Discovery of selective diacylglycerol lipase β inhibitors

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Chapter 2

A fluorescent surrogate substrate assay for high-throughput screening for DAGLβ

2.1 Introduction

The endocannabinoids anandamide (AEA)[1] and 2-arachidonoylglycerol (2-AG)[2] are endogenous signalling lipids that play a crucial role in human health and disease. The amount of 2-AG was found to be 170-fold higher than AEA in the brain, and its production is predominantly mediated by *sn*-1 specific diacylglycerol lipases (DAGLs).[3] There are two known isoforms of DAGL: DAGLα and DAGLβ.[4] DAGLα is the principal isoform to produce 2-AG in the central nervous system (CNS), where 2-AG functions as a retrograde messenger to activate cannabinoid receptor type 1 (CB_1R) on the presynaptic sites to inhibit neurotransmitter release on GABAergic and glutamatergic neurons.[5–7] By contrast, DAGLβ is the dominant isoform to produce 2-AG in the peripheral system during inflammation.[8,9] Selective DAGLβ inhibitors have been proposed as a potential treatment for inflammatory diseases with reduced potential for CNS mediated side effects, but they are currently lacking.[5]

Assay development is pivotal in drug discovery to identify compounds with desired activity for the drug target. A high-quality assay can alleviate the potential issues that might arise in later stages. Depending on the substrates employed in the assay, DAGL activity assays can be categorized into two groups: natural substrate-based assays and surrogate substrate-based assays. In 2003, Bisogno *et al.* developed a radiometric assay using *sn*-1-stearoyl-2-[^{14}C]arachidonoyl-glycerol ([^{14}C]-SAG) in the identification of DAGLs.[4] Although it is highly sensitive, the assay is labor intensive requiring the synthesis of the [^{14}C]-SAG substrate, lipid extraction, TLC separation, and quantification of the 2-[^{14}C]-AG product. An alternative way to avoid using radiolabeled SAG is to detect the formation of 2-AG by employing LC-MS analysis.[10] However, this method is still labor intensive. Moreover, neither of these two methods can monitor the formation of product in real-time. Inspired by the success of coupled enzyme glycerol assays for α/β-hydrolase domain ABHD6 and ABHD12[11], Van der Wel *et al.* developed a natural substrate-based fluorescence assay for DAGLα/β.[12] This assay can be used to monitor the reaction process in real-time and to determine the kinetic parameters, as well as to evaluate inhibitors for DAGL. However, the assay window for DAGLβ was narrow, which limited its application to evaluate the selectivity of DAGL inhibitors.

Surrogate substrate-based assays using either *para*-nitrophenol butyrate (PNPB)[13,14] or 6,8-difluoro-7-hydroxy-4-methylcoumarin (DiFMU) octanoate[15] are widely used in hit identification and initial SAR studies for DAGLα due to their cost-effectiveness. The progress of the reaction can be easily detected in real-time by measuring the absorbance in PNPB assay or fluorescence in DiFMU assay. However, the chemical structures of PNPB and DiFMU-octanoate are quite different from the structure of the natural substrate DAG, which may lead to distorted results of inhibitory activity, because of their different binding affinity.[12] Moreover, the assay window is relatively limited for DAGLβ.[16]

Recently, another commercially available lipase substrate, EnzChek™ lipase substrate was employed in a DAGLβ assay and showed an improved signal window.[16] The EnzChek lipase substrate (Figure 2.1) is a fluorescence-quenched substrate composed of a glycerol-like backbone, a green fluorophore BODIPY-C12 on the *sn*1 position, and a DABCYL-C12

quencher on the *sn*2 position. DAGL cleaves the *sn*1 ester bond to release the BODIPY fluorophore, whose fluorescence can be measured in real-time. It was envisioned that the EnzChek lipase assay could provide the opportunity to screen a library of compounds in a high throughput fashion and to evaluate their DAGL selectivity. In this Chapter, the EnzChek lipase substrate assay was, therefore, optimized and adapted for a high-throughput screening using recombinant human DAGLα and DAGLβ. A library of 12,587 compound was screened, resulting in the identification of eight confirmed hits.

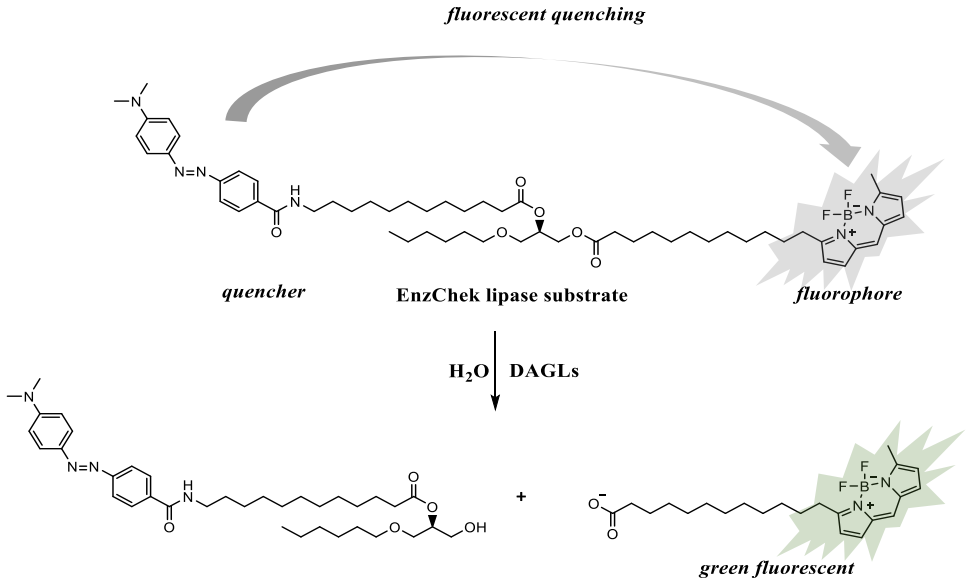


Figure 2.1 Schematic representation of the principle of EnzChek lipase substrate assay for DAGL.

2.2 Results and discussion

2.2.1 Optimization of EnzChek lipase substrate assay for DAGLα and DAGLβ

The EnzChek lipase substrate assay was adapted and optimized for recombinant human DAGLα and DAGLβ which were transiently overexpressed in human embryonic kidney (HEK293T) cells. The conditions of EnzChek lipase substrate assay were optimized in dark, flat-bottom 96-well plates. Initially, an assay buffer with 50 mM HEPES and 5% DMSO at pH 7.5 was used according to published methods.[16] It was observed that the fluorescence intensity increased in the first 5 minutes and then slowed down, resulting in the first linear part of the product-progression curve being too short to accurately calculating the initial velocity. Detergents are commonly used in biochemical assays to solubilize membrane proteins and hydrophobic substrates. DAGLα activity was previously reported to be observed in a narrow range of Triton X-100 concentrations, between 0.0075% to 0.075% in a natural substrate-based assay.[12] Therefore, diverse concentrations of Triton X-100 from 0% to 0.1% were assessed. 0.0025% Triton X-100 extended the linear part to at least 60 min for both DAGLs. This low concentration of Triton X-100 increased the overall activity of DAGLα about threefold

compared with that without Triton X-100, while the overall activity of DAGLβ remained unchanged. However, higher concentrations of Triton X-100 were intolerable, as they dramatically decreased the activity of DAGLα/β (Figure 2.2A, B). Previous studies observed that Ca^{2+} increased the activity of DAGL in a radiometric assay using substrate [^{14}C]-SAG.[4] To study the effects of cations on DAGL activity, $CaCl_2$ as well as other salts widely used in assay buffers, were investigated (Figure 2.2C, D). Ca^{2+} significantly decreased the activity of DAGLα but slightly increased the activity of DAGLβ when the concentrations of Ca^{2+} were at 5 mM and 10 mM. Mg^{2+} showed similar influence as Ca^{2+} on DAGL activities. However, monovalent ions Na^+ and K^+ had no significant influence on DAGLβ activity but decreased DAGLα activity at high concentrations of 100 mM and 150 mM. Due to the conflicting impacts on DAGL activity, no salt was added in the assay buffer. Subsequently, different protein concentrations were assessed (Figure 2.2E). The enzyme activity of DAGLα and DAGLβ increased up to 1 μg/mL and then declined. The signal-to-background (S/B) ratio was positively associated with enzyme activity. Therefore, the optimal concentration of protein used in the assay was determined to be 0.5 μg/mL for both DAGLα and DAGLβ.

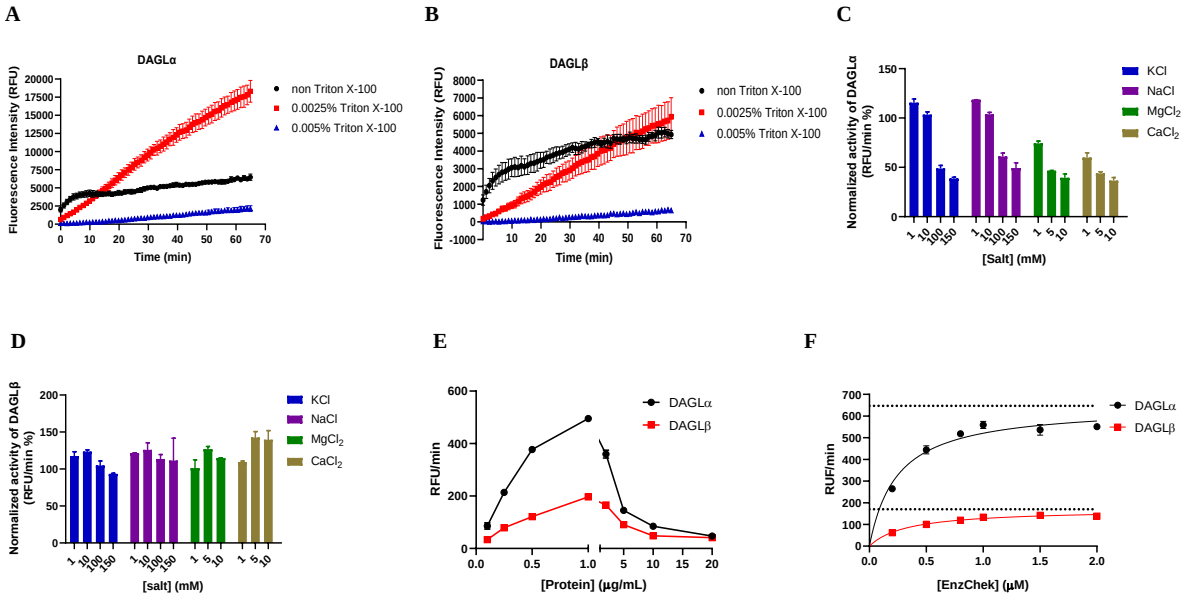


Figure 2.2 Optimization of EnzChek lipase substrate assay for DAGL. (A, B) Time course of EnzChek hydrolysis by DAGLα (A) and DAGLβ (B) as measured by fluorescence in assay buffers without and with different concentrations of Triton X-100. (C, D) The influence of salts in enzyme activity of DAGLα (C) and DAGLβ (D). (E) Rate of EnzChek hydrolysis as a function of protein concentration. (F) Michaelis-Menten kinetic curves. Rate of EnzChek hydrolysis by DAGLα (black) and DAGLβ (red) as a function of substrate concentration. All data were corrected for background fluorescence with mock membrane fraction observed at the same condition. Rates were determined in the linear interval of t = 10 min to t = 20 min. Data shown are mean ± SD (n = 2-4).

After optimizing the conditions of the assay, the Michaelis-Menten constant (K_M) was determined by measuring DAGLα/β activity at different substrate concentrations. Nonlinear regression analysis (Michaelis-Menten model) was used to calculate a V_{max} of 647 ± 38 RFU/min and a K_M of 0.24 ± 0.06 μM for DAGLα and a V_{max} of 170 ± 14 RFU/min and a K_M of 0.33 ± 0.09 μM for DAGLβ (Figure 2.2F). The EnzChek lipase substrate is highly

hydrophobic and has limited solubility in the aqueous assay buffer, leading to decreased enzyme activity when the substrate concentration exceeds 2 µM.

Subsequently, the assay was miniaturized to a dark flat-bottom 384-well plate to enhance the throughput. The concentration of EnzChek lipase substrate was increased from 0.2 µM in 96-well plate to 0.5 µM in 384-well plate, while the total assay volume was reduced from 100 µL to 30 µL. A time course of EnzChek hydrolysis was recorded for 180 min (Figure 2.3A), and similar product progression curves were observed as in 96-well plate. Endpoint measurement at 180 min, rather than kinetic measurements, were ultimately used. DAGL inhibitors, KT109[8] and DH376[7], were dose-dependently tested in the optimized assay, yielding pIC_{50} values of 8.90 ± 0.04 and 8.84 ± 0.08 for DAGLα, and 8.79 ± 0.08 and 8.66 ± 0.09 for DAGLβ, respectively (Figure 2.3B, C).

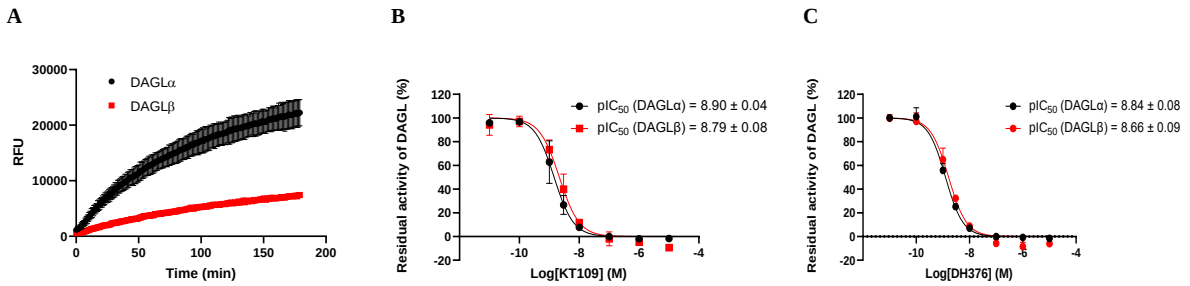


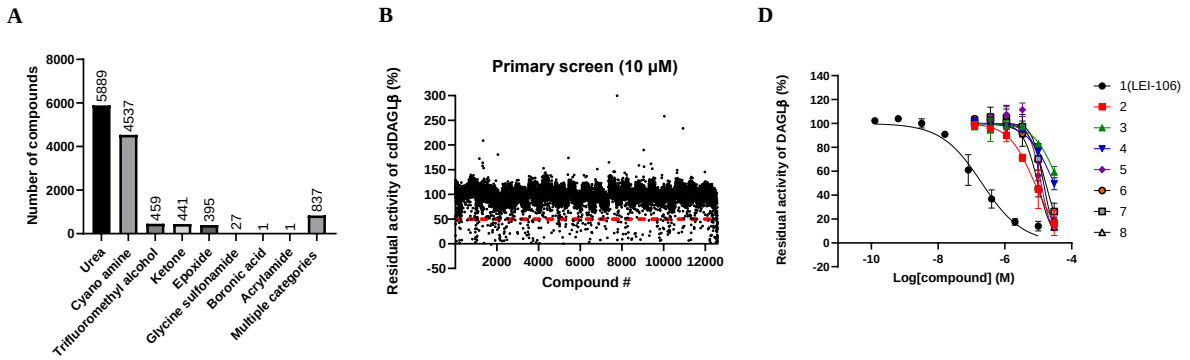
Figure 2.3 Miniaturization of DAGL EnzChek lipase substrate assay to 384-well plate format. (A). Time course of EnzChek hydrolysis by DAGL (Data shown are mean ± SD, n =3). (B, C). Dose-dependent curves of DAGL inhibitors KT109 (B) and DH376 (C) for DAGLα and DAGLβ (Data shown are mean ± SD, n = 1, N = 3).

2.2.2 High-throughput screening

EnzChek lipase substrate is commercially available but expensive. A large batch of EnzChek lipase substrate was, therefore, synthesized according to Scheme 2.1. The synthesized EnzChek lipase substrate displayed a K_M of 0.15 ± 0.05 µM for DAGLα and a K_M of 0.36 ± 0.13 µM for DAGLβ (Supplementary Figure S2.1), which was consistent with that of the commercial substrate. The S/B ratio of EnzChek lipase substrate assay for recombinant DAGLβ was around 2-3, which was not high enough for a HTS assay. Therefore, the catalytic domain (cd) of DAGLβ containing a His-MBP tag on the N-terminal (cdDAGLβ) was purified (Supplementary Figure S2.2) and applied in a HTS assay, which increased the S/B ratio more than 10-fold.

In total, 12,560 compounds from Enamine and 27 glycine sulfonamide analogs[13] synthesized previously in-house, were screened. An overview of chemotypes in the screened library is shown in Figure 2.4A. All compounds were screened at a single concentration of 10 µM in 384-well plates using 0.5 µg/mL cdDAGLβ. The concentration of EnzChek lipase substrate was adjusted to 0.25 µM, because it was sufficient for purified cdDAGLβ. The Z'-factor varied between 0.61 to 0.88, indicating a good screening quality (Z' > 0.6). Residual activity of cdDAGLβ (Figure 2.4B) was calculated for each compound, resulting in 277 primary actives showing > 50% inhibition at 10 µM, which reflects a hit rate of 2.2% (Figure 2.4C). All primary actives were screened again at 10 µM, which afforded 157 confirmed actives with a hit

rate of 1.2%. A deselection assay was performed to remove the compounds which may directly quench the fluorescence signal. EnzChek lipase substrate was incubated with cdDAGLβ to obtain the maximum fluorescence signal (after 5 h incubation), followed by adding the confirmed actives at a final concentration of 10 µM. Compounds decreasing the fluorescence signal by more than 30% were deselected, resulting in 156 remaining hits. Based on effect size, chemotype and clustering, 32 compounds were selected for dose-response determination. A list of eight hit compounds (**1**-**8**) was obtained based on potency, chemotype and the slope of dose-response curves.



C

	No. of compounds	Criterion	% Total
Total screened	12,587	-	100
Primary assay	277	> 50% inhibition at 10 µM	2.2
Confirmed assay	157	> 50% inhibition at 10 µM	1.2
Deselection assay	156	< 30% quenching at 10 µM	1.2
Selected for dose-response	32	efficacy, chemotype, clustering	0.25
Qualified hits	8	IC_{50}, hill slope	0.06

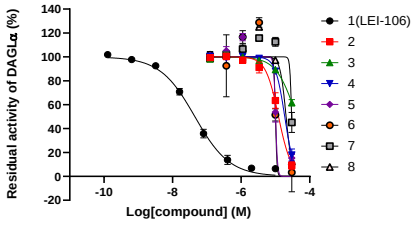


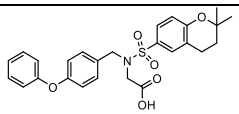
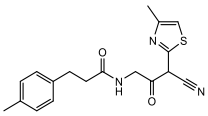
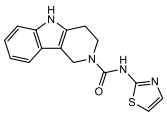
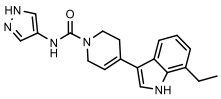
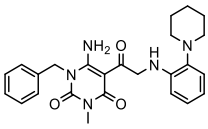
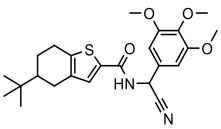
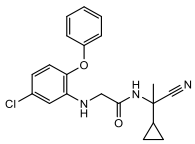
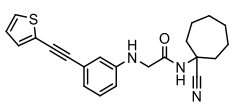
Figure 2.4 Overview of the high-throughput screening. (A) Overview of chemotypes of the screened library. (B) Residual activity of cdDAGLβ in primary screening. (C) Summary of the high-throughput screening for cdDAGLβ. (D, E) Dose-response curves of hits 1-8 for inhibiting recombinant human DAGLβ (D) and DAGLα (E) (Data shown are mean ± SD, n = 1, N = 3).

To confirm these hits, hit **1**, previously published as LEI-106[13], was resynthesized following the reported synthetic route.[13] Hit compounds **2**-**8** were purchased from Enamine. Molecular structure and purity of hits **1**-**8** were confirmed by ^{1}H NMR and HRMS. The potency of the eight hits for recombinant human DAGLβ (Figure 2.4D) and DAGLα (Figure 2.4E) were determined and summarized in Table 2.1.

Hit **1** (LEI-106), which is a glycine sulfonamide, displayed the highest potency for DAGL. It inhibited DAGL with a pIC_{50} of 6.69 ± 0.14 for DAGLβ and 7.35 ± 0.06 for DAGLα, respectively. Its potency for DAGLα in EnzChek lipase substrate assay fell within the same nanomolar range as the potency (pIC_{50} = 7.74) previously reported in a *para*-nitrophenyl butyrate (PNPB) assay[13], further confirming the reliability of EnzChek lipase substrate assay for evaluating inhibitor activity against DAGL. Hit **1** also exhibited the most optimal physicochemical properties, including molecular weight (MW), lipophilicity (cLogD) and topological polar surface area (tPSA).

Hits **2** and **5**, **3** and **4**, and **6-8** belong to the class of ketones, ureas, and cyano amides, respectively. They inhibited DAGLβ with pIC_{50} values ranging from 4.4 to 5.1. The activity of hits **5-8** for DAGLα could not be accurately determined due to the steepness of their dose-response curves, likely caused by aggregation or denaturation of the protein promoted by these compounds. Although less active compared to hit **1**, hit **2** could also be a potential starting point for developing a selective DAGLβ inhibitor in view of its potency, low molecular weight, and reasonable lipophilicity.

Table 2.1 Qualified hit list and corresponding physiochemical properties.[a]

ID	Structure	pIC_{50} (DAGLβ)	pIC_{50} (DAGL*α*)	MW (Da)	cLogP	tPSA (Å^2)	LipE (DAGLβ)	LipE (DAGL*α*)
1		6.69 ± 0.14	7.35 ± 0.06	481.56	1.9[b]	104	4.8	5.5
2		5.11 ± 0.10	4.91 ± 0.04	341.43	2.1	111	3.0	2.8
3		4.39 ± 0.10	4.38 ± 0.05	298.36	2.6	89	1.8	1.8
4		4.53 ± 0.06	4.73 ± 0.02	335.41	2.2	77	2.3	2.5
5		4.92 ± 0.09	n.d.	447.54	2.1	99	2.8	n.d.
6		5.01 ± 0.08	n.d.	442.57	4.5	109	0.5	n.d.
7		4.78 ± 0.08	n.d.	369.85	3.1	74	1.7	n.d.
8		4.84 ± 0.06	n.d.	377.51	3.9	93	0.9	n.d.

[a]The negative logarithm of half maximal inhibitory concentration (pIC_{50}) was determined with EnzChek lipase substrate assay. Molecular weight (MW), the calculated logarithm of the *n*-octanol-water partition coefficient (cLogP) and topological polar surface area (tPSA) were calculated using DataWarrior 5.0.0. Lipophilic efficiency (LipE) was calculated by formula LipE = pIC_{50} – cLogP. [b]cLogD equal to cLogP at pH 7.4 was used for Hit **1**.

2.3 Conclusion

In this Chapter, an EnzChek lipase substrate assay for DAGLα/β was successfully optimized and miniaturized into a 384-well plate format to screen a library of 12,587 compounds using purified catalytic domain of DAGLβ. This resulted in a hit list of eight compounds, which could be classified into four chemotypes, including glycine sulfonamide (hit **1**), ketones (hits **2**, **5**), ureas (hits **3**, **4**) and cyano amides (hits **6**-**8**). Hit **1**, also known as LEI-106, demonstrated the highest potency for DAGLβ and displayed favorable physiological properties. LEI-106 was, therefore, selected as a starting point for hit optimization in Chapter 3.

2.4 Acknowledgements

Dr. Hedwich Vlieg is kindly acknowledged for purifying the catalytic domain of DAGLβ. Julia Pols is acknowledged for making the running protocols and the training of Opentrons OT-2 in screening. Frans ter Brake is acknowledged for assisting in diluting the compounds in the screened library. Hans van den Elst is kindly acknowledged for HRMS measurements.

2.5 Experimental methods

Biology

Cell culture

HEK293T cells were cultured at 37 °C under 7% CO_2 in Dulbecco′s modified Eagle′s medium (DMEM) containing phenol red, Glutamax (2 mM), penicillin/streptomycin (200 µg/mL) and 10% newborn calf serum (Thermo Fischer). Cells were passaged twice a week by resuspension in fresh medium to appropriate confluence.

Transient Transfection

One day before transfection, HEK293T cells (~10^7) were seeded to 15 cm petri dishes. Before transfection, the medium was refreshed with a minimal amount of medium (13 mL). A mixture (3:1) of polyethyleneimine (PEI, 60 µg/dish) and plasmid DNA (20 µg/dish, human DAGLα-FLAG or DAGLβ-FLAG) was prepared in serum-free culture medium (2 mL/dish) and incubated at rt for 15 min. Transfection was done by dropwise addition of the PEI/DNA mixture to the cells. Transfection with the empty pcDNA3.1 vector was used for the mock control. After 24 h, the medium was refreshed and cells were harvested after 72 h by suspension in cold PBS. The suspension was centrifuged (200 g, 10 min, 4 °C) and supernatant was discarded. The cell pellets were flash frozen in liquid N_2 and stored at -80 °C until use.

Membrane preparation

Cell pellets were thawed on ice and suspended in cold lysis buffer (20 mM HEPES pH 7.2, 250 mM sucrose, 2 mM DTT, 1 mM $MgCl_2$, 2.5 U/mL Benzonase). The suspension was pipetted up and down and incubated on ice for 30 min. The cytosolic fraction and the membrane fraction were separated by ultra-centrifugation (93,000 g, 45 min, 4 °C, Beckman Coulter, Ti 70.1 rotor) or normal centrifugation (30,130 g, 3 h, 4 °C, Eppendorf Centrifuge 5430R). The pellet was washed with cold storage buffer (20 mM HEPES pH 7.2, 2 mM DTT) and suspended in cold storage buffer by pipetting through insulin needles. Protein concentrations were determined by Qubit® 2.0 fluorometer and the samples were diluted in cold storage buffer to 2.0 µg/µL. All samples were flash frozen in liquid N_2 and stored in small aliquots at -80 °C until use.

Cloning for insect cell expression and BACmid generation

For recombinant expression of the soluble DAGLβ catalytic domain with N-terminal 3C-cleavable His-MBP tags, the cDNA of human DAGLβ residues 265 to 627 was cloned into the pFastBac derived vector pCPF2.15 using the IVA cloning strategy.[17] Bacmids were generated by transforming this vector in to EmBacY cells (Geneva Biotech).

Protein expression in Sf9 cells

A P0 stock of baculovirus was generated by transfecting 8×10^5 Spodoptera frugiperda 9 (Sf9) cells with 10 µg of bacmid using CelFectin (Invitrogen) in SFM-II medium (Gibco) in a 6-well

plate. After at least 72 h of incubation at 28 °C, cell fluorescence was checked. The medium was harvested when at least 90% of cells showed YFP expression. This medium was used to generate a P1 viral stock by infecting 50 mL of Sf9 cells at 10^6 in Insect-Express medium (Lonza). The cells were incubated for 27 h at 28 °C while shaking. The medium was harvested and used to infect larger P2 cultures at 1 mL of P1 viral stock per 500 mL culture at 10^6 cells/mL. P2 cells were harvested 48-60 hours after infection by centrifugation (500 g, 10 min). Cell pellets were stored at -20 °C until use.

Purification of cdDAGLβ

The purification was performed at 4 °C. The cell pellets were resuspended in lysis buffer (25 mM HEPES pH 7.8, 300 mM NaCl, 1 mM $MgCl_2$, 2 mM DTT, 0.1% Triton X-100 and 25 U/mL Benzonase) and lysed by two cycles of freeze thaw. Lysates were cleared by centrifugation for 30 min at 15,000 g. The supernatant was incubated with amylose resin (New England Biolabs) for 1 h. Beads were washed extensively with wash buffer (25 mM HEPES pH 7.8, 300 mM NaCl, 2 mM DTT) followed by elution of the protein with elution buffer (25 mM HEPES pH 7.8, 300 mM NaCl, 2 mM DTT, 20 mM maltose and 0.025% octyl glucoside). The purified protein was concentrated to 1-2 μg/μL using a centrifugal filter (Millipore) and stored in small aliquots at -80 °C until use.

EnzChek lipase substrate assay for DAGLα and DAGLβ in 96-well plate

The membrane fractions of HEK293T cells transiently overexpressing human DAGLα or DAGLβ were diluted in assay buffer (50 mM HEPES pH 7.5, 4.5% DMSO, 0.0025% Triton X-100) to 2 μg/mL. 25 μL of protein solution was added to a black 96-well plate (Greiner Bio-One, REF 655076) and then 65 μL assay buffer was added. The membrane fraction from HEK293T cells transfected with empty pcDNA3.1 vector was used for the negative control (mock). The substrate EnzChek (1 mM in DMSO) was consecutively diluted in DMSO to 20 μM (50× dilution) and in assay buffer to 2 μM (10× dilution). 10 μL of substrate solution was added to the assay plate and the measurement was started immediately in CLARIOstar® (excitation 477-14 nm, emission 525-30 nm, gain = 1600, 60 or 72 sec/cycle for 61 cycles). The assay was performed in a final volume of 100 μL with 0.5 μg/mL protein and 0.2 μM EnzChek lipid substrate in 50 mM HEPES pH 7.5 with 5% DMSO and 0.0025% Triton X-100. Mock control was used for background subtraction. The initial linear part (t = 10 min to t = 20 min) was used to calculate the enzymatic rate (RFU/min). The assays varying in salts concentration, protein concentration and EnzChek concentration were adapted from this.

EnzChek lipase substrate assay for DAGLα and DAGLβ in 384-well plate

The membrane fractions from HEK293T cells transiently overexpressing human DAGLα and DAGLβ were diluted to 1.5 μg/mL in assay buffer 1 (50 mM HEPES pH 7.5, 5% DMSO, 0.0025% Triton X-100) and 10 μL was pipetted into a black 384-well plate (Greiner Bio-One, REF 781076). The membrane fraction from HEK293T cells transfected with empty pcDNA3.1 vector was used for the negative control (mock). Inhibitors were consecutively diluted in

DMSO to 600 µM and in assay buffer 2 (50 mM HEPES pH 7.5, 0.0025% Triton X-100) to 30 µM. A dilution series (8 concentrations) was prepared in assay buffer 1. 10 µL of inhibitor solution or assay buffer 1 was transferred to the enzyme samples in the assay plate. The plate was spun down at 1000 rpm for 1 min in Eppendorf Centrifuge 5810R and incubated at rt for 30 min. The EnzChek lipase substrate was consecutively diluted in DMSO to 30 µM and in assay buffer 2 to 1.5 µM. 10 µL of EnzChek solution (final concentration 0.5 µM) was added to each well. The plate was spun down at 1000 rpm for 1 min and incubated for 3 h at rt in the dark. The final concentrations of protein and substrate were 0.5 µg/mL and 0.5 µM, respectively. Endpoint fluorescence was measured in CLARIOstar® (excitation 477-14 nm, emission 525-30 nm, gain = 1600). DAGL and mock membrane fractions with DMSO were used as positive and negative controls respectively to calculate the windows of the assay. The mock membrane fraction with inhibitors at each concentration was used for background correction. Assay performance was assessed using Z' factor, which was calculated with the formula: $Z' = 1 - 3(\sigma_{pc} + \sigma_{nc})/(\mu_{pc} - \mu_{nc})$, where pc represents the positive control, nc represents the negative control, σ represents the standard deviation, and µ represents the mean value. Residual activity of DAGL was calculated using the equation: Residual activity (%) = $(\mu_{DAGL} - \mu_{mock})/(\mu_{pc} - \mu_{nc}) \times 100\%$, where μ_{DAGL} and μ_{mock} represent the fluorescence intensities of DAGL and mock with inhibitors, respectively. Residual activities were used to generate the dose-response curves using GraphPad Prism 9.0.0 (log(inhibitor) vs. normalized response with variable slope). All measurements were performed three times independently (n = 1, N = 3 or n = 4, N = 3 for controls, with $Z' \geq 0.6$).

EnzChek lipase substrate assay for cdDAGLβ in 384-well plate

All compounds (10 mM) in the screened library were diluted in DMSO to 300 µM, of which 1 µL was transferred to a black 384-well plate (Greiner Bio-One, REF 781076) by an Opentrons OT-2 robot. DMSO and 10 µM KT109 (n=8 for each assay plate) were added as positive and negative controls, respectively. Next, 19 µL of cdDAGLβ solution diluted in assay buffer (50 mM HEPES pH 7.5, 0.0025% Triton X-100, 0.79 µg/mL) was added. The plate was spun down at 1000 rpm for 1 min in Eppendorf Centrifuge 5810R and incubated at rt for 30 min. The EnzChek lipase substrate was consecutively diluted in DMSO to 15 µM and in assay buffer to 0.75 µM. 10 µL of EnzChek solution was added to each well. The plate was spun down at 1000 rpm for 1 min and incubated at rt for 3 h in the dark. The final concentrations of screened compound, cdDAGLβ, and EnzChek lipase substrate were 10 µM, 0.5 µg/mL, 0.25 µM, respectively. Endpoint fluorescence was measured in CLARIOstar® (excitation 477-14 nm, emission 525-30 nm, gain = 1600). Assay performance was assessed using Z' factor, which was calculated with the formula: $Z' = 1 - 3(\sigma_{pc} + \sigma_{nc})/(\mu_{pc} - \mu_{nc})$, where pc represents the positive control, nc represents the negative control, σ represents the standard deviation, and µ represents the mean value. Residual activity of DAGL was calculated via the equation: Residual activity (%) = $(\mu_{cdDAGL\beta} - \mu_{nc})/(\mu_{pc} - \mu_{nc}) \times 100\%$, where $\mu_{cdDAGL\beta}$ represents the fluorescence intensity of cdDAGLβ with inhibitors. The compounds which inhibited cdDAGLβ activity over 50% were considered as hits. The primary screening gave 277 hits with a hit rate of 2.2%. The

primary hits were screened again at 10 µM, resulting in 157 confirmed hits with a hit rate of 1.2%. For deselection, 19 µL of cdDAGLβ solution and 10 µL of EnzChek solution were incubated together at rt for 5 h in the dark before measuring the fluorescence. Subsequently, 1 µL of compound solution was added and the fluorescence was measured again. The compounds which inhibited the fluorescence over 30% were deselected, resulting in 156 remaining hits.

Chemistry

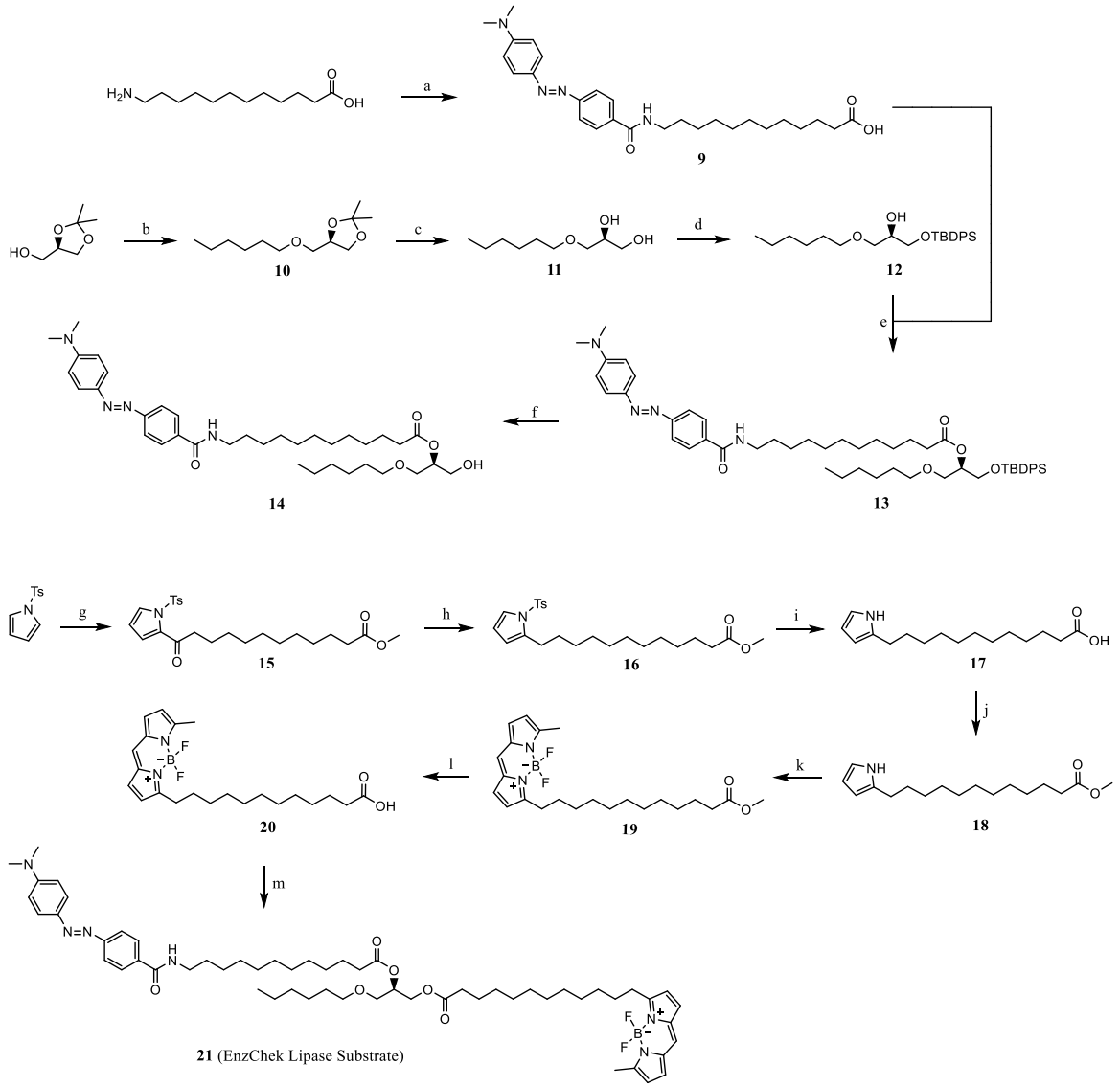
General remarks

All purchased chemicals were used without purification unless stated otherwise. All reactions were performed in oven-dried or flame-dried glassware. Anhydrous solvents were dried by activated 3 Å or 4 Å molecular sieves. Traces of water in starting materials were removed by co-evaporation with toluene if necessary. Thin layer chromatography (TLC) analysis was performed on Merck silica gel 60 F_{254} aluminium sheets and the compounds were visualized by using UV absorption at 254 nm and 366 nm and/or $KMnO_4$ staining (5 g/L $KMnO_4$ and 25 g/L K_2CO_3 in water). TLC plates were analysed with the Advion CMS Plate Express® connected to the Advion Expression® L-MS using 90% MeOH in H_2O with 0.1% formic acid as the solvent. Liquid chromatography-mass spectrometry (LC-MS) analyses were performed on a Thermo Finnigan LCQ Advantage MAX ion-trap mass spectrometer (ESI^+) coupled to a Surveyor HPLC system equipped with a C18 column (50 × 4.6 mm, 3 µm particle size, Macherey-Nagel) or a Thermo Finnigan LCQ Fleet ion-trap mass spectrometer (ESI^+) coupled to a Vanquish UHPLC system using H_2O, CH_3CN and 0.1% aq. TFA as eluents. Purification was performed on manual silica gel column chromatography (40-63 µm, 60 Å silica gel, Macherey-Nagel) or automated silica gel column chromatography (40-63 µm, 60 Å pre-packed silica gel, Screening Devices) on a Biotage Isolera™ Four 3.0 system. ^1H and ^{13}C spectra were recorded on a Bruker AV 400 MHz (400 MHz for ^1H and 101 MHz for ^{13}C) or AV 500 MHz spectrometer (500 MHz for ^1H and 126 MHz for ^{13}C) in deuterated solvents. Chemical shifts are reported in ppm with tetramethylsilane (TMS) or solvent resonance as the internal standard ($CDCl_3$: δ 7.26 for ^1H, δ 77.16 for ^{13}C; CD_3OD: δ 3.31 for ^1H, 49.00 for ^{13}C; DMSO-*d6*: δ 2.50 for ^1H, δ 39.52 for ^{13}C). Data is reported as follows: chemical shifts δ (ppm), multiplicity (s = singlet, d = doublet, dd = doublet of doublets, ddd = doublet of doublet of doublets, dt = doublet of triplets, t = triplet, td = triplet of doublets, tt = triplet of triplets, q = quartet, quintet = p, bs = broad singlet, m = multiplet), coupling constants *J* (Hz) and integration. High resolution mass spectrometry (HRMS) analysis was performed on a Thermo Finnigin LTQ Orbitrap mass spectrometer equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10 mL/min, capillary temperature 250 °C) with resolution R = 60000 at m/z 400 (mass range m/z = 150-2000) and dioctyl phthalate (m/z = 391.28428) as a lock mass.

Synthesis of EnzChek lipase substrate

EnzChek lipase substrate was synthesized as depicted in Scheme 2.1. Building block **9** was synthesized from dabcyl succinimidyl ester and 12-aminododecanoic acid and building block

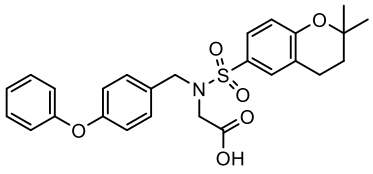
12 was obtained from (*R*)-(2,2-dimethyl-1,3-dioxolan-4-yl)methanol via sequential nucleophilic substitution (**10**), hydrolysis (**11**) and TBDPS protection[18]. Next, building block **9** was coupled to **12** to obtain ester **13**, whose TBDPS group was deprotected to gain *sn*2 ester **14**. During TBDPS deprotection, acetic acid was used to adjust the pH to avoid shifting of the *sn*2 ester bond towards the primary hydroxyl group. The synthesis of BODIPY green **20** started with the condensation of 1-tosyl-1*H*-pyrrole and 12-methoxy-12-oxododecanoic acid under the agency of TFAA[19] to obtain compound **15**. Next, the chemo-selective ketone reduction using ZnI_2 and $NaBH_3CN$[20] gained compound **16**. Intermediate **18** was obtained from **16** via sequential tosyl deprotection, saponification and esterification. **18** was condensed with 5-methyl-1*H*-pyrrole-2-carbaldehyde using $POCl_3$ and $BF_3{\cdot}Et_2O$[21] to obtain compound **19**, which was hydrolyzed under acidic condition to form bodipy **20**. Finally, dabcyl **14** and bodipy **20** were coupled to obtain EnzChek lipase substrate.



Scheme 2.1 Synthesis of EnzChek lipase substrate. Reagents and conditions: a) DABCYL NHS, DIPEA, DMF, rt; 79%; b) 1-bromohexane, NaH, DMF, rt, 71%; c) $TsOH{\cdot}H_2O$, MeOH, 45 °C, 74%; d) TBDPSCl, Et_3N, DMAP, DCM, rt, 80%; e) **9**, DIC, DMAP, DCM, rt, 51%; f) TBAF, AcOH, THF, rt, 66%; g) TFAA, 12-methoxy-12-oxododecanoic acid, DCM, reflux, 70%; h) $NaBH_3CN$, ZnI_2, DCE, 80 °C, 71%; i) 2 M aq. NaOH, MeOH, 80 °C,

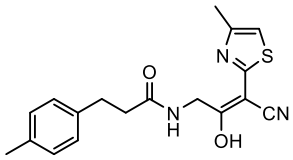
79%; j) DIC, DMAP, MeOH, DCM, rt, 81%; k) *i.* 5-methyl-1*H*-pyrrole-2-carbaldehyde, $POCl_3$, DCM, rt; *ii.* DIPEA, $BF_3 \cdot Et_2O$, rt, 64%; l) 4 M aq. HCl in THF, rt, 79%; m) **14**, DIC, DMAP, DCM, rt, 72%.

N-((2,2-Dimethylchroman-6-yl)sulfonyl)-*N*-(4-phenoxybenzyl)glycine (1, LEI-106)



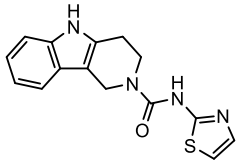
The compound was synthesized according to previously reported procedure.[13] ^{1}H NMR (400 MHz, MeOD+$CDCl_3$) δ 7.59 – 7.51 (m, 2H), 7.35 – 7.25 (m, 2H), 7.18 – 7.10 (m, 2H), 7.12 – 7.04 (m, 1H), 6.99 – 6.91 (m, 2H), 6.92 – 6.83 (m, 2H), 6.85 – 6.77 (m, 1H), 4.41 (s, 2H), 3.86 (s, 2H), 2.80 (t, *J* = 6.7 Hz, 2H), 1.82 (t, *J* = 6.8 Hz, 2H), 1.33 (s, 6H). ^{13}C NMR (101 MHz, MeOD+$CDCl_3$) δ 171.62, 158.54, 157.75, 157.31, 130.64, 130.46, 130.31, 130.22, 129.92, 127.40, 123.98, 122.03, 119.45, 119.08, 118.19, 76.25, 51.12, 47.15, 32.60, 27.01, 22.72. HRMS $[C_{26}H_{27}NO_6S+H]^+$: 482.16318 calculated, 482.16313 found.

N-(3-Cyano-3-(4-methylthiazol-2-yl)-2-oxopropyl)-3-(*p*-tolyl)propenamide (2)



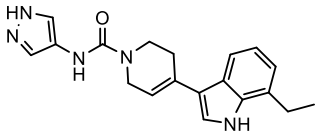
The compound was purchased from Enamine. ^{1}H NMR (500 MHz, $CDCl_3$+MeOD) δ 7.15 – 7.07 (m, 4H), 6.46 (t, *J* = 1.3 Hz, 1H), 4.35 (s, 2H), 2.98 – 2.91 (m, 2H), 2.59 – 2.54 (m, 2H), 2.36 (s, 3H), 2.31 (s, 3H). HRMS $[C_{18}H_{19}N_3O_2S+H]^+$: 342.12707 calculated, 342.12714 found.

N-(Thiazol-2-yl)-1,3,4,5-tetrahydro-2*H*-pyrido[4,3-*b*]indole-2-carboxamide (3)



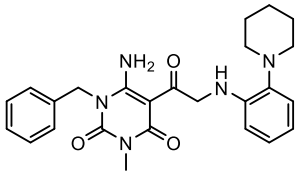
The compound was purchased from Enamine. ^{1}H NMR (500 MHz, MeOD+$CDCl_3$) δ 7.44 – 7.38 (m, 1H), 7.34 – 7.24 (m, 2H), 7.14 – 7.00 (m, 2H), 6.81 (t, *J* = 4.4 Hz, 1H), 4.75 (s, 2H), 3.92 (t, *J* = 5.5 Hz, 2H), 2.88 (t, *J* = 5.7 Hz, 2H). HRMS $[C_{15}H_{14}N_4OS+H]^+$: 299.09611 calculated, 299.09600 found.

4-(7-Ethyl-1*H*-indol-3-yl)-*N*-(1*H*-pyrazol-4-yl)-3,6-dihydropyridine-1(2*H*)-carboxamide (4)



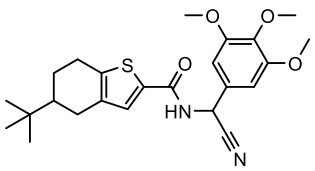
The compound was purchased from Enamine. ^{1}H NMR (500 MHz, $CDCl_3$+MeOD) δ 7.73 (d, *J* = 8.0 Hz, 1H), 7.66 (s, 2H), 7.23 (s, 1H), 7.12 (t, *J* = 7.5 Hz, 1H), 7.06 (d, *J* = 7.1 Hz, 1H), 6.19 (s, 1H), 4.28 – 4.15 (m, 2H), 3.77 (t, *J* = 5.5 Hz, 2H), 2.89 (q, *J* = 7.6 Hz, 2H), 2.73 – 2.56 (m, 2H), 1.37 (t, *J* = 7.5 Hz, 3H). HRMS $[C_{19}H_{21}N_5O+H]^+$: 336.18189 calculated, 336.18168 found.

6-Amino-1-benzyl-3-methyl-5-((2-(piperidin-1-yl)phenyl)glycyl)pyrimidine-2,4(1*H*,3*H*)-dione (5)



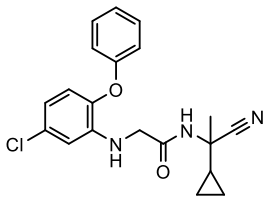
The compound was purchased from Enamine. ^{1}H NMR (500 MHz, $CDCl_3$) ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.45 – 7.39 (m, 2H), 7.39 – 7.34 (m, 1H), 7.29 – 7.25 (m, 4H), 7.02 – 6.95 (m, 2H), 6.70 – 6.63 (m, 2H), 5.88 (t, *J* = 4.0 Hz, 1H), 5.27 (s, 2H), 4.65 (d, *J* = 4.0 Hz, 2H), 3.45 (s, 3H), 3.17 – 2.50 (m, 4H), 1.74 (p, *J* = 5.5 Hz, 4H), 1.57 (s, 2H). LC-MS: HRMS $[C_{25}H_{29}N_5O_3+H]^+$: 448.23432 calculated, 448.23417 found.

5-(*tert*-Butyl)-*N*-(cyano(3,4,5-trimethoxyphenyl)methyl)-4,5,6,7-tetrahydrobenzo[*b*]thiophene-2-carboxamide (6)



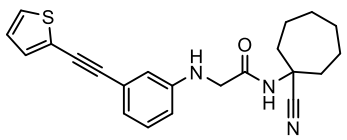
The compound was purchased from Enamine. ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.73 (s, 2H), 6.32 – 6.19 (m, 2H), 3.88 (s, 6H), 3.85 (s, 3H), 2.97 – 2.88 (m, 1H), 2.77 – 2.66 (m, 2H), 2.38 – 2.27 (m, 1H), 2.13 – 2.04 (m, 1H), 1.52 – 1.34 (m, 2H), 0.94 (s, 9H) (There is water in $CDCl_3$.). HRMS $[C_{24}H_{30}N_2O_4S+H]^+$: 443.19990 calculated, 443.19993 found.

2-((5-Chloro-2-phenoxyphenyl)amino)-*N*-(1-cyano-1-cyclopropylethyl)acetamide (7)



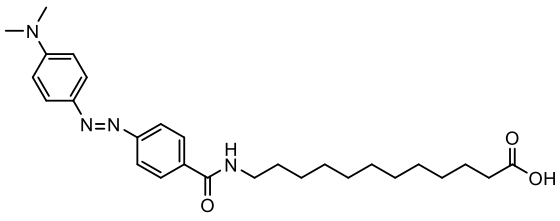
The compound was purchased from Enamine. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.38 – 7.32 (m, 2H), 7.16 – 7.10 (m, 1H), 6.98 – 6.94 (m, 2H), 6.83 – 6.72 (m, 3H), 6.61 (d, *J* = 2.3 Hz, 1H), 4.86 (t, *J* = 5.7 Hz, 1H), 3.88 – 3.78 (m, 2H), 1.77 (s, 3H), 1.25 – 1.18 (m, 1H), 0.76 – 0.55 (m, 4H). HRMS $[C_{20}H_{20}ClN_3O_2+H]^+$: 370.13168 calculated, 370.13167 found.

***N*-(1-Cyanocycloheptyl)-2-((3-(thiophen-2-ylethynyl)phenyl)amino)acetamide (8)**



The compound was purchased from Enamine. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.30 (dd, *J* = 5.2, 1.2 Hz, 1H), 7.28 (dd, *J* = 3.7, 1.2 Hz, 1H), 7.21 (t, *J* = 7.9 Hz, 1H), 7.03 – 6.99 (m, 2H), 6.77 (dd, *J* = 2.6, 1.4 Hz, 1H), 6.72 (s, 1H), 6.60 (ddd, *J* = 8.1, 2.5, 0.9 Hz, 1H), 4.29 (t, *J* = 5.4 Hz, 1H), 3.83 (d, *J* = 5.4 Hz, 2H), 2.32 (ddd, *J* = 14.2, 8.4, 2.2 Hz, 2H), 1.98 (ddd, *J* = 14.3, 9.6, 2.2 Hz, 2H), 1.75 – 1.47 (m, 8H). HRMS $[C_{22}H_{23}N_3OS+H]^+$: 378.16346 calculated, 378.16323 found.

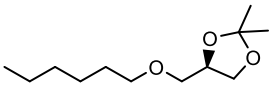
(*E*)-12-(4-((4-(Dimethylamino)phenyl)diazenyl)benzamido)dodecanoic acid (9)



To a stirred mixture of 2,5-dioxopyrrolidin-1-yl-4-((4-(dimethylamino)phenyl)diazenyl)benzoate (30 mg, 0.082 mmol, 1 eq) and 12-aminododecanoic acid (23 mg, 0.11 mmol, 1.3 eq) in anhydrous DMF (1.3 mL, 0.06 M) was

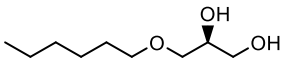
added DIPEA (43.0 µL, 0.246 mmol, 3 eq) and the mixture was stirred at rt for 3 days. The mixture was diluted in EtOAc and washed 1× with 0.05 M aq. HCl and 1× with H_2O. The organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified by silica gel column chromatography (0-3% MeOH in DCM) to afford the product as a yellow solid (30 mg, 0.064 mmol, 79%). ^{1}H NMR (400 MHz, MeOD) δ 7.91 – 7.84 (m, 3H), 7.83 – 7.78 (m, 3H), 6.78 – 6.72 (m, 2H), 3.37 (t, *J* = 7.3 Hz, 2H), 3.09 (s, 6H), 2.25 (t, *J* = 7.5 Hz, 2H), 1.65 – 1.52 (m, 4H), 1.39 – 1.19 (m, 14H). ^{13}C NMR (101 MHz, MeOD+$CDCl_3$) δ 177.20, 168.71, 155.38, 153.51, 143.89, 135.16, 128.44, 125.80, 122.38, 111.94, 40.63, 40.43, 34.54, 29.92, 29.90, 29.81, 29.75, 29.65, 29.52, 27.45, 25.35. LC-MS $[C_{27}H_{38}N_4O_3+H]^+$: 467.30 calculated, 467.40 found.

(*R*)-4-((Hexyloxy)methyl)-2,2-dimethyl-1,3-dioxolane (10)



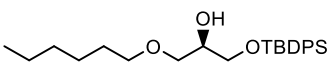
(*R*)-(2,2-Dimethyl-1,3-dioxolan-4-yl)methanol (50 mg, 0.38 mmol, 1 eq) was dissolved in anhydrous DMF (0.95 mL, 0.4 M) and cooled to 0 °C. NaH (60% w/w in mineral oil, 22.7 mg, 0.567 mmol, 1.5 eq) was added portion-wise and the mixture was stirred at 0 °C for 15 min. Subsequently, 1-bromohexane (63.7 µL, 0.454 mmol, 1.2 eq) was added and the mixture was stirred at 0 °C for 30 min after which it was allowed to warm to rt for 4 h. The reaction was quenched with MeOH (114 µL). The mixture was then diluted in water and extracted 3× with DCM. The combined organic layers were dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified by silica gel column chromatography (0-10% Et_2O in *n*-pentane) to afford the product as a colorless liquid (58 mg, 0.27 mmol, 71%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.27 (p, *J* = 6.0 Hz, 1H), 4.06 (dd, *J* = 8.2, 6.4 Hz, 1H), 3.73 (dd, *J* = 8.2, 6.4 Hz, 1H), 3.56 – 3.39 (m, 4H), 1.62 – 1.53 (m, 2H), 1.42 (s, 3H), 1.36 (s, 3H), 1.35 – 1.24 (m, 6H), 0.88 (t, *J* = 6.9, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 109.44, 74.85, 71.98, 71.91, 67.03, 31.76, 29.62, 26.86, 25.83, 25.52, 22.71, 14.13.

(*S*)-3-(Hexyloxy)propane-1,2-diol (11)



(*R*)-4-((Hexyloxy)methyl)-2,2-dimethyl-1,3-dioxolane (**10**, 58 mg, 0.27 mmol, 1 eq) and TsOH·H_2O (2.6 mg, 0.013 mmol, 0.05 eq) was dissolved in MeOH (0.67 mL, 0.4 M) at rt. The reaction mixture was stirred at 45 °C for 2 days. The reaction mixture was quenched with solid $NaHCO_3$ (4.5 mg, 0.053 mmol, 0.2 eq) and concentrated. The residue was purified by silica gel column chromatography (50-80% EtOAc in *n*-pentane) to afford the product as a colorless oil (35 mg, 0.20 mmol, 74%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.92 – 3.81 (m, 1H), 3.76 – 3.56 (m, 2H), 3.53 – 3.40 (m, 4H), 3.26 (d, *J* = 4.5 Hz, 1H), 2.98 (t, *J* = 5.9 Hz, 1H), 1.57 (p, *J* = 6.6 Hz, 2H), 1.39 – 1.20 (m, 6H), 0.89 (t, *J* = 7.0 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 72.44, 71.92, 70.72, 64.28, 31.74, 29.60, 25.81, 22.68, 14.12.

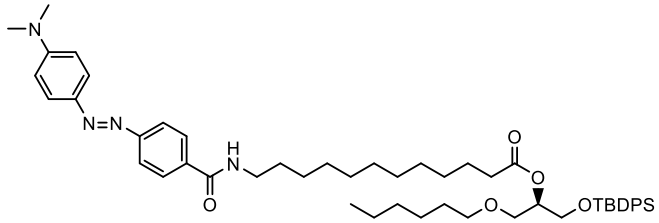
(*R*)-1-((*tert*-Butyldiphenylsilyl)oxy)-3-(hexyloxy)propan-2-ol (12)



To a solution of (*S*)-3-(hexyloxy)propane-1,2-diol (**11**, 53 mg, 0.30 mmol, 1 eq, co-evaporated with toluene twice before the reaction)

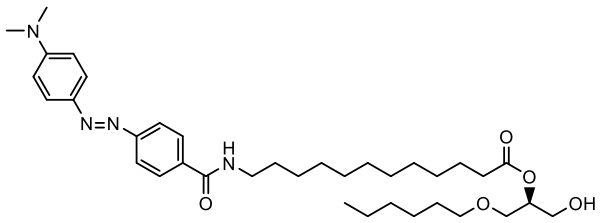
and 1*H*-imidazole (31 mg, 0.45 mmol, 1.5 eq) in anhydrous DMF (1.5 mL, 0.2 M) at 0 °C was added *tert*-butylchlorodiphenylsilane (86 µL, 0.33 mmol, 1.1 eq). The mixture was stirred at rt for 2 h. The mixture was diluted in water and extracted 3× with EtOAc. Combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified by silica gel column chromatography (0-5% EtOAc in *n*-pentane) to afford the product as a colorless oil (100 mg, 0.241 mmol, 80%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.69 – 7.64 (m, 4H), 7.45 – 7.34 (m, 6H), 3.88 (h, *J* = 5.4 Hz, 1H), 3.71 (d, *J* = 5.4 Hz, 2H), 3.54 – 3.45 (m, 2H), 3.43 (t, *J* = 6.8 Hz, 2H), 2.54 (d, *J* = 5.1 Hz, 1H), 1.59 – 1.51 (m, 2H), 1.34 – 1.23 (m, 6H), 1.06 (s, 9H), 0.88 (t, *J* = 7.0 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 135.67, 133.33, 129.88, 127.85, 71.76, 71.55, 70.85, 64.91, 31.81, 29.72, 26.95, 25.89, 22.72, 19.38, 14.18.

(*R*)-1-((*tert*-Butyldiphenylsilyl)oxy)-3-(hexyloxy)propan-2-yl (*E*)-12-(4-((4-(dimethylamino)phenyl)diazenyl)benzamido)dodecanoate (13)



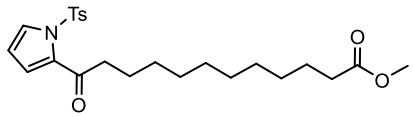
To a solution of (*E*)-12-(4-((4-(dimethylamino)phenyl)diazenyl)benzamido)dodecanoic acid (**9**, 68.0 mg, 0.146 mmol, 1 eq) and (*R*)-1-((*tert*-butyldiphenylsilyl)oxy)-3-(hexyloxy)propan-2-ol (**12**, 60.4 mg, 0.146 mmol, 1 eq) in anhydrous DCM (2 mL, 0.07 M) at 0 °C was added DIC (24.8 µL, 0.160 mmol, 1.1 eq) and DMAP (7.1 mg, 0.058 mmol, 0.4 eq). The mixture was allowed to warm to rt for overnight. The mixture was concentrated with Celite. The residue was purified by silica gel column chromatography (10-30% EtOAc in *n*-pentane) to afford the product as a red powder (64.5 mg, 75.0 µmol, 51%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.92 – 7.84 (m, 6H), 7.69 – 7.63 (m, 4H), 7.46 – 7.34 (m, 6H), 6.77 – 6.71 (m, 2H), 6.26 (t, *J* = 5.7 Hz, 1H), 5.12 (p, *J* = 5.1 Hz, 1H), 3.79 (d, *J* = 5.1 Hz, 2H), 3.60 (dd, *J* = 5.2, 1.8 Hz, 2H), 3.49 – 3.35 (m, 4H), 3.09 (s, 6H), 2.37 – 2.21 (m, 2H), 1.66 – 1.57 (m, 4H), 1.56 – 1.48 (m, 2H), 1.42 – 1.20 (m, 20H), 1.04 (s, 9H), 0.87 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.42, 167.14, 155.05, 152.87, 143.74, 135.70, 135.64, 134.98, 133.47, 133.42, 129.81, 129.79, 127.84, 127.79, 125.48, 122.32, 111.55, 72.84, 71.69, 69.00, 62.70, 40.39, 40.32, 34.57, 31.79, 29.81, 29.69, 29.64, 29.53, 29.46, 29.39, 29.25, 27.15, 26.84, 25.85, 25.09, 22.73, 19.36, 14.18.

(*S*)-1-(Hexyloxy)-3-hydroxypropan-2-yl (*E*)-12-(4-((4-(dimethylamino)phenyl)diazinyl)benzamido)dodecanoate (14)



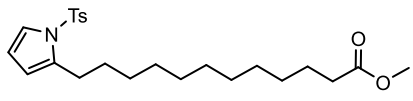
To a solution of (*R*)-1-((*tert*-butyldiphenylsilyl)oxy)-3-(hexyloxy)propan-2-yl(*E*)-12-(4-((4-(dimethylamino)phenyl)diazenyl)benzamido)dodecanoate (**13**, 65 mg, 0.075 mmol, 1 eq) in anhydrous THF (2.5 mL, 0.03 M) was added acetic acid (8.6 µL, 0.15 mmol, 2 eq) and TBAF (1 M in THF, 149 µL, 0.149 mmol, 2 eq) at 0 °C. The mixture was stirred at rt for overnight. The mixture was concentrated with Celite. The residue was purified by silica gel column chromatography (10-50% EtOAc in *n*-pentane) to afford the product as an orange-red oil (31 mg, 0.050 mmol, 66%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.91 – 7.84 (m, 6H), 6.78 – 6.72 (m, 2H), 6.31 (t, *J* = 5.6 Hz, 1H), 5.00 (p, *J* = 4.8 Hz, 1H), 3.80 (t, *J* = 4.6 Hz, 2H), 3.66 – 3.57 (m, 2H), 3.50 – 3.38 (m, 4H), 3.10 (s, 6H), 2.48 (t, *J* = 6.1 Hz, 1H), 2.35 (t, *J* = 7.9 Hz, 2H), 1.68 – 1.50 (m, 6H), 1.44 – 1.17 (m, 20H), 0.88 (m, *J* = 6.8 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.84, 167.19, 155.04, 152.87, 143.72, 134.96, 127.84, 125.48, 122.31, 111.55, 72.95, 71.97, 70.06, 62.98, 40.39, 40.31, 34.48, 31.73, 29.78, 29.60, 29.57, 29.55, 29.47, 29.40, 29.30, 29.15, 27.10, 25.80, 25.06, 22.70, 14.24.

Methyl 12-oxo-12-(1-tosyl-1*H*-pyrrol-2-yl)dodecanoate (15)



To a solution of 1-tosyl-1*H*-pyrrole (300 mg, 1.36 mmol, 1 eq) in anhydrous DCM (2.7 mL, 0.5 M) at 0 °C was added trifluoroacetic anhydride (942 µL, 6.78 mmol, 5 eq) followed by 12-methoxy-12-oxododecanoic acid (331 mg, 1.36 mmol, 1 eq). The reaction was heated to reflux for 40 h. The reaction was quenched with sat. $NaHCO_3$ and extracted 3× with DCM. Combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified by silica gel column chromatography (5-20% Et_2O in *n*-pentane) to afford the product as a white solid (425 mg, 0.950 mmol, 70%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.91 – 7.87 (m, 2H), 7.79 (dd, *J* = 3.2, 1.7 Hz, 1H), 7.33 – 7.28 (m, 2H), 7.03 (dd, *J* = 3.8, 1.7 Hz, 1H), 6.32 (t, *J* = 3.5 Hz, 1H), 3.66 (s, 3H), 2.68 – 2.63 (m, 2H), 2.41 (s, 3H), 2.30 (t, *J* = 7.5 Hz, 2H), 1.67 – 1.53 (m, 4H), 1.33 – 1.21 (m, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 189.18, 174.37, 144.71, 136.06, 133.45, 130.07, 129.37, 128.32, 123.25, 110.26, 51.49, 39.54, 34.14, 29.39, 29.36, 29.26, 29.20, 29.16, 24.99, 24.94, 21.73. LC-MS $[C_{24}H_{33}NO_5S+H]^+$: 448.22 calculated, 448.27 found.

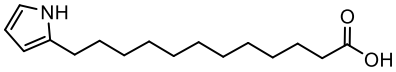
Methyl 12-(1-tosyl-1*H*-pyrrol-2-yl)dodecanoate (16)



To a solution of methyl 12-oxo-12-(1-tosyl-1*H*-pyrrol-2-yl)dodecanoate (**15**, 0.13 g, 0.29 mmol, 1 eq) in DCE (1.5

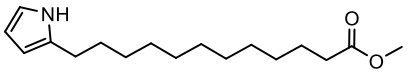
mL, 0.2 M) was added ZnI_2 (139 mg, 0.436 mmol, 1.5 eq) and $NaBH_3CN$ (137 mg, 2.18 mmol, 7.5 eq). The mixture was stirred at 80 °C for 4.5 h. The reaction was quenched with a solution of sat. aq. NH_4Cl and 6 M HCl (9:1, v:v, 10 mL), followed by extraction 3× with EtOAc. The combined organic layers were dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified by silica gel column chromatography (5-20% Et_2O in *n*-pentane) to afford the product as a white solid (89 mg, 0.21 mmol, 71%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.67 – 7.61 (m, 2H), 7.32 – 7.23 (m, 3H), 6.19 (t, *J* = 3.3 Hz, 1H), 6.00 – 5.96 (m, 1H), 3.67 (s, 3H), 2.63 (t, *J* = 7.7 Hz, 2H), 2.40 (s, 3H), 2.30 (t, *J* = 7.6 Hz, 2H), 1.61 (p, *J* = 7.1 Hz, 2H), 1.51 (p, *J* = 7.8 Hz, 2H), 1.35 – 1.19 (m, 14H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.49, 144.78, 136.68, 136.13, 130.05, 126.86, 122.26, 111.81, 111.34, 51.59, 34.24, 29.68, 29.64, 29.56, 29.51, 29.40, 29.39, 29.28, 28.75, 27.24, 25.09, 21.74. LC-MS $[C_{24}H_{35}NO_4S+H]^+$: 434.24 calculated, 434.27 found.

12-(1*H*-Pyrrol-2-yl)dodecanoic acid (17)



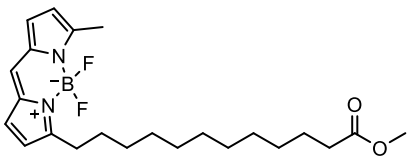
To a solution of methyl 12-(1-tosyl-1*H*-pyrrol-2-yl)dodecanoate (**16**, 289 mg, 0.667 mmol, 1 eq) in MeOH (2 mL, 0.33 M) was added 2 M aq. NaOH solution (1.67 mL, 3.33 mmol, 5 eq) and the mixture was stirred at 80 °C for 3 days. The mixture was diluted in 0.2 M aq. HCl solution and extracted 3× with EtOAc. Combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified by silica gel column chromatography (40-50% EtOAc in *n*-pentane) to afford the product as a white powder (140 mg, 0.527 mmol, 79%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.08 (bs, 1H), 7.93 (s, 1H), 6.68 – 6.59 (m, 1H), 6.12 (q, *J* = 2.8 Hz, 1H), 5.95 – 5.84 (m, 1H), 2.58 (t, *J* = 7.4 Hz, 2H), 2.34 (t, *J* = 7.5 Hz, 2H), 1.62 (h, *J* = 7.4 Hz, 4H), 1.38 – 1.19 (m, 14H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 180.38, 133.00, 116.08, 108.21, 104.93, 34.21, 29.78, 29.65, 29.63, 29.54, 29.50, 29.49, 29.33, 29.15, 27.84, 24.78. LC-MS $[C_{16}H_{27}NO_2+H]^+$: 266.21 calculated, 266.27 found.

Methyl 12-(1*H*-pyrrol-2-yl)dodecanoate (18)



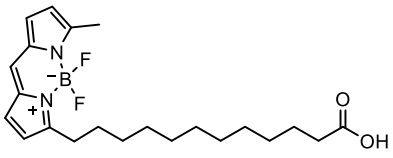
To a solution of 12-(1*H*-pyrrol-2-yl)dodecanoic acid (**17**, 76 mg, 0.29 mmol, 1 eq) and MeOH (116 µL, 2.86 mmol, 10 eq) in DCM (2.9 mL, 0.1 M) at 0 °C was added DIC (53.2 µL, 0.344 mol, 1.2 eq) and DMAP (21 mg, 0.17 mol, 0.6 eq). The mixture was allowed to warm to rt for overnight. The mixture was diluted water and extracted 3× with DCM. Combined organic layers were washed with 0.01 M aq. HCl solution, brine, dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified by silica gel column chromatography (5-10% EtOAc in *n*-pentane) to afford the product as a white solid (65 mg, 0.23 mmol, 81%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.01 (s, 1H), 6.67 – 6.60 (m, 1H), 6.12 (q, *J* = 2.9 Hz, 1H), 5.94 – 5.84 (m, 1H), 3.66 (s, 3H), 2.58 (t, *J* = 7.8 Hz 2H), 2.30 (t, *J* = 7.6 Hz, 2H), 1.67 – 1.55 (m, 4H), 1.39 – 1.21 (m, 14H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.49, 132.94, 116.04, 108.21, 104.86, 51.54, 34.19, 29.76, 29.61, 29.59, 29.50, 29.48, 29.45, 29.31, 29.21, 27.80, 25.03.

Methyl 12-(5,5-difluoro-7-methyl-5*H*-4λ^4,5λ^4-dipyrrolo[1,2-*c*:2',1'-*f*][1,3,2]diazaborinin-3-yl)dodecanoate (19)



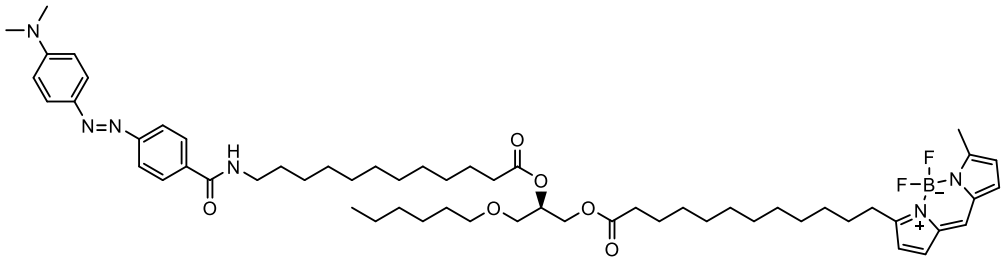
To a solution of methyl 12-(1*H*-pyrrol-2-yl)dodecanoate (**18**, 30.4 mg, 0.109 mmol, 1 eq) and 5-methyl-1*H*-pyrrole-2-carbaldehyde (13 mg, 0.12 mmol, 1.1 eq) in anhydrous DCM (1.1 mL, 0.1 M) was dropwise added $POCl_3$ (11.2 µL, 0.120 mmol, dissolved in anhydrous DCM) at 0 °C. The mixture was stirred at rt for 5 h. DIPEA (114 µL, 0.653 mmol, 6 eq) was added dropwise followed by stirring 20 min at 0 °C. Boron trifluoride diethyl etherate (81 µL, 0.65 mmol, 6 eq) was added subsequently at 0 °C. The reaction mixture was stirred at rt for overnight. More DIPEA (86 µL, 0.49 mmol, 4.5 eq) was added dropwise at 0 °C and stirred for 20 min. Subsequently, additional boron trifluoride diethyl etherate (60 µL, 0.49 mmol, 4.5 eq) were added at 0 °C. The mixture was stirred at rt for another overnight. The mixture diluted in brine and extracted 3× with DCM. Combined organic layers were dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified by silica gel column chromatography (10% Et_2O in *n*-pentane) to afford the product as a red oil (29 mg, 0.069 mmol, 64%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.05 (s, 1H), 6.95 (d, *J* = 4.2 Hz, 1H), 6.92 (d, *J* = 4.1 Hz, 1H), 6.33 (d, *J* = 4.2 Hz, 1H), 6.25 (d, *J* = 4.0 Hz, 1H), 3.66 (s, 3H), 2.99 (t, *J* = 7.9 Hz, 2H), 2.61 (s, 3H), 2.30 (t, *J* = 7.6 Hz, 2H), 1.73 (p, *J* = 7.5 Hz, 2H), 1.65 – 1.56 (m, 2H), 1.46 – 1.39 (m, 2H), 1.36 – 1.20 (m, 12H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 174.48, 163.52, 158.07, 134.71, 134.60, 130.27, 129.93, 126.94, 119.54 (d, $J_{C\text{-}F}$ = 2.9 Hz), 118.19 (d, $J_{C\text{-}F}$ = 3.0 Hz), 51.55, 34.25, 29.83, 29.70, 29.65, 29.61, 29.54, 29.36, 29.27, 28.98, 28.69, 25.09, 15.03.

12-(5,5-Difluoro-7-methyl-5*H*-4λ^4,5λ^4-dipyrrolo[1,2-*c*:2',1'-*f*][1,3,2]diazaborinin-3-yl)dodecanoic acid (20)



To a solution of methyl 12-(5,5-difluoro-7-methyl-5*H*-4λ^4,5λ^4-dipyrrolo[1,2-*c*:2',1'-*f*][1,3,2]diazaborinin-3-yl)dodecanoate (**19**, 38 mg, 0.091 mmol, 1 eq) was added 4 M aq. HCl solution in THF (4.5 mL, 198 eq). The mixture was stirred at rt for overnight. The mixture was diluted in water and extracted 3× with DCM. Combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified by silica gel column chromatography (0-3% MeOH in DCM) to afford the product as a red solid (29 mg, 0.072 mmol, 79%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.05 (s, 1H), 6.95 (d, *J* = 4.2 Hz, 1H), 6.92 (d, *J* = 4.1 Hz, 1H), 6.33 (d, *J* = 4.2 Hz, 1H), 6.25 (d, *J* = 4.1 Hz, 1H), 2.98 (t, *J* = 7.9 Hz, 2H), 2.61 (s, 3H), 2.34 (t, *J* = 7.5 Hz, 2H), 1.73 (p, *J* = 7.6 Hz, 2H), 1.63 (p, *J* = 7.5 Hz, 2H), 1.46 – 1.38 (m, 2H), 1.36 – 1.23 (m, 12H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 180.08, 163.53, 158.07, 134.71, 134.60, 130.28, 129.94, 126.95, 119.55 (d, $J_{C\text{-}F}$ = 3.4 Hz), 118.19 (d, $J_{C\text{-}F}$ = 3.0 Hz), 34.17, 29.70, 29.65, 29.60, 29.53, 29.35, 29.18, 28.98, 28.68, 24.81, 15.04.

(*R*)-1-((12-(5,5-Difluoro-7-methyl-5*H*-4λ^4,5λ^4-dipyrrolo[1,2-*c*:2',1'-*f*][1,3,2]diazaborinin-3-yl)dodecanoyl)oxy)-3-(hexyloxy)propan-2-yl (*E*)-12-(4-((4-(dimethylamino)phenyl)diazinyl)benzamido)dodecanoate (21, EnzChek lipase substrate)



To a solution of (*S*)-1-(hexyloxy)-3-hydroxypropan-2-yl (*E*)-12-(4-((4-(dimethylamino)phenyl)diazenyl)benzamido)dodecanoate (**14**, 31 mg, 0.050 mmol, 1 eq) and 12-(5,5-difluoro-7-methyl-5*H*-4λ^4,5λ^4-dipyrrolo[1,2-*c*:2',1'-*f*][1,3,2]diazaborinin-3-yl)dodecanoic acid **(20**, 22 mg, 0.055 mmol, 1.1 eq) in anhydrous DCM (2.5 mL, 0.02 M) at 0 °C was added DIC (8.5 µL, 0.055 mmol, 1.1 eq) and DMAP (2.4 mg, 0.020 mmol, 0.4 eq). The mixture was allowed to warm to rt for overnight. The mixture was diluted in water and extracted 3× with DCM. Combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated. The residue was purified by silica gel column chromatography (20-30 % EtOAc in *n*-heptane) to afford the product as an orange-red powder (36 mg, 0.036 mmol, 72%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.92 – 7.87 (m, 2H), 7.85 (s, 4H), 7.05 (s, 1H), 6.95 (d, *J* = 4.2 Hz, 1H), 6.92 (d, *J* = 4.1 Hz, 1H), 6.78 – 6.73 (m, 2H), 6.32 (d, *J* = 4.2 Hz, 1H), 6.25 (d, *J* = 4.1 Hz, 1H), 6.21 (t, *J* = 5.7 Hz, 1H), 5.24 – 5.16 (m, 1H), 4.33 (dd, *J* = 11.9, 3.7 Hz, 1H), 4.16 (dd, *J* = 11.9, 6.4 Hz, 1H), 3.57 – 3.50 (m, 2H), 3.49 – 3.37 (m, 4H), 3.10 (s, 6H), 2.98 (t, *J* = 7.9 Hz, 2H), 2.60 (s, 3H), 2.36 – 2.26 (m, 4H), 1.72 (p, *J* = 7.7 Hz, 2H), 1.63 – 1.50 (m, 6H), 1.46 – 1.21 (m, 36H), 0.88 (t, *J* = 6.9 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 173.61, 173.29, 167.14, 163.50, 158.07, 155.07, 152.91, 143.78, 135.01, 134.71, 134.60, 130.29, 129.95, 127.84, 126.95, 125.50, 122.34, 119.57 (d, $J_{C\text{-}F}$ = 3.4 Hz), 118.20 (d, $J_{C\text{-}F}$ = 3.4 Hz), 111.59, 71.87, 70.19, 69.06, 62.89, 40.42, 40.33, 34.48, 34.29, 31.76, 29.83, 29.73, 29.71, 29.65, 29.63, 29.60, 29.57, 29.48, 29.41, 29.39, 29.26, 29.20, 28.98, 28.69, 27.17, 25.82, 25.09, 25.05, 22.74, 15.04, 14.18. HRMS $[C_{58}H_{85}BF_2N_6O_6 + H]^+$: 1011.66741 calculated, 1011.66683 found.

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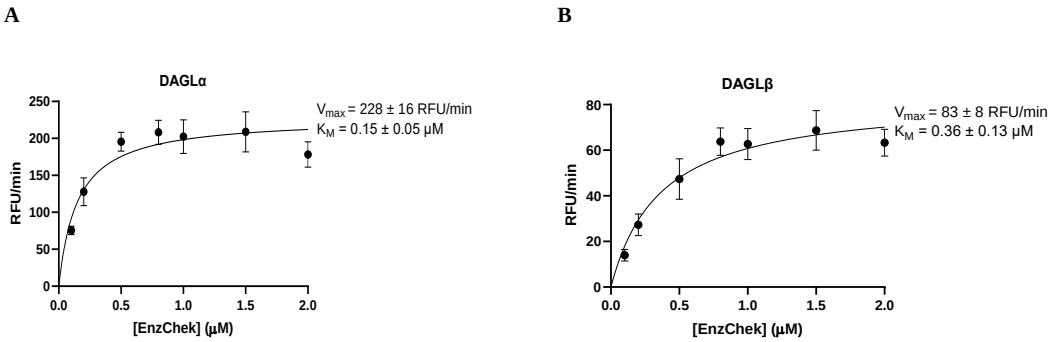
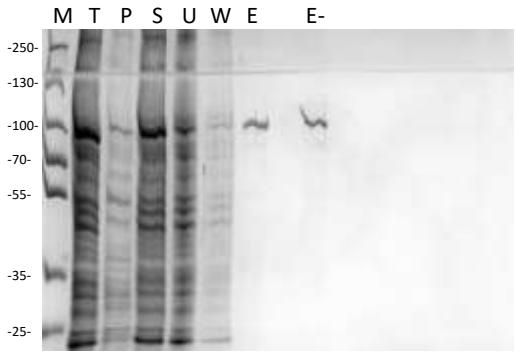
Supplementary Figures



Supplementary Figure S2.1 Michaelis-Menten kinetic curves. Rate of the synthesized EnzChek lipase substrate hydrolysis as a function of substrate concentration for DAGLα (A) and DAGLβ (B). All data were corrected for background fluorescence with mock membrane fraction observed at the same condition. Rates were determined in the linear interval of t = 10 min to t = 20 min. Data shown are mean ± SD (n = 5-6).



Supplementary Figure S2.2 Coomassie of different fractions during the purification of His-MBP-cdDAGLβ. Fractions on gel are **T**otal lysate, **P**ellet and **S**oluble fraction from lysate clearing, **U**nbound, the proteins that did not bind to the amylose resin, beads **W**ash fraction, the **E**luent with and without (**E-**) 2-mercaptoethanol in the sample buffer.