incubation at RT the samples (except the no UV samples) were irradiated with 350 nm light in a Caprobox™ for 5 minutes.

2 µL click mix (11 mM $CuSO_4$, 66 mM NaAsc, 2.2 mM THPTA, 10 µM Cy5-N_3 (not present in azide free mix)) was added to the samples. After incubation for 30 minutes at RT the samples were denatured with 5 µL 4* Laemmli buffer. After 30 minutes at RT the samples were loaded onto 12.5% 1.5 mm SDS gels (15 µL sample/well). Gels were run at 180V for 80-90 minutes. Gels were imaged with a ChemiDoc (Biorad, The Netherlands). After imaging all proteins loading of the wells was visualized by staining with Coomassie Brilliant Blue R-250 Dye and sequentially destaining with destaining fluid (50/40/10 H_2O/MeOH/AcOH). After leaving overnight in H_2O, pictures were made on the ChemiDoc (Biorad, The Netherlands).

Molecular Docking

The structure of CB_2R in complex with LEI-102 (8GUT) was retrieved from the Protein Data Bank using ICM Molsoft's inbuilt feature. This structure was converted to an ICM object, with 'optimize hydrogens' set to true, after which all but the R chain (the receptor) was removed. The probe LEI-121 was inserted as ICM objects via the 'New 3D Chemical' functionality. LEI-102 was extracted from the receptor object and a pocket box was defined around this molecule using default settings. Two probes were docked with the 'thoroughness' parameter set to 10, and the 10 best poses were retained. The resulting poses were manually inspected and visualised using the Open Source PyMOL application.

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