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Quenched Activity-based Probes

Design and Synthesis of Quenched Activity-based Probes for Diacylglycerol Lipase and α,β-Hydrolase Domain Containing Protein 6

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Abstract: Diacylglycerol lipases (DAGL) are responsible for the biosynthesis of the endocannabinoid 2-arachidonoylglycerol. The fluorescent activity-based probes DH379 and HT-01 have been previously shown to label DAGLs and to cross-react with the serine hydrolase ABHD6. Here, we report the synthesis and characterization of two new quenched activity-based probes **1** and **2**, the design of which was based on the structures of DH379 and HT-01, respectively. Probe **1** contains a BODIPY-FL and a 2,4-dinitroaniline moiety as a fluorophore–quencher pair, whereas probe **2** employs a Cy5-fluorophore and a cAB40-quencher. The fluorescence of both probes was quenched with relative quantum yields of 0.34 and 0.0081, respectively. The probes showed target inhibition as characterized in activity-based protein profiling assays using human cell- and mouse brain lysates, but were unfortunately not active in living cells, presumably due to limited cell permeability.

Introduction

The endocannabinoid signaling system (ECS) consists of the cannabinoid receptors, their endogenous ligands, that is, 2-arachidonoylglycerol (2-AG) and anandamide (AEA), and the enzymes regulating the levels of these ligands.[1] The endocannabinoid system is involved in a wide array of neurophysiological processes, including nociception, cognitive function and appetite. Enzymes involved in the ECS are consequently potential therapeutic targets for an array of human disorders, including pain, neurodegenerative diseases and obesity.[2–4] The endocannabinoid 2-AG is synthesized from diacylglycerol (DAG) by hydrolysis catalyzed by either *sn*-1-diacylglycerol lipase α or β (DAGLα and DAGLβ).[5] The DAG lipases belong to the serine hydrolase superfamily and selectively hydrolyze the *sn*-1 ester within diacylglycerols. DAGLα is mainly responsible for the generation of 2-AG in the central nervous system, whereas DAGLβ mostly acts in the periphery.[6] To date, isoform selective inhibitors have not been reported. DAGLα inhibitors have potential as drug candidates for obesity as well as neurodegenerative disorders.[7] Most of the reported covalent DAGL inhibitors also target α,β-hydrolase domain containing protein 6 (ABHD6).[8,9] ABHD6 is thought to control 2-AG levels post-synaptically.[10] The enzyme responsible for catalyzing the bulk 2-AG in the brain, monoacylglycerol lipase (MAGL), acts at presynaptic sites. To gain a better understanding of the regulation of 2-AG levels we need to study the activity of these enzymes in their native environment.

Activity-based protein profiling (ABPP) is a method to study native enzyme activity.[11] Activity-based probes (ABPs) form a covalent bond with active enzymes and thus report on the amount of active enzyme present in a biological system at a given time. Various fluorescent ABPs for DAGL and ABHD6, such as DH379[12] and HT-01,[8] have been reported.

Quenched ABPs (qABPs) have been developed to decrease the fluorescent signal arising from an unbound fluorescent probe.[13] A qABP consists of an electrophilic trap, a recognition element, a fluorophore (F) and a complementary quencher (Q) (Figure 1a). The quencher is part of the leaving group, which disassociates after the formation of the enzyme-probe complex. Bogyo and co-workers were the first to synthesize a qABP and developed a set of qABPs for cathepsins.[14–18] To date vari-

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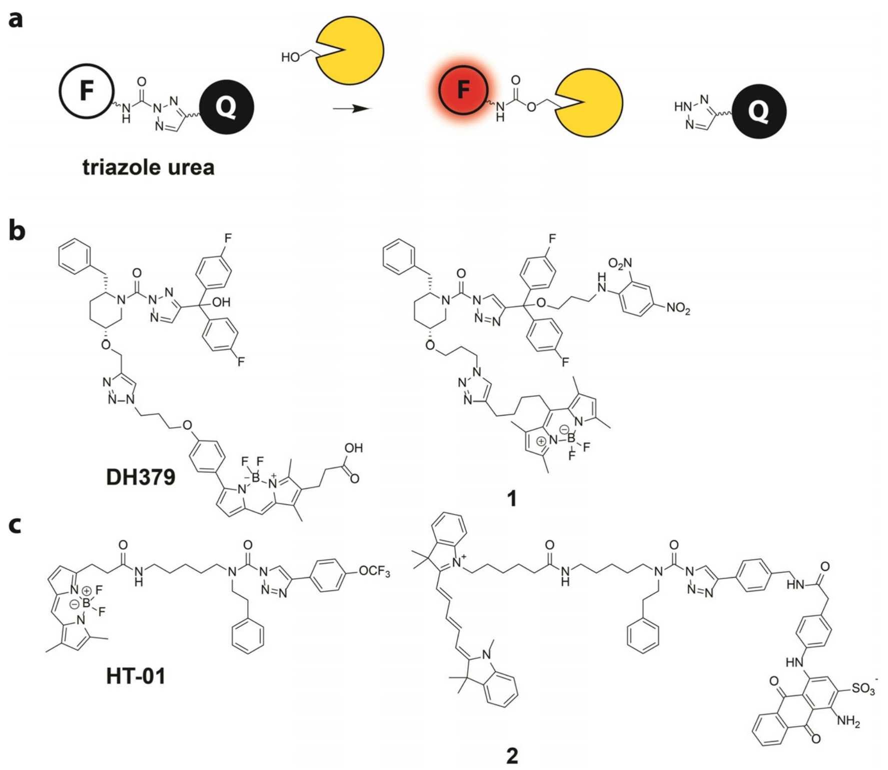


Figure 1. qABP design for DAGL and ABHD6. (a) General design of a triazole urea as qABP for serine hydrolases. (F) is the fluorophore and (Q) is the quencher. (b) Probe DH379 and probe **1**. (c) Probe HT-01 and probe **2**.

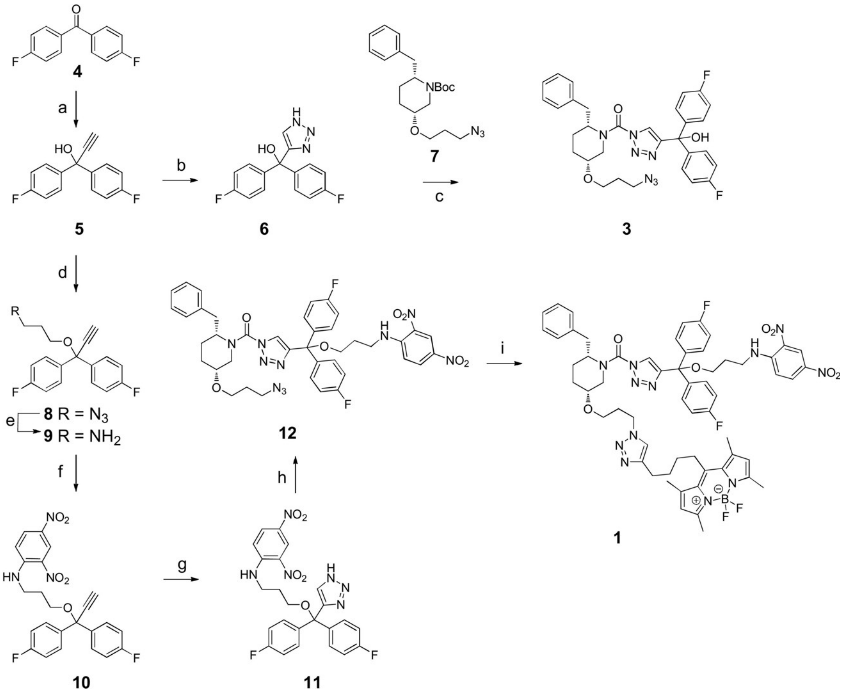
ous enzyme classes, including kinases,[19] serine proteases[20] and cysteine proteases such as legumain[21] and caspases,[22] have been targeted with qABPs. However, metabolic serine hydrolases have not yet been studied with qABPs. Here, we report on the synthesis and characterization of qABPs for the metabolic serine hydrolases DAGL and ABHD6.

Results and Discussion

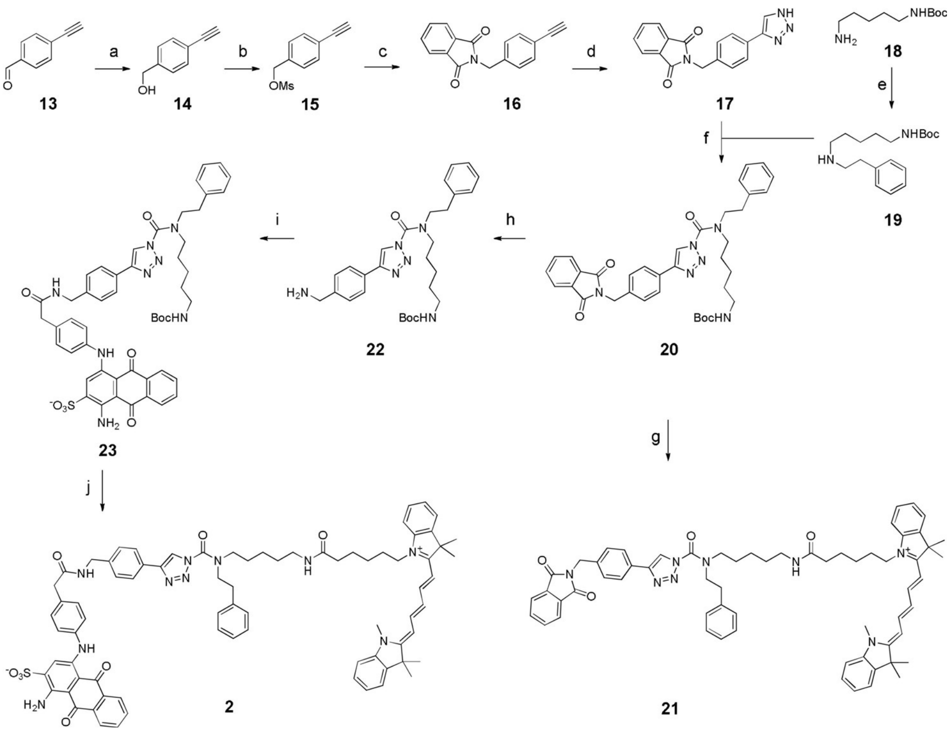
The design of our qABPs **1** and **2** is based on triazole ureas, DH379 and KT-01, respectively, both of which are ABPs for the serine hydrolases targeted in the here-reported studies. DH379 targets DAGLα, DAGLβ and ABHD6, while KT-01 labels DAGLβ and ABHD6 (Figure 1).[8,23] Both ABPs contain a triazole urea as an electrophilic trap, which is a commonly used warhead for serine hydrolases (Figure 1 a).[12,24] BODIPY-FL[25] and 2,4-DNA[26–30] were chosen as a fluorophore-quencher pair for probe **1**,[19,31] while Cy5 and cAB40 were selected as a fluorophore-quencher pair for qABP **2**.[32]

Synthesis of probe 1. The synthesis of probe **1** and DAGL inhibitor **3**, an azide analogue of inhibitor DH376, started with the nucleophilic addition of lithium TMS-acetylene onto 4,4′-difluorophenyl ketone **4** (Scheme 1), followed by deprotection in basic solution to give alcohol **5**. Copper(I)-catalyzed [2+3] azide alkyne cycloaddition (CuAAC) of **5** with hydrazoic acid, formed in situ from methanol and TMS-N_3 in DMF,[33,34] gave triazole **6** in moderate yield. Next, piperidine **7**, which was synthesized as previously reported,[12] was deprotected with acid and the resulting amine was treated with triphosgene and reacted with **6**. The two regioisomers thus formed were separated by column chromatography yielding inhibitor **3**. To obtain probe **1**, alkylation of alcohol **5** led to azide **8**, which was reduced by treatment with triphenylphosphine and water to give **9**. A nucleophilic aromatic substitution reaction between amine **9** and 2,4-dinitrofluorobenzene yielded the desired alkyne **10,** which contains dinitroaniline as a quencher. CuAAC of alkyne **10** with pivaloyloxymethyl-azide (POM-N_3) gave the POM-protected triazole, which was deprotected in basic solution to give compound **11**.[35] Similarly as described above for triazole **6**, triphosgene coupling of **11** with **7** gave intermediate **12** after separation of two regioisomers by column chromatography. A CuAAC reaction between azide **12** and acetylene-functionalized BODIPY-FL[25] furnished probe **1**.

Synthesis of probe 2. The qABP **2** is based on the fluorescent ABP HT-01 (Figure 1 c). Reduction of commercially available 4-ethynylbenzaldehyde (**13**) with sodium borohydride gave alcohol **14**, which was subsequently mesylated to provide **15** (Scheme 2). Next, nucleophilic substitution of the mesylate with phthalimide yielded compound **16**. A CuAAC reaction between alkyne **16** and TMS-azide resulted in triazole **17**. Separately, *N*-Boc-cadaverine (**18**) was nosylated and alkylated using phenethylbromide. Subsequent deprotection with ethylenediamine resulted in amine **19**. This amine was treated with triphosgene and coupled to triazole **17**, resulting in a mixture of two regioisomers, which were separated by column chromatography to give the N1 isomer **20**. The Cy5-analogue of HT-01 **21** was made by deprotecting **20** with TFA and coupling the resulting amine to a Cy5 activated ester. To make the probe **2**,



Scheme 1. Synthesis of probe **1**. Reagents and conditions: (a) *i.* TMS-acetylene, *n*BuLi, THF, −10–0 °C; *ii.* KOH, MeOH/THF, 0 °C, 74 %; (b) TMS-N_3, NaAsc, $CuSO_4$, DMF/MeOH, 60 °C, 59 %; (c) *i.* 7, 40 % TFA in DCM; *ii.* Triphosgene, DIPEA, THF, 0 °C; *iii.* 6, DIPEA, THF, 60 °C; (d) NaH, 3-azidopropanol tosylate, DMF, 0 °C—rt, 64 %; (e) PPh_3, H_2O/THF, 60 °C, 100 %; (f) 2,4-dinitrofluorobenzene, NEt_3, DMF, 95 %; (g) *i.* POM-azide, CuBr, TBTA, THF/H_2O; *ii.* KOH, MeOH, 67 %; (h) *i.* **7**, 40 % TFA in DCM; *ii.* Triphosgene, DIPEA, THF, 0 °C; *iii.* **11**, DIPEA, THF, 60 °C; (i) NaAsc, $CuSO_4$, DMF, MeOH, BODIPY-FL.[25]



Scheme 2. Synthesis of qABP **2** and control compound **21**. Reagents and conditions: (a) $NaBH_4$, EtOH, 99 %; (b) DCM, Et_3N, MsCl, 0 °C, 94 %; (c) potassium phthalimide, DMF, 92 %; (d) TMS-N_3, CuI, DMF/MeOH (5/1), reflux, 64 %; (e) *i.* NsCl, Et_3N, THF; *ii.* Ph$(CH_2)_2$Br, Cs_2CO_3, ACN, 80 °C; *iii.* PhSH, Cs_2CO_3, ACN, 71 %; (f) *i.* triphosgene, DIPEA, THF; *ii.* **17**, DMAP, DIPEA, THF, 27 %; (g) *i.* TFA/DCM. *ii.* Cy5-OSu, DIPEA, DMF, 44 %; (h) ethylenediamine, EtOH, 50 %; (i) HCTU, DIPEA, cAB40, DMF, 53 %; (j) *i.* 10 % TFA/DCM; *ii.* Cy5-NHS, DIPEA, DMF, 10 %.

the phthalimide **20** was deprotected with ethylenediamine. Amine **22** was coupled to the cAB40 quencher (for synthesis, see Experimental) and amide product **23** was deprotected by TFA, followed by coupling to Cy5, yielding the final product **2**.

Spectroscopic characterization. The absorbance spectrum of probe **1** was compared to BODIPY-FL. In ethanol, the probe and the parent BODIPY have identical absorbance maxima (Table 1). In aqueous solution the maximal absorbance of the probe is shifted towards the red (approximately 10 nm) while the parent BODIPY does not display such a redshift (Table 1). Similar shifts in absorbance profiles have been previously observed in comparable systems[26,27,36] and are indicative of a ground state complex. This suggests that the fluorescence of the probe is quenched to a certain extent by ground state complex formation. Probe **2** is also slightly redshifted in water compared to ethanol. To determine the amount of quenching, we determined the fluorescence quantum yield, which is defined as the ratio of the number of photons emitted to the number of photons absorbed by a fluorophore. Fluorescence quantum yields of qABPs **1** and **2** were determined relative to their parent fluorophores, that is, BODIPY-FL and Cy5 (Table 1). Probe **1** has a relative quantum yield of 0.34 (±3-fold quenched). Probe **2** has a relative quantum yield of 0.081 (±12-fold quenched).

Table 1. λ_{max} (absorbance maximum) and $\Phi^{relative}$ (relative quantum yield) of probe **1** and **2**.

Compound	λ_{max} in EtOH [nm]	λ_{max} in H_2O [nm]	$\Phi^{relative}$ in EtOH
BODIPY-FL	497	497	1.0[a]
Probe **1**	497	505	0.34[a]
Cy5	644	639	1.0[b]
Probe **2**	644	650	0.081[b]

[a] Relative to BODIPY-FL. [b] Relative to Cy5.

Biological characterization of probe 1. To determine whether qABP **1** inhibits human DAGLα, we used a colorimetric assay with *para*-nitrophenyl butyrate as surrogate substrate and membranes of HEK293T cells overexpressing recombinant human DAGLα.[37,38] Compound **3**, an azide analogue of DH376, was used as positive control and showed good inhibitory activity ($IC_{50} = 5$ nM), comparable to DH376 (Table 2). Intermediate **12**, containing only a quencher, retained high inhibitory activity, whereas qABP **1** was approximately ten-fold less active. Of note, probe **1** is about as active as the ABP DH379.

Table 2. Activity of DAGLα inhibitors in surrogate substrate assay.

Compound	pIC_{50} DAGLα
3	8.3±0.07
DH376	8.7±0.1
12	8.4±0.04
1	7.4±0.2
DH379	7.4±0.05

To investigate whether qABP **1** could covalently label human DAGLα, we employed a gel-based activity-based protein profiling (ABPP) assay for rapid and efficient visualization of endogenous serine hydrolase activity in native biological samples. Recombinant human DAGLα was over-expressed in HEK293T cells, which were lysed and treated with varying concentrations of qABP **1**. The proteins were resolved by SDS-PAGE and labeled proteins were visualized by in-gel fluorescence scanning. Human DAGLα was dose-dependently labeled by probe **1** (Figure 2a) and could be out-competed by the selective DAGL inhibitor, LEI105 (Figure 2b).[39]

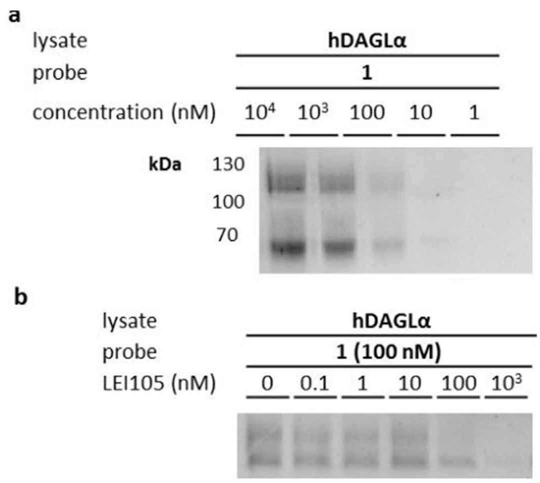


Figure 2. Activity-based protein profiling with probe **1**. (a) Lysate of recombinant human DAGLα over-expressed in HEK293T cells, treated with probe **1** (30 min, rt). (b) Competition of human DAGLα labeling by probe **1** with LEI105 (30 min pre-incubation with LEI105, followed by 20 min incubation with probe, rt).

Biological characterization of probe 2. To test whether probe **2** labeled ABHD6, lysates from two cell lines were used: human osteosarcoma U2OS cells stably overexpressing ABHD6 fused to green fluorescent protein (GFP) as well as wildtype mouse neuroblastoma Neuro2A (N2A) with endogenous ABHD6 expression. In the U2OS lysates, a strong signal from probe **2** was visible at approximately 70 kDa, corresponding to the MW of the ABHD6-GFP fusion protein (Figure 3a). Incubation of wildtype N2A lysates with probe **2** resulted in fluorescent labeling of several proteins, including a protein at 35 kDa, which was also labeled by the well-characterized ABHD6 probe DH379 (Figure 3b). These results suggest that probe **2** covalently labels mouse and human ABHD6.

Finally, live U2OS-ABHD6-GFP cells were treated with **2**, lysed and remaining ABHD6 activity was visualized post-lysis with DH379. Almost no fluorescent signal from **2** was detected and no decrease in ABHD6 activity was observed with DH379 labeling (Figure 4a), which suggested that probe **2** has limited cell permeability. Of note, control compound **21** was able to reduce ABHD6 activity in situ (Figure 4b), which suggested that the reduced cell permeability is caused by the quencher.

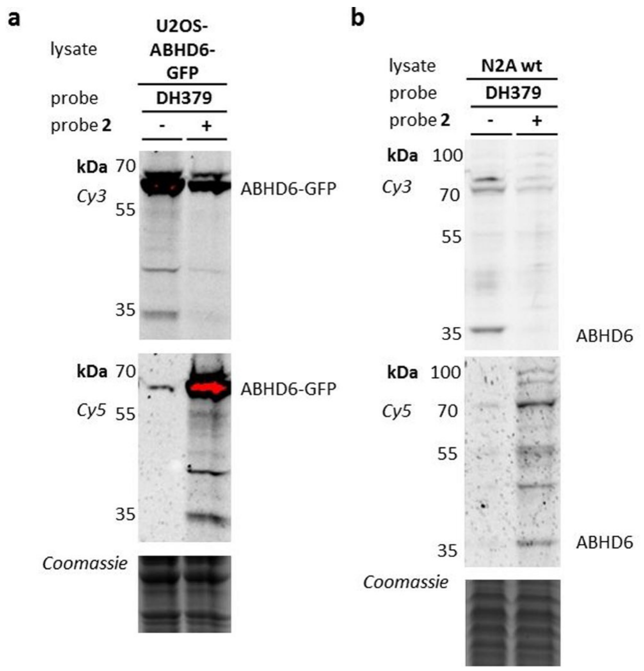


Figure 3. Activity-based protein profiling with probe **2**. (a) ABPP on U2OS-ABHD6-GFP lysate with probe DH379 (Cy3 signal, 1 μм, 20 min, rt), with or without pre-incubation with probe **2** (Cy5 signal, 10 μм, 30 min, 37 °C). (b) ABPP on N2A lysate with probe DH379 (Cy3 signal, 1 μм, 20 min, rt), with or without pre-incubation with probe **2** (Cy5 signal, 10 μм, 30 min, 37 °C). Gels with coomassie staining (lower panels) are shown as protein loading controls.

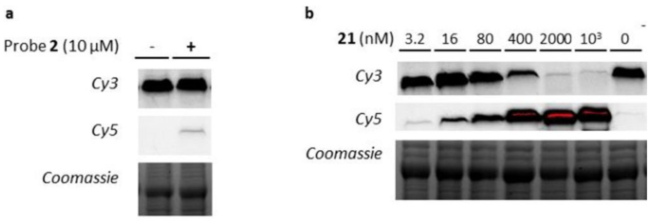


Figure 4. *In situ* treatment of U2OS-ABHD6-GFP. (a) Probe 2-treated cells (Cy5 signal, 1 h, 37 °C), lysed and labeled with DH379 (Cy3 signal, 20 min, 1 μм, rt). (b) Probe **21**-treated cells (Cy5 signal, 1 h, 37 °C), lysed and treated with DH379 (Cy3 signal, 20 min, 1 μм, rt). Gels with coomassie staining (lower panels) are shown as protein loading controls.

Conclusions

The qABPs **1** and **2** were successfully synthesized. Probe **1** showed characteristics of static quenching in aqueous solution and showed activity in a surrogate substrate assay using recombinant human DAGLα. Probe **1** could dose-dependently label human DAGLα in a gel-based activity-based protein profiling assay. Probe **2** did label endogenously expressed mouse ABHD6, but was not able to label ABHD6 in live cells. Further optimization of fluorescent properties and cell permeability of the probes is required to apply them in biological studies.

Experimental Section

Chemistry

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 (dried for a minimum of 24 h over activated molecular sieves of appropriate (3–4 Å) pore size). 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. Preparative TLC was performed on UNIPLATE Alumina GF 1000 μm plates. Compounds were visualized using UV-irradiation, a $KMnO_4$ stain (K_2CO_3 (40 g), $KMnO_4$ (6 g), H_2O (600 mL) and 10% NaOH (5 mL)). A stain for organic azides was used as follows: *i.* 10% PPh_3 in toluene, heating; *ii.* Ninhydrin in EtOH/AcOH, heating.[40] ^{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 50×4.60 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). General: 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 (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. IR spectra were recorded on a Shimadzu FTIR-8300 and are reported in cm^{-1}. Optical rotations were measured on a Propol automatic polarimeter (Sodium D-line, λ = 589 nm). Molecules shown are drawn using Chemdraw v16.0.

General procedure for the CuAAC reaction. To a solution of 1 equiv. of azide and 1.0 to 1.5 equiv. of BODIPY alkyne (red or green) in minimal DMF was added a freshly prepared solution of 0.15 equiv. NaAsc and 0.05 equiv. of $CuSO_4$ in H_2O. The resulting solution was stirred for 18 h. The reaction mixture was diluted with EtOAc and H_2O and extracted with EtOAc (3x). The combined organic layers were washed with H_2O (5x), brine, dried ($MgSO_4$), filtered and concentrated.

Pivaloyloxymethyl-azide (POM-N_3, Scheme 1**).** This procedure was adapted from literature.[41] To a solution of NaN_3 (0.44 g, 6.6 mmol) in H_2O (3.5 mL) was added POM-Cl (0.85 mL, 6 mmol). The resulting mixture was stirred vigorously at 90 °C for 18 h. The reaction mixture was diluted with water and extracted with DCM (3x). The combined organic layers were carefully concentrated to yield a colorless liquid (0.92 g, 5.9 mmol, 98%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.14 (s, 2H), 1.26 ppm (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 178.22, 74.52, 39.11, 27.13 ppm. IR: $\tilde{\nu}$ = 2102.41 (-N_3), 1737.86 cm^{-1} (C=O).

3-Azidopropanol tosylate (Scheme 1). To a solution of 3-bromopropanol (1.2 mL, 13.8 mmol) in water (40 mL) was added NaN_3 (1.8 g, 27.6 mmol). The resulting reaction mixture was stirred at 80 °C for 3 days, allowed to cool to rt and extracted with EtOAc (5x). The combined organic layers were washed with brine, dried (Na_2SO_4), filtered and carefully concentrated. The residue was dissolved in DCM and NEt_3 (3.8 mL, 27.6 mmol) was added. The resulting solution was cooled to 0 °C before addition of tosyl chloride (4.0 g, 21 mmol) and stirred for 18 h. The reaction mixture was diluted with H_2O and extracted with DCM (3x). The combined organic layers were dried (Na_2SO_4), filtered and concentrated over celite. Purification of the residue by silica gel column chromatography (9:1 PE:DCM) yielded a colorless liquid (2.3 g, 9 mmol, 65 %), which discolored slightly upon storage at rt. $\rho = \sim 1.5$ g mL^{-1}. TLC: $R_f = 0.3$ (1:9 DCM:pentane). IR: $\tilde{\nu} = 2095$ cm^{-1} (-N_3). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.83$ (d, $J = 8.2$ Hz, 2 H), 7.40 (d, $J = 8.0$ Hz, 2 H), 4.14 (t, $J = 5.9$ Hz, 2 H), 3.41 (t, $J = 6.5$ Hz, 2 H), 2.49 (s, 3 H), 1.92 ppm (p, $J = 6.2$ Hz, 2 H). ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 145.28$, 132.90, 130.17, 128.14, 67.20, 47.48, 28.66, 21.91 ppm.

1-Amino-4-((4-(carboxymethyl)phenyl)amino)-9,10-dioxo-9,10-dihydroanthracene-2-sulfonate (cAB40, Scheme 2**).** To a solution of bromaminic acid (2 g, 5 mmol) and 4-aminophenyl acetic acid (674 mg, 4.45 mmol) in water (75 mL) were added Na_2CO_3 (795 mg, 7.5 mmol) and $CuSO_4$ (120 mg, 0.75 mmol). The reaction mixture was refluxed for 16 h, washed with DCM (3 × 50 mL) and concentrated. MeOH was added to the residue and after filtration the filtrate was concentrated and purification of the residue by reversed phase silica gel column chromatography (H_2O > 1:4 MeOH:H_2O) yielded cAB40 as a blue solid (650 mg, 1.4 mmol, 32 %). ^{1}H NMR (400 MHz, MeOD): $\delta = 8.34$–8.32 (m, 2 H), 8.26 (s, 1 H), 7.88–7.77 (m, 2 H), 7.36 (d, $J = 8.4$ Hz, 2 H), 7.26 (d, $J = 8.4$ Hz, 2 H), 3.62 ppm (s, 2 H). LC-MS m/z: 452.9 $[M+2H]^+$.

1,1-Bis(4-fluorophenyl)prop-2-yn-1-ol (5). To a cooled (−10 °C) solution of ethynyltrimethylsilane (1.55 mL, 11 mmol) in dry THF (20 mL) was slowly added *n*BuLi (2.5 M, 4.5 mL, 11 mmol) and stirred for 1 h. Subsequently, a solution of 4,4′-difluorobenzophenone (**4**, 2.18 g, 10 mmol) in dry THF (16 mL) was added in 15 minutes. The resulting solution was stirred at −10 °C for 1 h and then 0 °C for 1 h. The reaction mixure was quenched by the addition of KOH (2 M in MeOH, large excess) and stirred for 18 h, poured into H_2O, adjusted to pH 6–7 with 1 M HCl and extracted with EtOAc (3x). The combined organic layers were dried ($MgSO_4$), filtered and concentrated. Purification of the residue by silica gel column chromatography (1:19 EtOAc:pentane) yielded a yellow oil (1.81 g, 7.4 mmol, 74 %). TLC: $R_f = 0.42$ (1:9 EtOAc:pentane). IR: $\tilde{\nu} = 3298$ (CC-H), 1601, 1504, 1221, 1157 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.60$–7.50 (m, 4 H), 7.07–6.96 (m, 4 H), 2.90 (s, 1 H), 2.79 ppm (s, 1 H). ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 163.76$, 127.99 (d, $J = 8.3$ Hz), 115.35 (d, $J = 21.7$ Hz), 76.10 ppm.

4,4′-(1-(3-Azidopropoxy)prop-2-yne-1,1-diyl)bis(fluorobenzene) (8). To a cooled (0 °C) solution of alkyne **5** (0.29 g, 1.2 mmol) in dry DMF (10 mL) was added NaH (60 % dispersion in mineral oil, 55 mg, 1.3 mmol) and the reaction mixture was stirred for 30 minutes. Next, 3-azidopropanol tosylate (0.44 g, 1.8 mmol) was added dropwise. The resulting yellow solution was stirred for 18 h at rt, diluted with EtOAc and H_2O and extracted with EtOAc (3x). The combined organic layers were washed with H_2O (3x), brine, dried ($MgSO_4$), filtered and concentrated over celite. Purification of the residue by silica gel column chromatography (1 % EtOAc in pentane) yielded a colorless viscous oil (0.25 g, 0.77 mmol, 64 %). TLC: $R_f = 0.57$ (1:19 EtOAc:pentane). IR: $\tilde{\nu} = 3297$ (CC-H), 2095 cm^{-1} (N_3). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.54$–7.43 (m, 4 H), 7.09–6.94 (m, 4 H), 3.54 (d, $J = 5.9$ Hz, 2 H), 3.46 (t, $J = 6.8$ Hz, 2 H), 2.92 (s, 1 H), 1.99–1.84 ppm (m, 2 H). ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 162.44$ (d, $J = 247.1$ Hz), 138.85, 128.50 (d, $J = 8.2$ Hz), 115.27 (d, $J = 21.6$ Hz), 82.82, 79.17, 78.23, 61.55, 48.74, 29.33 ppm. LC-MS m/z: 328 $[M+H]^+$.

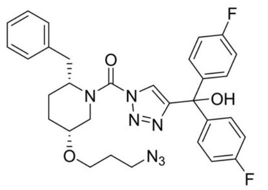
3-((1,1-Bis(4-fluorophenyl)prop-2-yn-1-yl)oxy)propan-1-amine (9). To a solution of **8** (0.25 g, 0.77 mmol) in 1:10 H_2O:THF was added PPh_3 (0.45 g, 1.7 mmol). The reaction mixture was stirred at 60 °C for 18 h, concentrated, diluted with EtOAc and H_2O, basified with 1 M NaOH and extracted with EtOAc (3x). The combined organic layers were washed with brine, dried ($MgSO_4$), filtered and concentrated. Purification of the residue by silica gel column chromatography (10 % methanolic solution of 3 % NH_3 in DCM) yielded a colorless film (234 mg, 0.77 mmol, 100 %). TLC: $R_f = 0.27$ (1:9 MeOH:DCM). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.57$–7.42 (m, 4 H), 7.07–6.92 (m, 4 H), 3.52 (t, $J = 6.1$ Hz, 2 H), 2.91 (s, 1 H), 2.86 (t, $J = 6.9$ Hz, 2 H), 1.86 (s, 2 H), 1.84–1.74 ppm (m, 2 H). ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 162.35$ (d, $J = 246.9$ Hz), 139.02 (d, $J = 3.1$ Hz), 128.45 (d, $J = 8.2$ Hz), 115.19 (d, $J = 21.6$ Hz), 83.03, 79.03, 78.05, 62.55, 39.48, 33.35 ppm. LC-MS m/z: 301.6 $[M+H]^+$.

***N*-(3-((1,1-Bis(4-fluorophenyl)prop-2-yn-1-yl)oxy)propyl)-2,4-dinitroaniline (10).** To a solution of **9** (234 mg, 0.77 mmol) in DMF (5 mL) was added NEt_3 (0.2 mL, 1.5 mmol) and 2,4-dinitrofluorobenzene (100 μL, 0.77 mmol). The reaction mixture was stirred for 18 h, diluted with Et_2O and brine and extracted with Et_2O (3x). The combined organic layers were washed with H_2O (5x), brine, dried ($MgSO_4$), filtered and concentrated to yield a viscous yellow oil (341 mg, 0.73 mmol, 95 %). TLC: $R_f = 0.33$ in (2:8 EtOAc:pentane). IR: $\tilde{\nu} = 3366$ (N-H), 3292 cm^{-1} (CC-H). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 9.10$ (d, $J = 2.7$ Hz, 1 H), 8.62 (t, $J = 5.5$ Hz, 1 H), 8.24 (dd, $J = 9.5$, 2.7 Hz, 1 H), 7.54–7.43 (m, 4 H), 7.05–6.92 (m, 5 H), 3.62 (m, 4 H), 2.96 (s, 1 H), 2.10 ppm (m, 2 H). ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 162.40$ (d, $J = 247.4$ Hz), 148.39, 138.52 (d, $J = 3.0$ Hz), 136.01, 130.39, 128.46 (d, $J = 8.2$ Hz), 124.38, 115.25 (d, $J = 21.6$ Hz), 114.03, 82.62, 79.40, 78.55, 61.70, 40.91, 28.89 ppm.

Bis(4-fluorophenyl)(1*H*-1,2,3-triazol-4-yl)methanol (6). To a degassed solution of alkyne **5** (1.2 g, 5 mmol) in 1:5 MeOH:DMF (20 mL) was added sequentially trimethylsilyl azide (1 mL, 7.4 mmol), $CuSO_4$ (1 M in water, 0.5 mL, 0.5 mmol) and NaAsc (1 M, 1.5 mL, 1.5 mmol). After prolonged heating (5 days) and several new portions of TMS-N_3 (3 × 1 mL), the reaction was quenched with H_2O. The reaction mixture was filtered over celite, concentrated, diluted with H_2O and extracted with Et_2O (3x). The combined organic layers were washed with H_2O (3x), brine, dried (Na_2SO_4), filtered and concentrated. Purification of the residue by silica gel column chromatography (10 > 30 % EtOAc in pentane) yielded a white solid with yellow discoloration. This solid was recrystallized from $CHCl_3$ to yield the title compound (835 mg, 2.91 mmol, 59 %). TLC: $R_f = 0.54$ (1:1 EtOAc:pentane). IR: $\tilde{\nu} = 3192$ (N-H), 1601, 1504 cm^{-1}. ^{1}H NMR (400 MHz, MeOD): $\delta = 7.58$ (s, 1 H), 7.42–7.29 (m, 4 H), 7.18–6.79 ppm (m, 4 H). ^{13}C NMR (100 MHz, DMSO): $\delta = 161.11$ (d, $J = 243.3$ Hz),143.12, 128.96 (d, $J = 8.2$ Hz), 114.38 (d, $J = 21.3$ Hz), 75.14 ppm. LC-MS m/z: 287.8 $[M+H]^+$.

***N*-(3-(Bis(4-fluorophenyl)(1*H*-1,2,3-triazol-4-yl)methoxy)propyl)-2,4-dinitroaniline (11).** To a degassed solution of **10** (110 mg, 0.24 mmol) and POM-azide (60 μL, 0.35 mmol) in THF:H_2O 10:3 (6.5 mL) was added CuBr (6.1 mg, 42 μmol) and the resulting suspension was sonicated shortly before being stirred at 60 °C for 4 days. The reaction mixture was allowed to cool to rt before KOH (2 M in MeOH) was added and the resulting solution was stirred for 30 minutes, concentrated, diluted with EtOAc and H_2O and extracted with EtOAc (3x). The combined organic layers were washed with a basic solution of 1 M EDTA (basified with NH_3(aq.), 2x), brine, dried ($MgSO_4$), filtered and concentrated. Purification of the residue by silica gel column chromatography (1:4 EtOAc: pentane) yielded a yellow film (81 mg, 0.167 mmol, 67%). TLC: $R_f = 0.33$ (1:1 EtOAc:-pentane). IR: $\tilde{\nu} = 3362$ (N-H aniline), 3100 cm^{-1} (N-H triazole). ^{1}H NMR (400 MHz, DMSO): $\delta = 15.03$ (s, 1H), 8.94–8.74 (m, 2H), 8.22 (dd, $J = 9.7$, 2.8 Hz, 1H), 7.50 (s, 1H), 7.39 (m, 4H), 7.21 (d, $J = 9.5$ Hz, 1H), 7.12 (m, 4H), 3.64–3.49 (m, 2H), 3.26 (m, 2H), 2.00–1.81 ppm (m, 2H). ^{13}C NMR (100 MHz, DMSO): $\delta = 161.18$ (d, $J = 245.2$ Hz), 148.10, 140.21, 134.71, 130.00, 129.4 (d, $J = 8$ Hz), 123.72, 115.28, 114.73 (d, $J = 21.3$ Hz), 61.24, 40.30, 28.50 ppm. LC-MS *m/z*: 510.8 $[M+H]^+$.

tert**Butyl (2*R*,5*R*)-5-(3-azidopropoxy)-2-benzylpiperidine-1-carboxylate (7).** To a cooled (0°C) solution of *tert*-butyl (2*R*,5*R*)-2-benzyl-5-hydroxypiperidine-1-carboxylate[23] (80 mg, 0.27 mmol) in dry DMF was added NaH (33 mg, 0.82 mmol) and stirred for 30 minutes. Subsequently, 3-azidopropanol tosylate (280 mg, 1.1 mmol) was added dropwise and the solution stirred at rt for 18 h. The mixture was diluted with Et_2O, poured into water and extracted with Et_2O (3x). The combined organic layers were washed with H_2O (2x), brine, dried (Na_2SO_4), filtered and concentrated. Purification of the residue by silica gel column chromatography (7.5% Et_2O in PE) yielded a colorless oil (66 mg, 0.18 mmol or 65%). TLC: $R_f = 0.44$ (1:9 Et_2O:pentane). $[\alpha]_D = -3.5$. IR: 2096 (-N_3), 1689 (Boc). ^{1}H NMR (400 MHz, CDCl3): $\delta = 7.37$–7.07 (m, 5H), 4.57–4.05 (m, 2H), 3.62 (m, 2H), 3.41 (m, 2H), 3.26 (m, 1H), 3.04–2.81 (m, 1H), 2.69 (m, 2H), 1.94 (m, 1H), 1.85 (m, 2H), 1.60 (m, 3H), 1.36 ppm (m, 9H). ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 129.29$, 128.55, 126.37, 79.72, 74.87, 65.35, 52.26, 48.56, 42.51, 36.03, 29.63, 28.31, 26.18 ppm. LC-MS *m/z*: 274.7 (M-Boc), 374.5 $[M+H]^+$.



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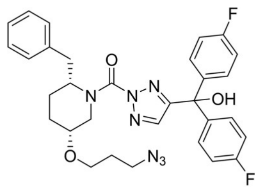

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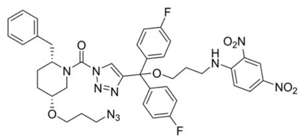
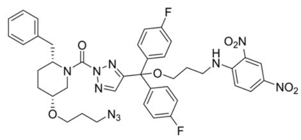
((2*R*,5*R*)-5-(3-Azidopropoxy)-2-benzylpiperidin-1-yl)(4-(bis(4-fluorophenyl)(hydroxy)methyl)-1*H*-1,2,3-triazol-1-yl)methanone (3) and ((2*R*,5*R*)-5-(3-azidopropoxy)-2-benzylpiperidin-1-yl)(4-(bis(4-fluorophenyl)(hydroxy)methyl)-2*H*-1,2,3-triazol-2-yl)methanone (24). 7 (46 mg, 0.12 mmol) was dissolved in 40% TFA in DCM and stirred for 15 minutes before the volatiles were removed under reduced pressure and coevaporated with toluene. The thus obtained TFA salt was dissolved in THF, treated with DIPEA (0.1 mL, 0.6 mmol) and cooled to 0°C before triphosgene (18 mg, 0.06 mmol) was added. The resulting solution was stirred for 30 minutes at 0°C. The reaction mixture was quenched with ice cold H_2O and extracted with EtOAc (3x). The combined organic layers were washed with H_2O, brine, dried (Na_2SO_4), filtered and concentrated. The crude carbamoyl chloride was dissolved in THF, treated with DIPEA (0.1 mL, 0.6 mmol), DMAP (16 mg, 0.12 mmol), 6 (35 mg, 0.12 mmol) and stirred at 60°C for 2 h. The reaction mixture was quenched with sat. aq. NH_4Cl and extracted with EtOAc (3x). The combined organic layers were washed with H_2O (2x), brine, dried (Na_2SO_4), filtered and concentrated. Purification of the residue by silica gel column chromatography (15 > 20% EtOAc in PE), to yield two regioisomers. N1 isomer, apolar fractions, 3: TLC: $R_f = 0.60$ (3:7 EtOAc:pentane). HRMS *m/z* calculated for $C_{31}H_{31}F_2N_7O_3$ $[M+Na]^+$: 610.2349, found: 610.2358. N2 isomer, polar fractions, 24: $R_f = 0.43$ (3:7 EtOAc:pentane). HRMS $_{m/z}$ calculated for $C_{31}H_{31}F_2N_7O_3$ $[M+Na]^+$: 610.2349, found: 610.2350.



12



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((2*R*,5*R*)-5-(3-Azidopropoxy)-2-benzylpiperidin-1-yl)(4-((3-((2,4-dinitrophenyl)amino)propoxy)bis(4-fluorophenyl)methyl)-1*H*-1,2,3-triazol-1-yl)methanone (12) and ((2*R*,5*R*)-5-(3-azidopropoxy)-2-benzylpiperidin-1-yl)(4-((3-((2,4-dinitrophenyl)amino)propoxy)bis(4-fluorophenyl)methyl)-2*H*-1,2,3-triazol-2-yl)methanone (25). Following the procedure for the preparation of 3, but from triazole 11 at 100 µmol scale. Purification by silica gel column chromatography (20 > 25% EtOAc in PE). Yellow film, 74% yield as a mixture of N1 and N2-isomers. N1, apolar fractions, 12: TLC: $R_f = 0.38$ (3:7 EtOAc:-pentane). HRMS *m/z* calculated for $C_{40}H_{40}F_2N_{10}O_7$ $[M+Na]^+$: 833.2942, found: 833.2947. N2, polar fractions, 25: TLF: $R_f = 0.26$ (3:7 EtOAc:pentane). HRMS *m/z* calculated for $C_{40}H_{40}F_2N_{10}O_7$ $[M+Na]^+$: 833.2942, found: 833.2956.

((2*R*,5*R*)-2-Benzyl-5-(3-(4-(4-(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)butyl)-1*H*-1,2,3-triazol-1-yl)propoxy)piperidin-1-yl)(4-((3-((2,4-dinitrophenyl)amino)propoxy)bis(4-fluorophenyl)methyl)-1*H*-1,2,3-triazol-1-yl)methanone (1). This compound was obtained by the general procedure for the CuAAC reaction on a 38 µmol scale. Purification of the residue by silica gel column chromatography (50 > 60% EtOAc in PE). TLC: $R_f = 0.25$ (3:2 EtOAc:pentane). N1 isomer, 1, HRMS *m/z* calculated for $C_{59}H_{63}BF_4N_{12}O_7$ $[M+H]^+$: 1139.5045, found: 1139.5076.

((2*R*,5*R*)-2-Benzyl-5-(3-(4-(4-(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)butyl)-1*H*-1,2,3-triazol-1-yl)propoxy)piperidin-1-yl)(4-((3-((2,4-dinitrophenyl)amino)propoxy)bis(4-fluorophenyl)methyl)-2*H*-1,2,3-triazol-2-yl)methanone (26). This compound was obtained by the general procedure for the CuAAC reaction on a 35 µmol scale. Purification of the residue by silica gel column chromatography (50 > 60% EtOAc in PE). TLC: $R_f = 0.25$ (3:2 EtOAc:pentane). N2 isomer, 26, HRMS *m/z* calculated for $C_{59}H_{63}BF_4N_{12}O_7$ $[M+H]^+$: 1139.5045, found: 1139.5067.

(4-Ethynylphenyl)methanol (14). To a solution of 4-ethynylbenzaldehyde (**13**, 390 mg, 3 mmol) in EtOH (6 mL) was added $NaBH_4$ (390 mg, 10.3 mmol). The reaction mixture was stirred for 10 minutes, quenched with water and extracted with DCM. The organic layer was washed with brine, dried ($MgSO_4$), filtered, and concentrated to yield **14** (389 mg, 3 mmol, 99%) as a yellow oil. TLC: $R_f = 0.61$ (6:4 pentane:EtOAc). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.48$ (d, $J = 8.2$ Hz, 2H), 7.31 (d, $J = 8.1$ Hz, 2H), 4.68 (s, 2H), 3.08 (s, 1H), 1.90 ppm (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 141.67$, 132.42, 126.85, 121.38, 83.59, 77.36, 64.94 ppm.

4-Ethynylbenzyl methanesulfonate (15). To a cooled (0°C) solution of **14** (373 mg, 2.82 mmol) in dry DCM (15 mL) were added Et_3N (0.59 mL, 4.23 mmol) and MsCl (262 µL, 3.38 mmol). The reaction mixture was stirred for 45 minutes, washed with water, extracted with DCM, dried ($MgSO_4$), filtered and concentrated to yield **15** (558 mg, 2.7 mmol, 94%). TLC: $R_f = 0.66$ (6:4 pentane:EtOAc). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.53$ (d, $J = 8.3$ Hz, 2H), 7.38 (d, $J = 8.3$ Hz, 2H), 5.23 (s, 2H), 4.57 (s, 1H), 3.14 (s, 1H), 2.94 ppm (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 134.07$, 132.70, 128.74, 123.37, 82.93, 78.57, 70.80, 38.47 ppm. LC-MS *m/z*: 211.0 $[M+H]^+$.

2-(4-Ethynylbenzyl)isoindoline-1,3-dione (16). To a cooled (0 °C) solution of **15** (546 mg, 2.6 mmol) in dry DMF (10 mL) was added phthalimide potassium salt (722 mg, 3.9 mmol). The reaction mixture was stirred on ice for 2 h and at rt for 18 h. After addition of water, the product precipitated. The suspension was filtered and the solid was dissolved in DCM, washed with HCl (0.1 M), brine, dried ($MgSO_4$), filtered and concentrated to yield **16** (625 mg, 2.4 mmol, 92 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.85 (m, 2H), 7.72 (m, 2H), 7.44 (d, J = 8.4 Hz, 2H), 7.38 (d, J = 8.4 Hz, 2H), 4.84 (s, 2H), 3.06 ppm (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 137.10, 134.25, 132.58, 132.15, 128.69, 123.58, 77.63, 41.43 ppm.

2-(4-(1*H*-1,2,3-Triazol-4-yl)benzyl)isoindoline-1,3-dione (17). To a degassed solution of **16** (102 mg, 0.4 mmol) and TMS-azide (79 µL, 0.6 mmol) in DMF:MeOH (4:0.8 mL) was added CuI (5 mg, 25 µmol). The reaction mixture was refluxed for 18 h, concentrated and purification of the residue by silica gel column chromatography (6:4 pentane:EtOAc) yielded **17** (78 mg, 0.26 mmol, 64 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.05–7.66 (m, 6H), 7.52 (m, 2H), 7.00 (s, 1H), 4.89 ppm (s, 2H). LC-MS *m/z*: 305.2 $[M+H]^+$.

***tert*Butyl (5-((2-nitro-*N*-phenethylphenyl)sulfonamido)pentyl)carbamate.** To a solution of *N*-Boc-cadaverine (372 mg, 1.84 mmol) in THF (8 mL) were added 2-nitrobenzenesulfonyl chloride (408 mg, 1.84 mmol) and Et_3N (0.38 mL, 2.76 mmol). The cloudy reaction mixture was stirred for 75 minutes, quenched with water (40 mL) and extracted with EtOAc (3 × 20 mL). The combined organic layers were washed with water (60 mL), brine (60 mL), dried ($MgSO_4$), filtered and concentrated. The residue was dissolved in CH_3CN (16 mL) and Cs_2CO_3 (1798 mg, 5.52 mmol) and phenethylbromide (0.38 mL, 2.76 mmol) were added. The solution was stirred at 80 °C for 6 h. Another equivalent of phenethylbromide (0.38 mL, 2.76 mmol) was added and stirred at 80 °C for 18 h. The mixture was poured into water (50 mL) and extracted with EtOAc (3 × 25 mL). The combined organic layers were washed with water (50 mL), brine (50 mL), dried (MgSO4), filtered, and concentrated. Purification of the residue by silica gel column chromatography (2:8 > 3:7 EtOAc:pentane) yielded the title compound (781 mg, 1.6 mmol, 87 %) as a yellow oil. TLC: R_f = 0.75 (1:1 pentane:EtOAc). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.95 (d, J = 7.6 Hz, 1H), 7.67–7.58 (m, 3H), 7.26–7.15 (m, 5H), 4.52 (br s, 1H), 3.50 (t, J = 8.0 Hz, 2H), 3.33 (t, J = 7.6 Hz, 2H), 3.07–3.06 (m, 2H), 2.84 (t, J = 8.0 Hz, 2H), 1.57 (m, 2H), 1.49–1.43 (m, 11H), 1.27 ppm (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) 156.06, 148.10, 138.11, 133.67, 133.48, 131.69, 130.73, 128.84, 128.69, 126.75, 124.24, 79.07, 48.87, 47.68, 40.35, 35.19, 29.69, 28.51, 27.82, 23.75 ppm. LC-MS *m/z*: 492.1 $[M+H]^+$.

***tert*Butyl (5-(phenethylamino)pentyl)carbamate (19).** To a solution of *tert*-butyl (5-((2-nitro-*N*-phenethylphenyl)sulfonamido)pentyl)carbamate (781 mg, 1.59 mmol) in CH_3CN (15 mL) were added Cs_2CO_3 (1.57 g, 4.77 mmol) and PhSH (244 µL, 2.38 mmol). The reaction mixture was stirred for 18 h, poured into water (100 mL) and extracted with DCM (3 × 50 mL). The combined organic layers were dried ($MgSO_4$), filtered and concentrated. Purification of the residue by silica gel column chromatography (1:9 MeOH:DCM > 1:9 MeOH:DCM + 1 % Et_3N) yielded **19** (400 mg, 1.3 mmol, 82 %) as a clear oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.33–7.27 (m, 2H), 7.21 (dt, J = 5.9, 1.4 Hz, 3H), 4.66 (br s, 1H), 3.74 (br s, 1H), 3.09 (m, 2H), 3.00–2.84 (m, 4H), 2.68 (m, 2H), 1.56 (m, 2H), 1.44 (s, 11H), 1.40–1.23 ppm (m, 2H). LC-MS *m/z*: 307.2 $[M+H]^+$.

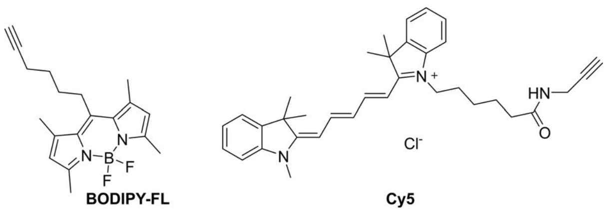
*tert*Butyl (5-(4-(4-((1,3-dioxoisoindolin-2-yl)methyl)phenyl)-*N*-phenethyl-1*H*-1,2,3-triazole-1-carboxamido)pentyl)carbamate (20). To a cooled (0 °C) solution of 19 (98 mg, 0.32 mmol) in dry THF (3 mL) were added DIPEA (167 µL, 0.96 mmol) and triphosgene (47 mg, 0.16 mmol). The reaction mixture was stirred on ice for 1 h, quenched with water and extracted with EtOAc (3 × 15 mL). The combined organic layers were washed with brine, dried ($MgSO_4$), filtered and concentrated. The residue was dissolved in dry THF (3 mL) and DMAP (39 mg, 0.32 mmol), DIPEA (167 µL, 0.96 mmol) and 17 (97 mg, 0.32 mmol) were added and stirred at 60 °C for 3 h. The reaction was quenched by the addition of NH_4Cl (sat. aq.), extracted with EtOAc (3 × 15 mL), washed with water, brine, dried ($MgSO_4$), filtered and concentrated. Purification of the residue by silica gel column chromatography (7:3 pentane:EtOAc) yielded 20 (55 mg, 86 µmol, 27 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.39 (s, 1H), 7.86 (m, 2H), 7.80 (m, 2H), 7.71 (m, 2H), 7.51 (m, 2H), 7.31–7.07 (m, 5H), 4.88 (s, 2H), 4.63 (s, 1H), 3.96 (m, 1H), 3.73 (m, 1H), 3.63–3.45 (m, 2H), 3.23–2.90 (m, 3H), 1.84–1.66 (m, 2H), 1.66–1.53 (m, 1H), 1.43 ppm (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 156.15, 146.11, 136.85, 134.17, 132.15, 129.32, 128.99, 128.81, 126.80, 126.70, 126.51, 126.26, 123.52, 121.17, 120.95, 51.35, 49.21, 41.42, 40.38, 35.14, 33.58, 29.86, 28.51, 26.99, 24.06, 23.71 ppm. LC-MS *m/z*: 637.2 $[M+H]^+$. HRMS *m/z* calculated for $C_{36}H_{40}N_6O_5$ $[M+H]^+$: 637.3133, found: 637.3134.

***tert*Butyl (5-(4-(4-(aminomethyl)phenyl)-*N*-phenethyl-1*H*-1,2,3-triazole-1-carboxamido)pentyl)carbamate (22).** To a solution of **20** (27 mg, 0.04 mmol) in EtOH (1 mL) was added ethylene diamine (4.3 µL, 0.06 mmol). The reaction mixture was stirred for 18 h, concentrated and purification of the residue by silica gel column chromatography (1:9 MeOH:DCM > 1:9 MeOH:DCM + 1 % Et_3N) yielded **22** (10 mg, 20 µmol, 50 %). A side reaction was nucleophilic addition on the urea by the ethylenediamine, requiring the deprotection to be stopped before full conversion was reached. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.38 (s, 1H), 7.71 (m, 2H), 7.30 (m, 2H), 7.29–7.12 (m, 5H), 4.60 (br s, 1H), 3.99 (s, 2H), 3.72–3.39 (m, 4H), 3.16–3.00 (m, 4H), 1.75 (br s, 2H), 1.55–1.43 (m, 11H), 1.34–1.23 ppm (m, 2H). LC-MS *m/z*: 507.0 $[M+H]^+$.

1-Amino-4-((4-(2-((4-(1-((5-((*tert*-butoxycarbonyl)amino)pentyl)(phenethyl)carbamoyl)-1*H*-1,2,3-triazol-4-yl)benzyl)amino)-2-oxoethyl)phenyl)amino)-9,10-dioxo-9,10-dihydroanthracene-2-sulfonate (23). To a solution of cAB40 (8.5 mg, 0.02 mmol) and HCTU (7.7 mg, 0.02 mmol) in dry DMF (1 mL) was added DIPEA (3.6 µL, 0.02 mmol). The reaction mixture was stirred for 15 minutes before addition of 22 (10 mg in 1 mL DMF, 0.02 mmol). The reaction mixture was stirred for 3 h, concentrated and purification of the residue by silica gel column chromatography (5:5:1 DCM:pentane:MeOH + 1 % AcOH) yielded 23 (10 mg, 11 µmol, 53 %) as a blue solid. ^{1}H NMR (500 MHz, $CDCl_3$/MeOD): δ = 8.31 (t, J = 9.0 Hz, 2H) 8.27 (s, 1H), 7.84–7.74 (m, 4H), 7.35–7.13 (m, 12H), 4.46 (br s, 2H), 3.67–3.57 (m, 4H), 3.37 (s, 2H), 3.16–2.99 (m, 6H), 1.77 (br s, 2H), 1.43–1.33 (m, 11H), 1.31–1.26 ppm (m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$/MeOD) 183.97, 172.03, 140.92, 138.67, 134.53, 134.07, 133.09, 132.80, 131.04, 130.46, 128.90, 128.76, 128.13, 126.44, 126.31, 126.15, 124.29, 123.43, 54.73, 51.37, 46.77, 43.18, 42.86, 42.64, 29.58, 28.34 ppm. LC-MS *m/z*: 940.93 $[M+H]^+$.

1-Amino-4-((4-(2-((4-(1-((5-(6-(3,3-dimethyl-2-((1*E*,3*E*)-5-((*Z*)-1,3,3-trimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-3*H*-indol-1-ium-1-yl)hexanamido)pentyl)(phenethyl)carbamoyl)-1*H*-1,2,3-triazol-4-yl)benzyl)amino)-2-oxoethyl)phenyl)amino)-9,10-dioxo-9,10-dihydroanthracene-2-sulfonate (2). A solution of 23 (9 mg, 9.6 µmol) in DCM (9 mL) and TFA (1 mL) was stirred for 45 minutes. The solvent was evaporated and co-evaporated with toluene. The crude was dissolved in dry DMF (5 mL), Cy5-OSu ester (5.5 mg, 9.6 µmol) and DIPEA (5.2 µL, 0.03 mmol) were added and the reaction mixture was stirred for 4 h, concentrated and purified by semi-preparative HPLC to yield 2 (1.3 mg, 1.0 µmol, 10 %) as a blue solid. HRMS *m/z* calculated for $C_{77}H_{80}N_{10}O_8S$ $[M+H]^+$: 1305.5954, found: 1305.5941.

1-(6-((5-(4-(4-((1,3-Dioxoisoindolin-2-yl)methyl)phenyl)-*N*-phenethyl-1*H*-1,2,3-triazole-1-carboxamido)pentyl)amino)-6-oxohexyl)-3,3-dimethyl-2-((1*E*,3*E*)-5-((*Z*)-1,3,3-trimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-3*H*-indol-1-ium (21). A solution of 20 (21 mg, 0.035 mmol) in DCM (0.9 mL) and TFA (0.1 mL) and stirred for 1 h. The solvent was evaporated and co-evaporated with toluene. The crude was dissolved in dry DMF (11 mL), Cy5-OSu ester (19 mg, 0.035 mmol) and DIPEA (11.9 µL, 0.09 mmol) were added and the reaction mixture was stirred for 18 h, poured into H_2O and extracted with EtOAc (3x). The combined organic layers were washed with water, brine, dried (Na_2SO_4), filtered and concentrated. Purification of the residue by silica gel column chromatography (EtOAc >1:19 MeOH:DCM) yielded the title compound (15 mg, 15 µmol, 44%) as a blue solid. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.40 (s, 1H), 7.98–7.65 (m, 7H), 7.50 (m, 2H), 7.45–6.93 (m, 15H), 6.66 (s, 1H), 6.28 (s, 1H), 4.88 (s, 2H), 4.10 (s, 2H), 3.94 (s, 1H), 3.86–3.62 (m, 2H), 3.56 (s, 4H), 3.40–3.12 (m, 2H), 3.12–2.91 (m, 2H), 2.49 (s, 1H), 2.00–1.50 (m, 22H), 1.50–1.20 ppm (m, 7H). ^{13}C NMR (125 MHz, $CDCl_3$): δ = 173.51, 172.29, 168.17, 142.96, 142.00, 141.27, 140.69, 134.21, 132.20, 129.31, 129.15, 128.79, 128.76, 126.28, 125.60, 124.89, 123.56, 122.18, 111.20, 110.19, 52.23, 51.33, 49.49, 49.33, 48.95, 44.87, 41.46, 29.83, 28.18, 27.04, 26.50, 25.43, 25.22 ppm. HRMS *m/z* calculated for $C_{63}H_{69}N_8O_4^+$ $[M]^+$: 1001.5436, found: 1001.5449.



Spectroscopic characterization

UV-VIS spectra (400–700 nm) were recorded on a Shimadzu PharmaSpec UV-1700 UV/Visible spectrophotometer in a cuvette with 1 cm light path. Fluorescence spectra were recorded on a Shimadzu RF-5301PC spectrofluorophotometer in a quartz cuvette with four polished windows with a light path of 1 cm. The spectrofluorophotometer was set at high sensitivity and to record spectra with a 1 nm sampling interval. For BODIPY-FL and probe **1**, the wavelength of excitation was set to 497 nm and emission spectra were recorded from 500–700 nm with a slit width of 1.5 nm. For Cy5 and probe **12**, the wavelength of excitation was set to 646 nm and emission spectra were recorded from 650–800 nm with a slit width of 3 nm. Absorbance and fluorescence spectra were recorded at five concentrations in 2 mL of 96% EtOH. The slopes of the absorbance versus fluorescence plot were used to determine the relative fluorescence quantum yields (gradient probe divided by gradient parent fluorophore).

Biological assays

Surrogate substrate assay. The surrogate substrate assay was performed as published previously,[38] with the following adaptions: 100 µL per well as final volume and an endpoint measurement of the absorbance. Briefly, the assay was performed in a transparent flat bottomed 96 wells plate. The membranes used in these experiments were derived from HEK293T cells overexpressing human DAGLα. The assay was performed in 50 mM HEPES buffered to pH 7.0. Inhibitors were incubated for 20 min at rt, followed by addition of *p*-nitrophenol butyrate. The final *p*-nitrophenol butyrate concentration was 300 µM. The amount of hydrolysis of *p*-nitrophenol butyrate was determined from the absorbance at 420 nm after 30 min incubation at rt. All measurements were performed with 5% DMSO present and a final protein concentration of 0.2 mg mL^{-1}. Negative controls consisted of mock transfected membranes or 10 µM Orlistat inhibited hDAGLα membranes. All measurements were performed in duplo (N = 2, *n* = 2).

Cell culture. Cells were cultured at 37 °C under 7% CO_2 in DMEM containing phenol red, GlutaMax, 10% (v/v) New Born Calf Serum (Thermo Fisher), penicillin and streptomycin (200 µg mL^{-1} each; Duchefa). For selection and maintenance of stable expression cell lines, complete DMEM was supplemented with G418 (0.4 mg mL^{-1}). Medium was refreshed every 2–3 days and cells were passaged twice a week at 80–90% confluence by resuspension in fresh medium (Neuro2A, HEK293T) or by trypsinization (U2OS).

Plasmids. The hDAGLα plasmid was constructed as described before.[38] 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. The plasmid was 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. HEK293T cells were grown to ≈70% confluency in 15 cm dishes. Prior to transfection, culture medium was refreshed (15 mL). A 3:1 (m:m) mixture of polyethyleneimine (PEI, 60 µg/well) and plasmid DNA (20 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 (2 mL/well) to the cells. 24 h post-transfection, the medium was refreshed and after 48 h cells were harvested.

U2OS_ABHD6-GFP stable expression. Full-length human cDNA of ABHD6 (Source Bioscience) was cloned into mammalian expression vector pcDNA3.1, containing genes for ampicillin and neomycin resistance. The inserts were cloned in frame with a C-terminal FLAG- and GFP-tag. Plasmids were isolated from transformed XL-10 Z-competent cells (Maxi Prep kit: QiaGen) and sequenced at the Leiden Genome Technology Center. Sequences were analyzed and verified (CLC Main Workbench). One day prior to transfection U2OS cells were seeded to a 6 wells plates (≈0.5 million cells/well). Prior to transfection, culture medium was aspirated and a minimal amount of medium was added. A 3:1 (m m^{-1}) mixture of polyethyleneimine (PEI) (3.75 µg/well) and plasmid DNA (11.25 µg/well) was prepared in serum free culture medium and incubated (15 min, RT). Transfection was performed by dropwise addition of the PEI/DNA mixture to the cells. After 24 h, transfection efficiency was determined by fluorescence microscopy and transfection medium was exchanged for selection medium containing 800 µg mL^{-1} G418. 48 h Post-transfection single cells were seeded to 96 wells plates in 100 µL selection medium. After 14 days, plates were inspected for cell growth, clones were checked for ABHD6-GFP expression by fluorescence microscopy (GFP channel). From here on, cells were grown in maintenance medium containing 400 µg mL^{-1} G418, and expanded in 12- and 6-wells plates and 10 cm dishes.

Inhibitor treatment. The medium was aspirated and 0.5 mL serum-free medium containing the inhibitor was added. After incubation for 1 h at 37 °C, medium was removed and PBS added, removed, trypsin buffer was added, quenched with 1 mL medium and the cells were harvested by pipetting. After centrifugation (5 min, 1000 *g*), the medium was removed, the cells were resuspended in PBS, centrifuged again (5 min, 1000 *g*) and the pellets flash frozen with N_2 (l) and stored at −80 °C.

Whole cell lysate preparation. 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^{-1} benzonase) and incubated on ice (15–30 min). The cell lysate was diluted to 2 mg mL^{-1} (Neuro2A) in cold storage buffer (20 mM Hepes, pH 7.2, 2 mM DTT). Protein concentrations were determined by a Quick Start™ Bradford Protein Assay (Bio-Rad) and diluted samples were flash frozen in liquid nitrogen and stored at −80 °C until further use.

Activity-based protein profiling. Cell lysate (15 μL, 2.0 mg mL^{-1}) was pre-incubated with vehicle or inhibitor (0.375 μL 40 *x* inhibitor stock, 30 min, rt or 37 °C) followed by an incubation with the activity-based probe (1 μM DH379 or 100 nM **1**, 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.).

Conflict of interest

The authors declare no conflict of interest.

Keywords: activity-based protein profiling · endocannabinoid · enzymes · fluorescent probes · quenched activity-based probes

[1] I. Katona, T. F. Freund, *Annu. Rev. Neurosci.* **2012**, *35*, 529 – 558.
[2] R. A. Kohnz, D. K. Nomura, *Chem. Soc. Rev.* **2014**, *43*, 6859 – 6869.
[3] J. L. Blankman, B. F. Cravatt, *Pharmacol. Rev.* **2013**, *65*, 849 – 871.
[4] V. Di Marzo, *Nat. Rev. Drug Discovery* **2008**, *7*, 438 – 455.
[5] T. Bisogno, F. Howell, G. Williams, A. Minassi, M. G. Cascio, A. Ligresti, I. Matias, A. Schiano-Moriello, P. Paul, E.-J. Williams, U. Gangadharan, C. Hobbs, V. Di Marzo, P. Doherty, *J. Cell Biol.* **2003**, *163*, 463 – 468.
[6] M. Reisenberg, P. L. Singh, G. Williams, P. Doherty, *Philos. Trans. R. Soc. London Ser. B* **2012**, *367*, 3264 – 3275.
[7] V. Di Marzo, *Nat. Neurosci.* **2011**, *14*, 9 – 15.
[8] K.-L. Hsu, K. Tsuboi, A. Adibekian, H. Pugh, K. Masuda, B. F. Cravatt, *Nat. Chem. Biol.* **2012**, *8*, 999 – 1007.
[9] W. R. Marrs, J. L. Blankman, E. A. Horne, A. Thomazeau, Y. H. Lin, J. Coy, A. L. Bodor, G. G. Muccioli, S. S.-J. Hu, G. Woodruff, S. Fung, M. Lafourcade, J. P. Alexander, J. Z. Long, Weiwei Li, et al., *Nat. Neurosci.* **2010**, *13*, 951 – 957.
[10] J. R. Savinainen, S. M. Saario, J. T. Laitinen, *Acta Physiol.* **2012**, *204*, 267 – 276.
[11] N. Li, H. S. Overkleeft, B. I. Florea, *Curr. Opin. Chem. Biol.* **2012**, *204*, 267 – 276.
[12] D. Ogasawara, H. Deng, A. Viader, M. P. Baggelaar, A. Breman, H. den Dulk, A. M. C. H. van den Nieuwendijk, M. Soethoudt, T. van der Wel, J. Zhou, H. S. Overkleeft, M. Sanchez-Alavez, S. Mori, W. Nguyen, B. Conti, et al., *Proc. Natl. Acad. Sci. USA* **2016**, *113*, 26 – 33.
[13] S. Lee, K. Park, K. Kim, K. Choi, I. C. Kwon, *Chem. Commun.* **2008**, 4250 – 4260.
[14] G. Blum, S. R. Mullins, K. Keren, M. Fonovič, C. Jedeszko, M. J. Rice, B. F. Sloane, M. Bogyo, *Nat. Chem. Biol.* **2005**, *1*, 203 – 209.
[15] G. Blum, G. von Degenfeld, M. J. Merchant, H. M. Blau, M. Bogyo, *Nat. Chem. Biol.* **2007**, *3*, 668 – 677.
[16] M. Verdoes, L. E. Edgington, F. A. Scheeren, M. Leyva, G. Blum, K. Weiskopf, M. H. Bachmann, J. A. Ellman, M. Bogyo, *Chem. Biol.* **2012**, *19*, 619 – 628.
[17] M. Verdoes, K. O. Bender, E. Segal, W. A. van der Linden, S. Syed, N. P. Withana, L. E. Sanman, M. Bogyo, *J. Am. Chem. Soc.* **2013**, *135*, 14726 – 14730.
[18] M. Garland, J. J. Yim, M. Bogyo, *Cell Chem. Biol.* **2016**, *23*, 122 – 136.
[19] Q. Zhang, H. Liu, Z. Pan, *Chem. Commun.* **2014**, *50*, 15319 – 15322.
[20] S. Serim, P. Baer, S. H. L. Verhelst, *Org. Biomol. Chem.* **2015**, *13*, 2293 – 2299.
[21] L. E. Edgington, M. Verdoes, A. Ortega, N.P. Withana, J. Lee, S. Syed, M. H. Bachmann, G. Blum, M. Bogyo, *J. Am. Chem. Soc.* **2013**, *135*, 174 – 182.
[22] Y. Shaulov-Rotem, E. Merquiol, T. Weiss-Sadan, O. Moshel, Seth Salpeter, D. Shabat, F. Kaschani, M. Kaiser, G. Blum, *Chem. Sci.* **2016**, *7*, 1322 – 1337.
[23] H. Deng, S. Kooijman, A. M. C. H. van den Nieuwendijk, D. Ogasawara, T. van der Wel, F. van Dalen, M. P. Baggelaar, F. J. Janssen, R. J. B. H. N. van den Berg, H. den Dulk, B. F. Cravatt, H. S. Overkleeft, P. C. N. Rensen, M. van der Stelt, *J. Med. Chem.* **2017**, *60*, 428 – 440.
[24] A. Adibekian, B. R. Martin, C. Wang, K.-L. Hsu, D. A. Bachovchin, S. Niessen, H. Hoover, B. F. Cravatt, *Nat. Chem. Biol.* **2011**, *7*, 469 – 478.
[25] M. Verdoes, U. Hillaert, B. I. Florea, M. Sae-Heng, M. D. P. Risseeuw, D. V. Filippov, G. A. van der Marel, H. S. Overkleeft, *Bioorg. Med. Chem. Lett.* **2007**, *17*, 6169 – 6171.
[26] K. K. Sadhu, S. Mizukami, S. Watanabe, K. Kikuchi, *Chem. Commun.* **2010**, *46*, 7403.
[27] K. K. Sadhu, S. Mizukami, S. Watanabea, K. Kikuchi, *Mol. Biosyst.* **2011**, *7*, 1766.
[28] H. Li, J. Kang, L. Ding, F. Lü, Y. Fang, *J. Photochem. Photobiol. A* **2008**, *197*, 226 – 231.
[29] K.-S. Focsaneanu, J. C. Scaiano, *Photochem. Photobiol. Sci.* **2005**, *4*, 817.
[30] M. Johnston, S. R. Bhatt, S. Sikka, R. W. Mercier, J. M. West, A. Makriyannis, S. J. Gatley, R. I. Duclos, Jr., *Bioorg. Med. Chem. Lett.* **2012**, *22*, 4585 – 4592.
[31] S. A. Farber, *Science* **2001**, *292*, 1385 – 1388.
[32] F. E. Jernigan, D. S. Lawrence, *Chem. Commun.* **2013**, *49*, 6728 – 6730.
[33] J. Haase in *Large-Scale Preparation and Usage of Azides. Organic Azides: Syntheses and Applications* (Eds.: S. Bräse, K. Banert). Wiley-VCH, Weinheim, **2010**, chap. 2.
[34] R. C. Woudenberg, *Doctoral Dissertations Available from Proquest.* AAI3289247, **2007**.
[35] C. A. Blencowe, D. W. Thornthwaite W. Hayes, A. Russell, *Org. Biomol. Chem.* **2015**, *13*, 8703 – 8707.
[36] K. E. Bullok, D. W. Thornthwaite, W. Hayes, A. T. Russell, *Biochemistry* **2007**, *46*, 4055 – 4065.
[37] D. L. Pedicord, M. J. Flynn, C. Fanslau, M. Miranda, L. Hunihan, B. J. Robertson, B. C. Pearce, X.-C. Yu, R. S. Westphal, Y. Blat, *Biochem. Biophys. Res. Commun.* **2011**, *411*, 809 – 814.
[38] M. P. Baggelaar, F. J. Janssen, A. C. M. van Esbroeck, H. den Dulk, M. Allarà, S. Hoogendoorn, R. McGuire, B. I. Florea, N. Meeuwenoord, H. van den Elst, G. A. van der Marel, J. Brouwer, V. Di Marzo, H. S. Overkleeft, M. van der Stelt, *Angew. Chem. Int. Ed.* **2013**, *52*, 12081 – 12085; *Angew. Chem.* **2013**, *125*, 12303 – 12307.
[39] M. P. Baggelaar, P. J. P. Chameau, V. Kantae, J. Hummel, K.-L. Hsu, F. Janssen, T. van der Wel, M. Soethoudt, H. Deng, H. den Dulk, M. Allarà, B. I. Florea, V. Di Marzo, W. J. Wadman, C. G. Kruse, et al., *J. Am. Chem. Soc.* **2015**, *137*, 8851 – 8857.
[40] B. Cegielska, K. M. Kacprzak, *Chem. Anal.* **2009**, *54*, 807 – 812.
[41] K. S. England, A. Tumber, T. Krojer, G. Scozzafava, S. S. Ng, M. Daniel, A. Szykowska, K. Che, F. von Delft, N. A. Burgess-Brown, A. Kawamura, C. J. Schofield, P. E. Brennan, *Med. Chem. Commun.* **2014**, *5*, 1879 – 1886.

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