Activity-based proteomics of the endocannabinoid system
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Chapter 6

Two-step activity-based protein profiling of diacylglycerol lipase[1]

Introduction

Endocannabinoids are key regulators of neurotransmitter release in the central nervous system (CNS). They are involved in virtually every aspect of brain function, including modulation of synaptic plasticity and (patho)physiological processes, such as anxiety, fear and neuroinflammation.[2] 2-Arachidonoylglycerol (2-AG) is one of the most important endocannabinoids and activates the cannabinoid CB_1 and CB_2 receptors. 2-AG is synthesized by two diacylglycerol lipases (DAGLα (120 kDa) and DAGLβ (70 kDa)).[3] Both enzymes belong to the family of serine hydrolases, which share the same catalytic Ser-His-Asp triad to hydrolyze the *sn*-1 ester of 1-acyl-2-arachidonoylglycerides to generate 2-AG. A method to measure endogenous DAGL activity in biological samples is therefore important to understand endocannabinoid physiology.

Activity-based protein profiling (ABPP) is a powerful technique for monitoring enzyme activity in living systems using chemical probes.[4] These activity-based probes (ABPs) covalently and irreversibly bind to the active site of an enzyme and this interaction can be subsequently monitored using different techniques depending on the reporter group.[5] Several fluorescent ABPs have been reported to study the two isoforms of DAGLs. For example, HT-01 (**Fig. 1**), a DAGL probe based on 1,2,3-triazole urea inhibitors developed by the Cravatt laboratory, was used to study endogenous DAGLβ in (primary) macrophages.[6] In addition, DH379 (**Fig. 1**), based on the potent DAGL inhibitor DH376, was developed as a tailored fluorescent probe for DAGLα and DAGLβ.[7] However, reporter groups may affect the affinity and selectivity of the probes as well as cell permeability and metabolic stability. These issues are avoided by ligation of the reporter group to the probe after covalent binding of the target. Bioorthogonal chemistry enables the design of chemical probes with a minimalist handle for the conjugation of a reporter group after the probe target has been bound.[8] These two-step bioorthogonal probes also provide flexibility, as different reporter groups can be attached to the same probe. Different pairs of bioorthogonal reactants are

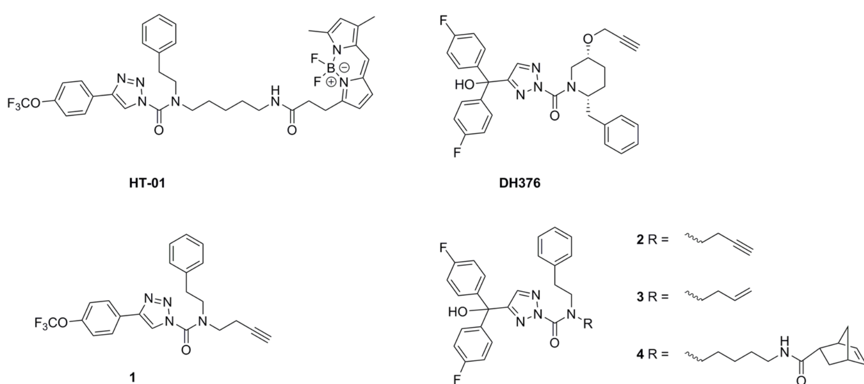

Figure 1. Design of two-step labeling probes **1** - **4** based on HT-01 and DH376.

currently available.[9] For two-step activity-based probes, the most popular pair is the azide-alkyne couple. These handles are reacted using the copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC), often called "click" reaction. Both azides and alkynes are compact handles, chemically stable and synthetically accessible. An example of a two-step bioorthogonal probe for DAGL is DH376, which carries an alkyne handle (**Fig. 1**).

The CuAAC is relatively slow and requires toxic Cu(I) as a catalyst, therefore the inverse electron-demand Diels-Alder (IEDDA) ligation is sometimes used as an alternative. The reactants are an electron-rich dienophile and electron poor diene, usually a tetrazine. Tetrazines attached to fluorophores can serve as both the bioorthogonal reactive group and the fluorescence quencher, creating fluorescence "turn-on" reporters ideal for imaging.[10,11] An additional advantage is that no catalyst is required for the IEDDA.

Seven different two-step ABPs (**1** - **4**, **21** - **23**) are reported for DAGLs based on the scaffolds of HT-01 and DH376 (**Fig. 1**). Probe **1** is based on the HT-01 scaffold and has an alkyne handle. Probes **2** - **4** are hybrid probes of DH376 and HT-01. Probes **3** and **4** were designed to enable the use of the IEDDA reaction,[12,13] employing an alkene and a more strained norbornene, respectively. Probes **21** - **23** are triazole regioisomers of probes **1** - **3**, respectively.

Results and discussion

Synthesis. The synthesis of the triazole urea probes **1** – **4** started with the synthesis of the triazole scaffolds **6** and **8**, which are subsequently converted to the triazole ureas by coupling to an amine using triphosgene.[14] For the synthesis of probe **1**, 4-trifluoromethoxyphenylacetylene (**5**) was reacted with TMS-N_3 under Cu^I-catalyzed [2+3] cycloaddition conditions to give triazole **6** (**Scheme 1**).

Scheme 1. Synthesis of triazoles. Reagents and conditions: (**a**) TMS-N_3, CuI, DMF/MeOH, 100 °C, o/n, 27%; (**b**) 4-fluorophenylmagnesium bromide, THF, 74%.

Scheme 2. Synthesis of amines. Reagents and conditions: (**a**) *i*. NsCl, Et_3N, THF; ii. $Ph(CH_2)_2Br$, Cs_2CO_3, CH_3CN, 80 °C, **10**: 93%; **13**: 70%; **16**: 92%; (**b**) PhSH, Cs_2CO_3, CH_3CN, **11**: 74%; **14**: 81%; **20**: 41%; (**c**) TFA/DCM 1:9, 100%; (**d**) DIPEA, DMF, 33%.

The triazole scaffold of compounds **2** - **4** was synthesized by performing a Grignard reaction on ester **7** to yield triazole **8**.

The amine building blocks (**11**, **14**, **20**), which were used to construct the urea warheads, were synthesized using the following reaction sequences (**Scheme 2**). 1-Amino-3-butyne (**9**) was first nosylated and reacted with phenethyl bromide to yield **10**, which was subsequently deprotected to give **11** in 69% yield over three steps. Amines **14** and **16** were synthesized in a similar fashion from 3-butenylamine (**12**) and N-Boc-cadaverine (**15**), respectively. The Boc-group of **16** was removed with acid to yield **17**, which was coupled to the activated norbornene ester **18** to give the amide **19** as the endo-isomer. Removal of the nosyl group yielded amine **20**.

With the triazoles (**6**, **8**) and amines (**11**, **14** and **20**) in hand, the final compounds (probes **1** - **4**) were obtained using a triphosgene coupling (**Scheme 3**).[14] The amines were first converted to the corresponding carbamoyl chlorides and subsequently reacted with the triazoles. Generally, this yielded two regioisomers:

Scheme 3 (chemical scheme; labels: a, b, N1 regioisomer, N2 regioisomer)

Scheme 3. Triphosgene coupling for synthesis of triazole ureas. Reagents and conditions: (**a**) triphosgene, DIPEA, THF, 0 °C; (**b**) DIPEA, DMAP, THF, 60 °C. **1**: 37%; **21**: 41%; **22**: 33%; **2**: 40%; **23**: 29%; **3**: 11%; **4**: 37% (Table 2).

N1 and N2.

To assign the separate compounds as either N1-regioisomer or N2-regioisomer, it was anticipated that the NMR chemical shift of the triazole carbon could be used (**Table 1**, **2**). To this end, theoretical chemical shifts were computed with density functional theory (DFT) for simplified structures of the triazole urea scaffold (**Table 1**). For structures with either a phenyl or a methyl directly attached to the triazole, the chemical shift in DMSO was calculated for the lowest energy conformer of either the N1 or N2 regioisomer. This resulted in theoretical chemical shift differences of approximately 10 ppm between the triazole carbon of the regioisomers with a shift of ±125 ppm for the N1 regioisomers and ±135 ppm for the N2 regioisomers.

Next, the experimental chemical shifts of the regioisomeric pairs (**1** & **21**, **2** & **22**, **3** & **23** and **4**) were measured (**Table 2**). In line with the theoretical calculations, the experimental chemical shift differed ~10 ppm between the two separated isomers. The triazole proton is highly characteristic (broad, downfield peak) and HSQC experiments were used to confidently assign the triazole carbon peak in the ^{13}C aromatic region. For the polar regioisomers (slower migration on TLC, left column of **Table 1**) the chemical shift is ~125 ppm and the same carbon in the apolar regioisomers (right column of **Table 1**) is shifted downfield to around 135 ppm. This trend is observed for each pair of regioisomers (**Table 2**; the corresponding apolar regioisomer of probe **4** was not obtained in sufficient yield for NMR analysis). The compounds (**Table 2**) can therefore be assigned as N1 or N2 isomers depending on their chemical shift. This assignment is in agreement with earlier reported regioisomers of other triazole ureas as determined with a crystal structure[6] or NMR measurements.[7,15]

Table 1. Computed values of the ^{13}C shift of the triazole carbon (indicated with the red circle). Simplified structures of N1 and N2 regioisomers for DFT calculations are shown.

Structure	Theoretical δ (ppm)
	122 (R = Ph) 124 (R = Me)
	135 (R = Ph) 138 (R = Me)

Table 2. Final compounds obtained from triphosgene couplings (Scheme 3) and the ^{13}C NMR chemical shift of the triazole carbon of 1,2,3-triazole urea regioisomers (measured in DMSO).

Entry	Structure	^{13}C ppm	Entry	Structure	^{13}C ppm
1		126	21		134
22		123	2		135
23		123	3		135
			4		135

Biochemical analysis

The potency and selectivity of the probes **1** - **4** and their regioisomers (**21** - **23**) were initially screened in mouse membrane proteomes using competitive ABPP with MB064 (**Fig. 2**).

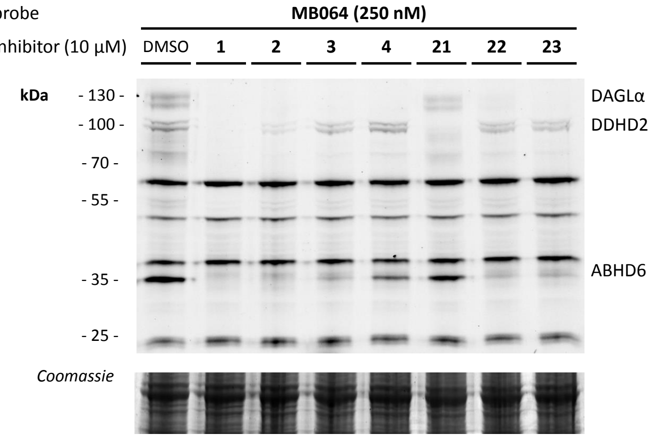

Figure 2. Activity-based protein profiling of probes **1** – **4** and their regioisomers (**21** - **23**) in mouse brain membrane proteome in competition with probe MB064.

Probe **1** (10 µM) inhibited DAGLα, DDHD domain-containing protein 2 (DDHD2) and ABHD6, as judged from the disappearing bands on the gel. Its regioisomer **21** was inactive against DAGLα and ABHD6, but did inhibit DDHD2. Probe **2** (and the regioisomer **22**) also inhibited DAGLα, DDHD2 and ABHD6. Probe **3** (and the regioisomer **23**) showed a similar labeling pattern as probe **2**, but were less active against DDHD2. The norbornene-substituted probe **4** was highly potent and selective for DAGLα in mouse brain, therefore this probe was further profiled in mouse brain proteome against ABPs MB064, FP-TAMRA and DH379 (**Fig. 3a**,**b**). Probe **4** showed a dose-dependent inhibition of DAGLα and DAGLβ with a pIC_{50} of 8.3 ± 0.3 and 8.6 ± 0.1, respectively. In addition, *in situ* experiments were performed with probe **4** (**Fig. 3c**) using the human cell line U2OS transiently transfected with recombinant human DAGLα. Live cells were treated with **4** and post-lysis labeled with MB064. Probe **4** was able to cross the cell membrane and label human DAGLα, albeit with a ten-fold lower potency compared to *in vitro* mouse brain proteome (**Fig. 3f**). Of note, probe **4** also inhibited the post-lysis labeling of endogenous ABHD6 with pIC_{50} of 8.5 ± 0.3. This discrepancy between *in situ* and *in vitro* potency has been previously observed for other covalent, irreversible serine hydrolase inhibitors.[7,16]

Finally, *in situ* two-step labeling was performed with fluorogenic BODIPY-tetrazine **24** (**Fig. 4**, see experimental for synthesis).[11] U2OS cells were transfected with either human DAGLα or catalytically

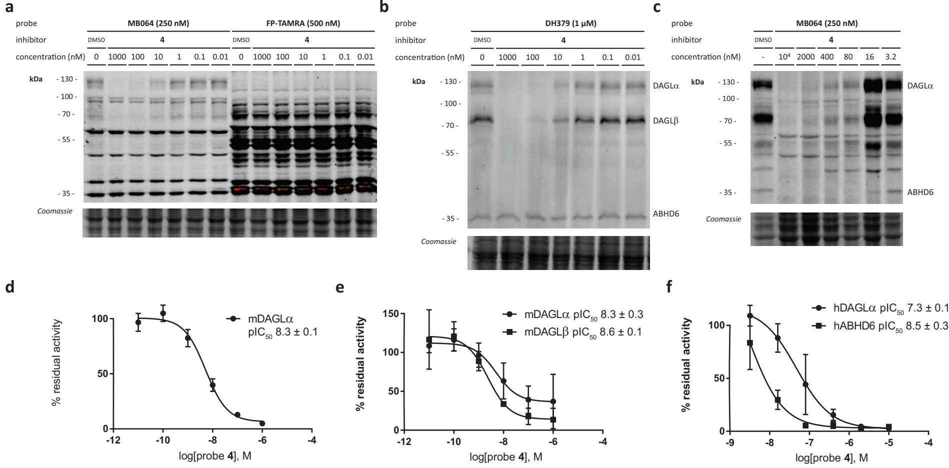

Figure 3. Activity-based protein profiling of probe **4** in mouse brain membrane proteome against (**a**) MB064 and FP-TAMRA and (**b**) DH379. (**c**) *In situ* treatment of U2OS cells transfected with DAGLα. (**d**) Quantification of residual DAGLα activity as measured with MB064 in mouse brain. (**e**) Quantification of residual DAGLα and DAGLβ activity as measured with DH379 in mouse brain. (**f**) Quantification of residual DAGLα and ABHD6 activity as measured with MB064 in U2OS-DAGLα cells.

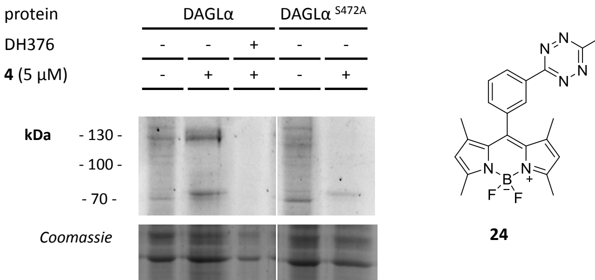

Figure 4. *In situ* two-step labeling of DAGLα overexpressed in U2OS cells with probe **4** and BODIPY-tetrazine **24** (10 μM).

inactive DAGLα^{S472A}. Cells were pre-treated with DH376 as a negative control. Treatment of cells expressing DAGLα or DAGLα^{S472A} with tetrazine **24** only showed some background labeling (**Fig. 4**), whereas treatment with norbornene probe **4**, followed by *in situ* treatment with tetrazine **24**, resulted in a strong fluorescent band just below 130 kDa, which was prevented by pre-treatment of cells with DH376 and absent in the DAGLα^{S472A} control. This experiment demonstrated that norbornene probe **4** reacted with the catalytic serine of DAGLα in live cells and can be labeled with a tetrazine fluorophore.

Conclusion

Norbornene probe **4** was successfully synthesized as a two-step ABP for visualization of DAGLα using an IEDDA ligation in living cells. The IEDDA reaction is complementary to the CuAAC reaction for labeling DAGLα, but is preferred for *in situ* imaging of enzyme activity. However, additional probes with improved activity and selectivity should be made to study endogenous DAGL activity. For live cell imaging, fluorogenic tetrazines with longer wavelengths than **24** are required.[17] It is envisioned that live cell imaging of DAGL activity will enable the study of this endocannabinoid enzyme's localization and processing during differentiation and other cellular processes.

Experimental

Synthesis

General methods. Reagents were purchased from Sigma Aldrich, Acros or Merck and used without further purification unless noted otherwise. Reactions under dry conditions were performed using oven or flame-dried glassware and dry solvents, which were dried for a minimum of 24 h over activated molecular sieves of appropriate pore size (3 - 4Å). Traces of water were removed from starting compounds by co-evaporation with toluene. All moisture sensitive reactions were performed under an argon or nitrogen atmosphere. Flash chromatography was performed using SiliCycle silica gel type SilicaFlash P60 (230 – 400 mesh). HPLC purification was performed on a preparative LC-MS system (Agilent 1200 series) with an Agilent 6130 Quadruple MS detector. TLC analysis was performed on Merck silica gel 60/Kieselguhr F254, 0.25 mm. Compounds were visualized using UV-irradiation and/or a $KMnO_4$ stain (K_2CO_3 (40 g), $KMnO_4$ (6 g), H_2O (600 mL) and 10% NaOH (5 mL)). ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker AV-400 MHz spectrometer at 400 (^{1}H) and 100 (^{13}C) MHz using $CDCl_3$, CD_3OD or $(CD_3)_2SO$ as solvent, unless stated otherwise. Spectra were analyzed using MestReNova 11.0.3. Chemical shift values are reported in ppm with tetramethylsilane or solvent resonance as the internal standard ($CDCl_3$, δ 7.26 for ^{1}H, δ 77.16 for ^{13}C; CD_3OD, δ 3.31 for ^{1}H, δ 49.00 for ^{13}C; $(CD_3)_2SO$, δ 2.50 for ^{1}H, δ 39.52 for ^{13}C). Data are reported as follows: chemical shifts (δ), multiplicity (s = singlet, d = doublet, dd = double doublet, td = triple doublet, t = triplet, q = quartet, m = multiplet, br = broad), coupling constants J (Hz), and integration. LC-MS analysis was performed on a Finnigan Surveyor HPLC system with a Gemmi C18 50x4.60 mm column (detection at 200-600 nm), coupled to a Finnigan LCQ Adantage Max mass spectrometer with ESI. The applied buffers were H_2O, MeCN and 1.0% TFA in H_2O (0.1% TFA end concentration). High resolution mass spectra (HRMS) were recorded by direct injection on a q-TOF mass spectrometer (Synapt G2-Si) equipped with an electrospray ion source in positive mode with leu-enkephalin H^+ (m/z = 556.2771) as an internal lock mass. The instrument was calibrated prior to measurement using the MS/MS spectrum of [glu^1]-fibrinopeptide B. Molecules shown are drawn using ChemDraw v16.0.

4-(4-(Trifluoromethoxy)phenyl)-2*H*-1,2,3-triazole (6).
To a solution of 4-trifluoromethoxyphenylacetylene (**5**, 0.61 mL, 4.0 mmol) in a mixture of DMF (27 mL) and MeOH (5.3 mL) were added CuI (75 mg, 0.4 mmol) and azidotrimethylsilane (0.8 mL, 6 mmol). The reaction mixture was stirred at 100 °C o/n, quenched with brine (20 mL), extracted with DCM (3 x 50 mL), dried ($MgSO_4$), filtered and concentrated. Purification of the residue by silica gel

column chromatography (1:4 EtOAc:pentane) yielded the title compound (0.25 g, 1.1 mmol, 27%). ^{1}H NMR (400 MHz, MeOD) δ 8.25 – 8.11 (br s, 1H), 7.93 (d, J = 8.8 Hz, 2H), 7.34 (d, J = 7.9 Hz, 2H).^{13}C NMR (100 MHz, MeOD) δ 150.30, 130.44, 128.56, 123.18, 122.56, 120.64. LC-MS m/z: 230.1 $[M+H]^{+}$.

Methyl 1*H*-1,2,3-triazole-5-carboxylate (7). This protocol is based on literature procedure.[18] A mixture of azidotrimethylsilane (2.6 mL, 20 mmol) and methyl propiolate (1.8 mL, 20 mmol) was heated for 4 h at 90 °C, concentrated and coevaporated with MeOH to yield the title compound (1.74 g, 14 mmol, 68%). ^{1}H NMR (400 MHz, CD_3OD) δ 8.36 (s, 1H), 3.92 (s, 3H). ^{13}C NMR (100 MHz, CD_3OD) δ 162.61, 139.63, 131.92, 52.53.

***bis*(4-Fluorophenyl)(2*H*-1,2,3-triazol-4-yl)methanol (8)**. To a cooled (0 °C) solution of **7** (0.69 g, 5.4 mmol) in THF (70 mL) was added 4-fluorophenylmagnesium bromide (2.0 M in THF, 9.5 mL, 19 mmol). The reaction mixture was stirred for 2 h at rt, quenched with NH_4Cl (sat. aq.) and extracted with DCM. The combined organic layers were washed with brine, dried ($MgSO_4$), filtered and concentrated. Purification of the residue by silica gel column chromatography (20 > 40% EtOAc in pentane) yielded the title compound (1.2 g, 4.0 mmol, 74%). ^{1}H NMR (300 MHz, CD_3OD) δ 7.58 (s, 1H), 7.40 – 7.29 (m, 4H), 7.09 – 6.97 (m, 4H). ^{13}C NMR (75 MHz, CD_3OD) δ 174.84, 165.07, 130.38, 130.27, 115.64, 115.35, 103.14, 103.12, 100.62.

***N*-(But-3-yn-1-yl)-2-nitro-*N*-phenethylbenzenesulfonamide (10)**. This protocol is based on literature procedure.[6] To a solution of 1-amino-3-butyne (**9**, 0.52 g, 7.5 mmol) in DCM (30 mL) were added *O*-nitrophenylsulfonyl chloride (1.7 g, 7.5 mmol) and Et_3N (1.6 mL, 11 mmol). The reaction mixture was stirred for 4 h, poured into H_2O and extracted with EtOAc. The combined organic layers were washed with H_2O, brine, dried (Na_2SO_4), filtered and concentrated. The residue was dissolved in CH_3CN (60 mL) and Cs_2CO_3 (7.3 g, 23 mmol) and phenethyl bromide (3.0 mL, 22 mmol) were added. The reaction mixture was stirred at 80 °C for 2 h, poured into H_2O and extracted with EtOAc. The combined organic layers were washed with H_2O, brine, dried (Na_2SO_4), filtered and concentrated. Purification of the residue by silica gel column chromatography (0 > 25% EtOAc in pentane) yielded the title compound (2.5 g, 7.0 mmol, 93%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.95 (dd, J = 7.6, 1.7 Hz, 1H), 7.71 – 7.57 (m, 3H), 7.27 – 7.14 (m, 5H), 3.64 – 3.50 (m, 4H), 2.93 – 2.83 (m, 2H), 2.46 (td, J = 7.2, 2.7 Hz, 2H), 2.00 (t, J = 2.7 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 147.99, 137.81, 133.73, 133.25, 131.88, 130.62, 128.79, 128.66, 126.75, 124.29, 80.66, 70.74, 49.58, 46.46, 35.07, 19.09.

***N*-(But-3-en-1-yl)-2-nitro-*N*-phenethylbenzenesulfonamide (13)**. To a solution of 3-butenylamine hydrochloride (**12**, 0.23 g, 2.1 mmol) in THF (8.4 mL) were added *O*-nitrophenylsulfonyl chloride (0.46 g, 2.1 mmol) and DIPEA (0.5 mL, 3 mmol). The reaction mixture was stirred o/n, poured into H_2O and extracted with EtOAc. The combined organic layers were washed with H_2O, brine, dried ($MgSO_4$), filtered and concentrated. The residue was dissolved in CH_3CN (17 mL) and Cs_2CO_3 (2.1 g, 6.3 mmol) and phenethyl bromide (1.4 mL, 11 mmol) were added. The reaction mixture was stirred at 80 °C for 2 h, poured into H_2O and extracted with EtOAc. The combined organic layers were washed with H_2O, brine, dried ($MgSO_4$), filtered and concentrated. Purification of the residue by silica gel column chromatography (5 > 30% EtOAc in pentane) yielded the title compound (0.53 g, 1.5 mmol, 70%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.96 (dd, J = 7.5, 1.8 Hz, 1H), 7.71 – 7.57 (m, 3H), 7.30 – 7.14 (m, 5H), 5.70 (ddt, J = 17.1, 10.2, 6.8 Hz, 1H), 5.12 – 4.99 (m, 2H), 3.58 – 3.50 (m, 2H), 3.46 – 3.38 (m, 2H), 2.90 – 2.82 (m, 2H), 2.32 (q, J = 7.2 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 138.08, 134.26, 133.72, 133.54, 131.74, 130.79, 128.86, 128.73, 126.79, 124.28, 117.62, 49.01, 47.17, 35.15, 32.80.

***N*-Phenethylbut-3-yn-1-amine (11)**. To a solution of **10** (2.5 g, 7.0 mmol) in CH_3CN (70 mL) were added Cs_2CO_3 (6.8 g, 21 mmol) and benzenethiol (1.2 mL, 12 mmol). The reaction mixture was stirred o/n, quenched with $NaHCO_3$ (sat. aq., 200 mL) and extracted with DCM (2 x 150 mL). The combined organic layers were dried (Na_2SO_4), filtered and concentrated. Purification of the residue by silica gel column chromatography (neutralized column with Et_3N, 0 > 10% MeOH in DCM) yielded the title compound (0.90 g, 5.2 mmol, 74%). ^1H NMR (400 MHz, $CDCl_3$) δ 7.32 – 7.25 (m, 2H), 7.21 (m, 3H), 2.89 (dd, J = 8.4, 6.5 Hz, 2H), 2.80 (m, 4H), 2.36 (td, J = 6.7, 2.6 Hz, 2H), 1.95 (t, J = 2.6 Hz, 1H), 1.47 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 139.93, 128.75, 128.50, 126.21, 82.41, 69.56, 50.68, 47.87, 36.42, 19.58.

***N*-Phenethylbut-3-en-1-amine (14)**. To a solution of **13** (0.54 g, 1.5 mmol) in CH_3CN (15 mL) were added Cs_2CO_3 (1.5 g, 4.5 mmol) and benzenethiol (0.23 mL, 2.3 mmol). The reaction mixture was stirred o/n, quenched with $NaHCO_3$ (sat. aq.) and extracted with DCM. The combined organic layers were dried ($MgSO_4$), filtered and concentrated. Purification of the residue by silica gel column chromatography (1 > 20% MeOH in DCM) yielded the title compound (0.21 g, 1.2 mmol, 81%). ^1H NMR (300 MHz, CD_3OD) δ 7.34 – 7.12 (m, 5H), 5.75 (ddt, J = 17.1, 10.2, 6.8 Hz, 1H), 5.12 – 4.83 (m, 3H), 2.81 (m, 4H), 2.65 (t, J = 7.2 Hz, 2H), 2.24 (q, J = 7.1 Hz, 2H). ^{13}C NMR (75 MHz, CD_3OD) δ 140.73, 136.92, 129.65, 129.55, 127.31, 117.04, 51.81, 49.36, 36.48, 34.50.

***tert*-Butyl (5-((2-nitro-*N*-phenethylphenyl)sulfonamido)pentyl)carbamate (16)**. To a solution of N-Boc-cadaverine (15, 0.70 g, 3.5 mmol) in THF (14 mL) were added 2-nitrobenzenesulfonyl chloride (0.77 g, 3.5 mmol) and Et_3N (0.73 mL, 5.2 mmol). The reaction mixture was stirred for 1.5 h, poured into H_2O (60 mL) and extracted with EtOAc (3x 30 mL). The combined organic layers were washed with H_2O, brine, dried ($MgSO_4$), filtered and concentrated. The residue was dissolved in CH_3CN (28 mL) and Cs_2CO_3 (3.4 g, 10 mmol) and phenethyl bromide (2.2 mL, 16 mmol) were added. The reaction mixture was stirred at 80 °C o/n, poured into H_2O (70 mL) and extracted with EtOAc (3 x 35 mL). The combined organic layers were washed with H_2O, brine, dried ($MgSO_4$), filtered and concentrated. Purification of the residue by silica gel column chromatography (20 > 30% EtOAc in pentane) yielded the title compound (1.6 g, 3.2 mmol, 92%). ^1H NMR (400 MHz, $CDCl_3$) δ 7.95 (dd, J = 7.4, 1.9 Hz, 1H), 7.71 – 7.57 (m, 3H), 7.29 – 7.23 (m, 2H), 7.22 – 7.13 (m, 3H), 4.53 (br s, 1H), 3.54 – 3.45 (t, J = 8.0 Hz, 2H), 3.33 (t, J = 7.6 Hz, 2H), 3.07 (d, J = 6.4 Hz, 2H), 2.93 – 2.79 (t, J = 8.0 Hz, 2H), 1.57 (p, J = 7.6 Hz, 2H), 1.44 (m, 11H), 1.33 – 1.20 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 156.08, 148.12, 138.13, 133.69, 133.50, 131.72, 130.75, 128.87, 126.77, 124.26, 79.07, 48.90, 47.71, 40.39, 35.22, 29.72, 27.85, 23.78. LC-MS m/z: 391.9 (M-Boc), 513.9 $[M+Na]^+$.

***N*-(5-Aminopentyl)-2-nitro-*N*-phenethylbenzenesulfonamide (17)**. To a solution of **16** (0.72 g, 1.5 mmol) in DCM (13.5 mL) was added TFA (1.4 mL). The reaction mixture was stirred for 1 h, concentrated and co-evaporated with toluene (3 x) to yield the title compound as TFA adduct (0.74 mg, 1.5 mmol, 100%). ^1H NMR (400 MHz, $CDCl_3$) δ 7.84 (dd, J = 7.4, 1.8 Hz, 1H), 7.69 – 7.54 (m, 3H), 7.29 – 7.07 (m, 5H), 6.4 (br s, 2H), 3.46 (t, J = 7.9 Hz, 2H), 3.32 (t, J = 7.2 Hz, 2H), 2.97 (s, 2H), 2.79 (t, J = 7.9 Hz, 2H), 1.66 (t, J = 7.7 Hz, 2H), 1.57 (q, J = 7.4 Hz, 2H), 1.34 (q, J = 7.8 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 148.06, 137.93, 133.81, 133.19, 132.07, 130.29, 128.85, 128.74, 126.84, 124.37, 49.14, 47.56, 40.11, 35.07, 27.49, 26.75, 23.01. LC-MS m/z: 391.9 $[M+H]^+$, 782.8 $[2M + H]^+$.

2,5-Dioxopyrrolidin-1-yl (1*S*,4*S*)-bicyclo[2.2.1]hept-5-ene-2-carboxylate (18). To a solution of 5-norbornene-2-carboxylic acid (Sigma Aldrich, mixture of endo and exo, predominantly endo, 0.60 mL, 4.9 mmol) in DCE (50 mL) were added EDC (3.8 g, 20 mmol) and N-hydroxysuccinimide (2.3 g, 20

mmol). The reaction mixture was stirred o/n, washed with 1 M HCl (3 x 40 mL), dried ($MgSO_4$), filtered and concentrated. Purification of the residue by silica gel column chromatography (30% EtOAc in pentane) yielded the title compound as a 1 : 0.3 mixture of endo and exo isomers (1.1 g, 4.5 mmol, 92%). TLC: R_f = 0.45 (1:2 EtOAc:pentane). NMR assignment for major isomer: ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.24 (dd, J = 5.7, 3.1 Hz, 1H), 6.12 (dd, J = 5.7, 2.9 Hz, 1H), 3.40 (m, 1H), 3.25 (dt, J = 9.0, 3.8 Hz, 1H), 2.99 (d, J = 1.6 Hz, 1H), 2.80 (s, 4H), 2.04 – 1.98 (m, 1H), 1.53 – 1.48 (m, 2H), 1.39 – 1.32 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.99, 138.15, 132.17, 49.66, 46.46, 42.53, 40.61, 29.57, 25.61.

(1*S*,2*S*,4*S*)-*N*-(5-((2-Nitro-*N*-phenethylphenyl)sulfonamido)pentyl)bicyclo[2.2.1]hept-5-ene-2-carboxamide (19). To a solution of **18** (0.34 g, 1.5 mmol) in DMF (10 mL) were added **17** (0.74 g, 1.5 mmol) and DIPEA (0.76 mL, 4.4 mmol). The reaction mixture was stirred for 2 h, poured into a 1:1 mixture of 1 M HCl and brine (100 mL) and extracted with Et_2O (2 x 50 mL). The combined organic layers were washed with brine (3 x 50 mL), dried ($MgSO_4$), filtered and concentrated. Purification of the residue by silica gel column chromatography (1:1 pentane:EtOAc) yielded two stereoisomers: endo and exo. Title compound **19** (endo isomer): 0.23 g, 0.46 mmol, 33%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.94 (dd, J = 7.5, 1.4 Hz, 1H), 7.72 – 7.56 (m, 3H), 7.31 – 7.10 (m, 5H), 6.23 (dd, J = 5.7, 3.1 Hz, 1H), 5.96 (dd, J = 5.8, 2.8 Hz, 1H), 5.51 (s, 1H), 3.55 – 3.45 (m, 2H), 3.34 (t, J = 7.4 Hz, 2H), 3.16 (dt, J = 13.2, 6.6 Hz, 3H), 2.94 – 2.79 (m, 4H), 1.98 – 1.86 (m, 1H), 1.58 (p, J = 7.5 Hz, 2H), 1.52 – 1.41 (m, 3H), 1.35 – 1.26 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.57, 148.13, 138.08, 137.87, 133.63, 133.54, 132.39, 131.76, 130.69, 128.84, 128.72, 126.79, 124.29, 50.14, 48.92, 47.62, 46.31, 44.91, 42.84, 39.27, 35.18, 30.05, 29.10, 27.70, 23.71.
Exo isomer: 0.14 g, 0.26 mmol, 18%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.98 – 7.91 (m, 1H), 7.72 – 7.56 (m, 3H), 7.29 – 7.14 (m, 5H), 6.18 – 6.05 (m, 2H), 5.61 (s, 1H), 3.55 – 3.44 (m, 2H), 3.40 – 3.32 (m, 2H), 3.28 – 3.19 (m, 2H), 2.91 (dd, J = 3.0, 1.4 Hz, 2H), 2.88 – 2.80 (m, 2H), 2.01 – 1.85 (m, 4H), 1.71 (d, J = 8.3 Hz, 1H), 1.60 (t, J = 7.4 Hz, 2H), 1.55 – 1.48 (m, 2H), 1.33 (dd, J = 5.9, 2.9 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.87, 138.29, 138.09, 136.20, 133.66, 133.54, 131.77, 130.76, 128.86, 128.74, 126.81, 124.31, 48.90, 47.59, 47.37, 46.46, 44.83, 41.71, 39.41, 35.17, 30.64, 29.83, 27.69, 23.69.

(1*S*,2*S*,4*S*)-*N*-(5-(Phenethylamino)pentyl)bicyclo[2.2.1]hept-5-ene-2-carboxamide (20). To a solution of **19** (0.23 g, 0.46 mmol) in CH_3CN (6 mL) were added Cs_2CO_3 (0.45 g, 1.4 mmol) and thiophenol (70 µL, 0.70 mmol). The reaction mixture was stirred o/n, quenched with $NaHCO_3$ (sat. aq., 10 mL) and extracted with DCM (3 x 30 mL). The combined organic layers were dried ($MgSO_4$), filtered and concentrated. Purification of the residue by silica gel column chromatography (silica neutralized with Et_3N, 1:19 MeOH:DCM) yielded the title compound (62 mg, 0.19 mmol, 41%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.32 – 7.27 (m, 2H), 7.26 – 7.18 (m, 3H), 6.21 (dd, J = 5.7, 3.1 Hz, 1H), 5.94 (dd, J = 5.7, 2.8 Hz, 1H), 5.90 (t, J = 6.3 Hz, 1H), 4.30 (s, 2H), 3.24 – 3.11 (m, 3H), 3.05 (m, 2H), 2.96 – 2.76 (m, 4H), 1.91 (ddd, J = 11.7, 9.3, 3.7 Hz, 1H), 1.72 (p, J = 7.5 Hz, 2H), 1.55 – 1.23 (m, 8H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.24, 139.99, 137.75, 132.33, 128.75, 128.53, 126.23, 51.21, 50.09, 49.68, 46.29, 44.87, 42.79, 39.36, 36.30, 29.98, 29.67, 29.61, 24.68.

***N*-(But-3-yn-1-yl)-*N*-phenethyl-4-(4-(trifluoromethoxy)phenyl)-1*H*-1,2,3-triazole-1-carboxamide (1) and *N*-(but-3-yn-1-yl)-*N*-phenethyl-4-(4-(trifluoromethoxy)phenyl)-2*H*-1,2,3-triazole-2-carboxamide (21)**. To a cooled (0 °C) solution of **11** (85 mg, 0.49 mmol) in THF (5 mL) were added DIPEA (0.26 mL, 1.5 mmol) and triphosgene (77 mg, 0.26 mmol). The reaction mixture was stirred at 0 °C for 1 h, quenched with H_2O and extracted with EtOAc. The combined organic layers were washed with brine, dried ($MgSO_4$), filtered and concentrated. The residue was dissolved in THF (5 mL) and DIPEA (0.26 mL, 1.5 mmol), DMAP (61 mg, 0.5 mmol) and **6** (0.12 g, 0.5 mmol) were added. The

reaction mixture was stirred for 4 h at 60 °C, quenched with NH_4Cl (sat. aq.) and extracted with EtOAc. The combined organic layers were washed with brine (2x), dried (Na_2SO_4), filtered and concentrated. Purification of the residue by silica gel column chromatography (5 > 10% EtOAc in pentane) yielded the title compounds. LC-MS m/z: 428.9 $[M+H]^+$. **1** (apolar): 79 mg, 0.18 mmol, 37%. ^1H NMR (400 MHz, $(CD_3)_2SO$, 90 °C) δ 8.85 (s, 1H), 8.10 – 8.02 (m, 2H), 7.49 – 7.39 (m, 2H), 7.35 – 7.15 (m, 5H), 3.85 (dd, J = 8.6, 6.6 Hz, 2H), 3.70 (t, J = 7.0 Hz, 2H), 3.00 (t, J = 7.6 Hz, 2H), 2.75 (d, J = 2.2 Hz, 1H), 2.65 – 2.56 (m, 2H). ^{13}C NMR (150 MHz, $(CD_3)_2SO$, 20 °C) δ 148.89, 148.25, 144.66, 144.54, 138.51, 138.09, 128.92, 128.89, 128.74, 128.50, 127.48, 126.46, 126.40, 123.00, 122.65, 122.56, 121.68, 120.95, 119.25, 117.55, 81.43, 81.23, 73.30, 72.95, 51.19, 50.00, 48.15, 47.11, 34.24, 32.61, 18.19, 16.51. HRMS *m/z* calculated for $C_{22}H_{19}F_3N_4O_2$ $[M+Na]^+$: 451. 1352, found: 451.1360. **21** (polar): 84 mg, 0.20 mmol, 41%. ^1H NMR (400 MHz, $(CD_3)_2SO$, 90 °C) δ 8.57 (s, 1H), 8.11 – 8.01 (m, 2H), 7.53 – 7.43 (m, 2H), 7.38 – 7.12 (m, 5H), 3.77 (dd, J = 8.9, 6.5 Hz, 2H), 3.63 (t, J = 7.1 Hz, 2H), 2.99 (t, J = 7.7 Hz, 2H), 2.73 (s, 1H), 2.59 (td, J = 7.0, 2.7 Hz, 2H). ^{13}C NMR (150 MHz, $(CD_3)_2SO$, 20 °C) δ 149.37, 149.35, 148.94, 147.14, 138.53, 138.04, 134.06, 133.99, 128.85, 128.59, 128.42, 128.26, 128.09, 126.39, 122.58, 121.72, 120.88, 119.18, 117.48, 81.42, 81.09, 73.05, 72.83, 51.08, 50.09, 48.15, 47.21, 34.32, 32.56, 18.22, 16.44. HRMS *m/z* calculated for $C_{22}H_{19}F_3N_4O_2$ $[M+Na]^+$: 451.1352, found: 451.1355.

4-(bis(4-Fluorophenyl)(hydroxy)methyl)-*N*-(but-3-yn-1-yl)-*N*-phenethyl-1*H*-1,2,3-triazole-1-carboxamide (22) and 4-(bis(4-fluorophenyl)(hydroxy)methyl)-*N*-(but-3-yn-1-yl)-*N*-phenethyl-2H-1,2,3-triazole-2-carboxamide (2). To a cooled (0 °C) solution of **11** (78 mg, 0.45 mmol) in THF (5 mL) were added DIPEA (0.26 mL, 1.5 mmol) and triphosgene (77 mg, 0.26 mmol). The reaction mixture was stirred at 0 °C for 1.5 h, quenched with H_2O and extracted with EtOAc. The combined organic layers were washed with brine, dried ($MgSO_4$), filtered and concentrated. The residue was dissolved in THF (5 mL) and DIPEA (0.26 mL, 1.5 mmol), DMAP (61 mg, 0.5 mmol) and **8** (0.13 g, 0.45 mmol) were added. The reaction mixture was stirred for 4 h at 60 °C, quenched with NH_4Cl (sat. aq.) and extracted with EtOAc. The combined organic layers were washed with brine (2x), dried (Na_2SO_4), filtered and concentrated. Purification of the residue by silica gel column chromatography (12 > 20% EtOAc in pentane) yielded the title compounds. LC-MS *m/z*: 487.0 $[M+H]^+$. **22** (apolar): 72 mg, 0.15 mmol, 33%. ^1H NMR (400 MHz, $(CD_3)_2SO$, 90 °C) δ 8.01 (s, 1H), 7.48 – 7.36 (m, 4H), 7.31 – 7.07 (m, 8H), 6.59 (d, J = 2.2 Hz, 1H), 3.79 (dd, J = 8.7, 6.6 Hz, 2H), 3.67 (t, J = 7.0 Hz, 2H), 2.95 (t, J = 7.6 Hz, 2H), 2.72 (s, 1H), 2.58 (td, J = 7.0, 2.7 Hz, 2H). ^{13}C NMR (100 MHz, $(CD_3)_2SO$, 90 °C) δ 162.08, 159.66, 152.82, 148.60, 142.09, 137.78, 128.63, 128.54, 128.20, 127.92, 125.91, 123.23, 113.99, 113.77, 80.84, 74.67, 72.04, 50.17, 47.24, 33, 17. HRMS *m/z* calculated for $C_{28}H_{24}F_2N_4O_2$ $[M+Na]^+$: 509.1760, found: 509.1760. **2** (polar): 88 mg, 0.18 mmol, 40%. ^1H NMR (400 MHz, $(CD_3)_2SO$, 90 °C) δ 7.97 (s, 1H), 7.43 – 7.34 (m, 4H), 7.30 – 7.06 (m, 8H), 6.77 (s, 1H), 3.69 (dd, J = 8.8, 6.7 Hz, 2H), 3.53 (t, J = 7.2 Hz, 2H), 2.89 (d, J = 7.9 Hz, 2H), 2.69 (d, J = 1.8 Hz, 1H), 2.46 (s, 2H). ^{13}C NMR (100 MHz, $(CD_3)_2SO$, 90 °C) δ 162.13, 159.71, 155.41, 149.02, 141.64, 137.83, 134.78, 128.53, 128.45, 128.13, 127.87, 125.84, 114.09, 113.87, 80.79, 74.98, 71.92, 65.99, 50.10, 47.48. HRMS *m/z* calculated for $C_{28}H_{24}F_2N_4O_2$ $[M+Na]^+$: 509.1760, found: 509.1766.

4-(*bis*(4-Fluorophenyl)(hydroxy)methyl)-*N*-(but-3-en-1-yl)-*N*-phenethyl-1*H*-1,2,3-triazole-1-carboxamide (23) and 4-(*bis*(4-fluorophenyl)(hydroxy)methyl)-*N*-(but-3-en-1-yl)-*N*-phenethyl-2*H*-1,2,3-triazole-2-carboxamide (3). To a cooled (0 °C) solution of **14** (0.11 g, 0.62 mmol) in THF (6.2 mL) were added DIPEA (0.3 mL, 1.7 mmol) and triphosgene (96 mg, 0.32 mmol). The reaction mixture was stirred at 0 °C for 1 h, quenched with H_2O and extracted with EtOAc. The combined organic layers were washed with brine, dried ($MgSO_4$), filtered and concentrated. The residue was dissolved in THF (6.2 mL) and DIPEA (0.3 mL, 1.7 mmol), DMAP (76 mg, 0.62 mmol) and **8** (0.18 g, 0.62 mmol) were

added. The reaction mixture was stirred o/n at 60 °C, quenched with NH_4Cl (sat. aq.) and extracted with EtOAc. The combined organic layers were washed with brine (2x), dried (Na_2SO_4), filtered and concentrated. Purification of the residue by silica gel column chromatography (10 > 25% EtOAc in pentane) yielded the title compounds. **23** (apolar): 89 mg, 0.18 mmol, 29%. NMR assignment major rotamer: ^1H NMR (400 MHz, $(CD_3)_2SO$) δ 7.8 (s, 1H), 7.49 – 6.83 (m, 13H), 5.94 – 5.52 (m, 1H), 5.25 – 4.91 (m, 2H), 3.81 – 3.45 (m, 4H), 3.07 – 2.79 (m, 2H), 2.38 (dd, J = 50.6, 8.3 Hz, 2H). ^{13}C NMR (100 MHz, $(CD_3)_2SO$) δ 162.89, 160.47, 153.62, 149.41, 142.99, 138.98, 138.45, 135.55, 135.15, 129.53, 129.45, 129.16, 128.88, 126.88, 124.26, 123.84, 117.75, 117.63, 115.05, 114.84, 75.36, 51.23, 50.45, 49.25, 47.86, 34.71, 33.06, 31.59, 21.20. HRMS *m/z* calculated for $C_{28}H_{26}F_2N_4O_2$ [M+H]$^+$: 489.2097, found: 489.2098. **3** (polar): 32 mg, 65 µmol, 11%. ^1H NMR (400 MHz, $(CD_3)_2SO$) δ 8.03 (s, 1H), 7.40 – 6.87 (m, 13H), 5.62 (m, 1H), 5.25 – 4.76 (m, 2H), 3.68 – 3.48 (m, 3H), 3.30 (s, 1H), 2.84 (d, J = 78.8 Hz, 2H), 2.26 (dd, J = 109.5, 7.4 Hz, 2H). ^{13}C NMR (100 MHz, $(CD_3)_2SO$) δ 162.91, 160.48, 156.10, 149.90, 142.60, 138.98, 138.38, 135.60, 135.04, 129.42, 129.34, 128.98, 128.82, 126.82, 117.59, 117.45, 115.14, 114.93, 75.68, 50.93, 50.42, 49.08, 48.16, 34.65, 33.09, 32.73, 31.52, 29.47. HRMS *m/z* calculated for $C_{28}H_{26}F_2N_4O_2$ [M+Na]$^+$: 511.1916, found: 511.1924.

***N*-(5-((1*S*,2*S*,4*S*)-bicyclo[2.2.1]hept-5-ene-2-carboxamido)pentyl)-4-(bis(4-fluorophenyl)(hydroxy)methyl)-*N*-phenethyl-2*H*-1,2,3-triazole-2-carboxamide (4)**. To a cooled (0 °C) solution of **20** (36 mg, 0.11 mmol) in THF (1 mL) were added DIPEA (58 µL, 0.33 mmol) and triphosgene (15 mg, 0.05 mmol). The reaction mixture was stirred for 1.5 h, quenched with H_2O (10 mL) and extracted with EtOAc (2 x 10 mL). The combined organic layers were washed with brine (2 x 10 mL), dried (Na_2SO_4), filtered and concentrated. The residue was dissolved in THF (2 mL) and DIPEA (58 µL, 0.33 mmol), DMAP (15 mg, 0.11 mmol) and **8** (33 mg, 0.11 mmol) were added. The reaction mixture was stirred for 3.5 h at 60 °C, quenched with NH_4Cl (sat. aq., 10 mL) and extracted with EtOAc (2 x 10 mL). The combined organic layers were washed with H_2O (10 mL), brine (10 mL), dried (Na_2SO_4), filtered and concentrated. Purification of the residue by silica gel column chromatography (3:2 PE:EtOAc) yielded the title compound as the lower TLC spot (26 mg, 0.04 mmol, 37%). ^1H NMR (400 MHz, $(CD_3)_2SO$, 80 °C) δ 7.95 (s, 1H), 7.37 (m, 4H), 7.29 – 7.07 (m, 9H), 6.07 (dd, J = 5.7, 3.0 Hz, 1H), 5.81 (dd, J = 6.0, 2.7 Hz, 1H), 3.70 (t, J = 7.6 Hz, 1H), 3.59 (s, 1H), 3.45 (s, 1H), 3.30 (s, 1H), 3.07 – 2.71 (m, 6H), 1.73 (ddd, J = 12.6, 9.4, 3.8 Hz, 1H), 1.66 – 1.15 (m, 13H). ^{13}C NMR (100 MHz, $(CD_3)_2SO$, 80 °C) δ 162.19, 159.76, 155.28, 149.18, 148.63, 142.22, 141.79, 136.26, 134.59, 131.87, 128.71, 128.61, 128.53, 128.30, 128.22, 128.02, 127.96, 126.02, 125.95, 114.21, 114.11, 113.99, 113.90, 49.01, 45.26, 43.19, 41.79, 38.02, 28.55, 28.50, 28.47, 28.29, 23.21. HRMS *m/z* calculated for $C_{37}H_{39}F_2N_5O_3$ [M+Na]$^+$: 662.2913, found: 662.2923.

Scheme 4. Synthesis of BODIPY-tetrazine. Reagents and conditions: (**a**) *i*. TFA (catalytic), DCM; *ii*. DDQ; *iii*. DIPEA, $BF_3 \cdot OEt_2$; 42%; (b) *i*. $Zn(OTf)_2$, CH_3CN, NH_2NH_2, 60 °C; *ii*. $NaNO_2$; 0.3%.

3-(5,5-Difluoro-1,3,7,9-tetramethyl-5*H*-4λ4,5λ4-dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-10-yl) benzonitrile (27). This protocol is based on literature procedure.[11] To a solution of 3-formylbenzonitrile (**25**, 0.81 g, 6.1 mmol) and 2,4-dimethylpyrrole (**26**, 1.4 mL, 13 mmol) in DCM (160 mL) were added four drops of TFA. After stirring at rt for 30 min, a solution of DDQ (1.4 g, 6.1 mmol) in DCM (160 mL) was added, followed by DIPEA (12.5 mL, 73 mmol) and $BF_3{\cdot}OEt_2$ (12.5 mL, 100 mmol). The reaction was stirred o/n, diluted with water and extracted with DCM. The combined organic layers were dried ($MgSO_4$), filtered and concentrated. The residue was purified by column chromatography (75 > 100% toluene in pentane) to yield the title compound (0.89 g, 2.6 mmol, 42%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.89 – 7.51 (m, 4H), 6.01 (s, 2H), 2.56 (s, 6H), 1.36 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 156.70, 142.63, 138.09, 136.65, 132.92, 132.83, 131.91, 131.13, 130.27, 121.94, 117.99, 113.65, 14.82, 14.77.

5,5-Difluoro-1,3,7,9-tetramethyl-10-(3-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-5H-4λ4,5λ4-dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinine (24). Adapted from literature procedure, omitted DMF as a co-solvent.[11] To a suspension of **27** (175 mg, 0.5 mmol) and $Zn(OTf)_2$ (91 mg, 0.25 mmol) in CH_3CN (0.26 mL) in a sealed microwave tube under argon was added hydrazine (0.8 mL, 25 mmol). The reaction mixture was stirred at 60 °C o/n and allowed to cool down to rt before addition of $NaNO_2$ (0.7 g in 5 mL H_2O). The mixture was acidified with 1 M HCl and extracted with DCM. The combined organic layers were dried ($MgSO_4$), filtered and concentrated. The residue was purified with column chromatography (0 > 0.5% CH_3CN in toluene, followed by 4:1 pentane:EtOAc) 0.3% isolated yield after preparative HPLC. NMR data agrees with literature values.[11] 1H NMR (500 MHz, $(CD_3)_2SO$) δ 8.70 – 8.57 (m, 1H), 8.41 – 8.32 (m, 1H), 7.88 (t, J = 7.8 Hz, 1H), 7.75 (dt, J = 7.7, 1.4 Hz, 1H), 6.22 (s, 2H), 3.01 (s, 3H), 2.48 (s, 6H), 1.41 (s, 6H). HRMS *m/z* calculated for $C_{22}H_{21}BF_2N_6$ $[M+H]^+$: 419.1962, found: 419.1962.

Computational methods

The triazole urea structures were initially optimized by a conformer distribution search included in the Spartan 10 program.[19] The conformer distribution was calculated in the gas phase at the DFT level of theory using B3LYP as hybrid functional and 6-31G(d) as basis set. The resulting structure library was further refined using the Gaussian 09 program revision A.02,[20] with the use of the ωB97XD long-range corrected hybrid functional and 6-311+G(d,p) as basis set. Optimization was done in gas-phase and subsequently corrections for solvent effects were done by use of a polarizable continuum model (PCM), using DMSO as solvent parameter. Gas-phase free energies were computed using the quasi-harmonic approximation in the gas phase according to the work of Truhlar - the quasi-harmonic approximation is the same as the harmonic oscillator approximation except that vibrational frequencies lower than 100 cm^{-1} were raised to 100 cm^{-1} as a way to correct for the breakdown of the harmonic oscillator model for the free energies of low-frequency vibrational modes. The denoted free Gibbs energy was calculated using Equation (1) in which ΔE_{gas} is the gas-phase energy (electronic energy), ΔG^T_{gas} (T = 298.15 K and pressure = 1 atm.) is the sum of corrections from the electronic energy to free Gibbs energy in the harmonic oscillator approximation also including zero-point-vibrational energy, and ΔG^T_{solv} is their corresponding free solvation Gibbs energy.

$$\Delta G^T_{\text{in solution}} = \Delta E_{gas} + \Delta G^T_{gas} + \Delta G^{solv} = \Delta E_{gas} \qquad (1)$$

All found minima were checked for negative frequencies. Based on the lowest energy structures according to the optimization described above, the chemical shifts were calculated with the use of the Gauge-Independent Atomic Orbital (GIAO) method using WC04/6-311+G(2d,p) and a PCM model with as solvent DMSO. No additional scaling was used for the denoted chemical shifts.

Biochemical methods

Mouse brain membrane proteome. Mouse brains (C57Bl/6) were isolated according to guidelines approved by the ethical committee of Leiden University (DEC#13191), frozen in N_2 (l), and stored at -80 °C until use. Tissues were thawed on ice, dounce homogenized in appropriate volumes (2-4 mL) of cold lysis buffer (20 mM HEPES, pH 7.2, 2 mM DTT, 250 mM sucrose, 1 mM $MgCl_2$, 2.5 U/mL benzonase) and incubated on ice (15 min), followed by two low-speed spins (3 min, 1,400–2,500 *g*, 4 °C) to remove debris. The supernatant fraction was collected for further use. The membrane and cytosolic fractions of cell or tissue lysates were separated by ultracentrifugation (93,000 *g*, 45 min, 4 °C). The supernatant was collected (cytosolic fraction) and the membrane pellet was resuspended in cold storage buffer (20 mM HEPES, pH 7.2, 2 mM DTT) by thorough pipetting and passage through an insulin needle. Protein concentrations were determined by a Quick Start™ Bradford Protein Assay and samples were diluted to 2.0 mg/mL with cold storage buffer, aliquoted, flash frozen in N_2 (l) and stored at -80 °C until further use.

SDS-PAGE. Mouse brain proteome or cell lysate (15 µL, 2.0 or 1.0 mg/mL, membrane fraction or whole lysate) was pre-incubated with vehicle or inhibitor (0.375 µL 40 x inhibitor stock, 30 min, rt) followed by an incubation with the activity-based probe (0.375 µL 40 x probe stock, 20 min, rt). Final concentrations for the inhibitors are indicated in the main text and figure legends. Reactions were quenched with 4x Laemmli buffer (5 µL, 240 mM Tris (pH 6.8), 8% (w/v) SDS, 40% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 0.04% (v/v) bromophenol blue). 10 or 20 µg per reaction was resolved on a 10% acrylamide SDS-PAGE gel (180 V, 75 min). Gels were scanned using Cy2, Cy3 and Cy5 multichannel settings on a ChemiDoc MP (Bio-Rad) and stained with Coomassie after scanning. Fluorescence was normalized to Coomassie staining and quantified with Image Lab v5.2.1 (Bio-Rad). IC_{50} curves were fitted with Graphpad Prism® v7 (Graphpad Software Inc.).

Cell culture. U2OS cells were cultured at 37 °C under 7% CO_2 in DMEM containing phenol red, stable glutamine, 10% (v/v) New Born Calf Serum (Thermo Fisher), and penicillin and streptomycin (200 µg/mL each; Duchefa). Medium was refreshed every 2-3 days and cells were passaged twice a week at 80-90% confluence by trypsinization, followed by resuspension in fresh medium. Cells lines were purchased from ATCC and were regularly tested for mycoplasma contamination. Cultures were discarded after 2-3 months of use.

Plasmids. The DAGLα and DAGLα-S472A plasmids (both containing a FLAG tag) were constructed as described before.[21] Briefly, full length human cDNA of hDAGL-α was purchased from Biosource and cloned into mammalian expression vector pcDNA3.1, containing genes for ampicillin and neomycin resistance. A FLAG-linker was made from primers and cloned into the vector at the C-terminus of hDAGL-α. Two step PCR mutagenesis was performed to substitute the active site serine for an alanine in the hDAGL-α-FLAG, to obtain hDAGL-α-S472A-FLAG. All plasmids were grown in XL-10 Z-competent cells and prepped (Maxi Prep, Qiagen). The sequences were confirmed by sequence analysis at the Leiden Genome Technology Centre.

Transfection. U2OS cells were grown to ~70% confluency in 6-well plates. Prior to transfection, culture medium was aspirated and 1 mL medium was added per well. A 3:1 (m:m) mixture of polyethyleneimine (PEI, 4.5 µg/well) and plasmid DNA (1.5 µg/well) was prepared in serum free culture medium and incubated for 10 min at rt. Transfection was performed by dropwise addition of the PEI/DNA mixture (200 µL/well) to the cells. 24 h post-transfection, the medium was refreshed

and after 48 h cells were harvested or used for *in situ* treatments.

***In situ* tetrazine labeling**. 48 h after transfection, the cells were washed with serum free medium (3 x 1 mL). The cells were treated with either DMSO as vehicle or DH376 (2.5 μM final concentration, 0.05% DMSO) for 1 h at 37 °C, followed by either vehicle or 4 (5 μM final concentration, 1 h 37 °C, 0.1% DMSO). The cells were washed with serum-free medium and treated with either vehicle or tetrazine 24 (10 μM final concentration, 0.1% DMSO) for 1 h at 37 °C. The cells were subsequently washed with PBS and harvested by scraping into PBS. The cells were pelleted by centrifugation (5 min, 1000 *g*), the supernatant was discarded and cell pellets were frozen in N_2 (l) and stored at -80 °C until sample preparation for SDS-PAGE. Cell pellets were thawed on ice, resuspended in cold lysis buffer (20 mM HEPES, pH 7.2, 2 mM DTT, 250 mM sucrose, 1 mM $MgCl_2$, 2.5 U/mL benzonase) and incubated on ice (15-30 min). Protein concentrations were determined by a Quick Start™ Bradford Protein Assay. The cell lysate was diluted to 1 mg/mL in cold storage buffer (20 mM HEPES, pH 7.2, 2 mM DTT) before being prepared for SDS-PAGE analysis as described above.

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