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Article

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Structure-activity relationship studies of α-ketoamides as inhibitors of the phospholipase A and acyltransferase (PLAAT) enzyme family

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Abstract

The phospholipase A and acyltransferase (PLAAT) family of cysteine hydrolases consists of five members, which are involved in the Ca^{2+}-independent production of *N*-acylphosphatidylethanolamines (NAPEs). NAPEs are lipid precursors for bioactive *N*-acylethanolamines (NAEs) that are involved in various physiological processes such as food intake, pain, inflammation, stress and anxiety. Recently, we identified α-ketoamides as the first pan-active PLAAT inhibitor scaffold that reduced arachidonic acid levels in PLAAT3-overexpressing U2OS cells and in HepG2 cells. Here, we report the structure-activity relationships of the α-ketoamide series using activity-based protein profiling. This led to the identification of **LEI-301**, a nanomolar potent inhibitor for the PLAAT family members. **LEI-301** reduced NAE levels, including anandamide, in cells overexpressing PLAAT2 or PLAAT5. Collectively, **LEI-301** may help to dissect the physiological role of the PLAATs.

Introduction

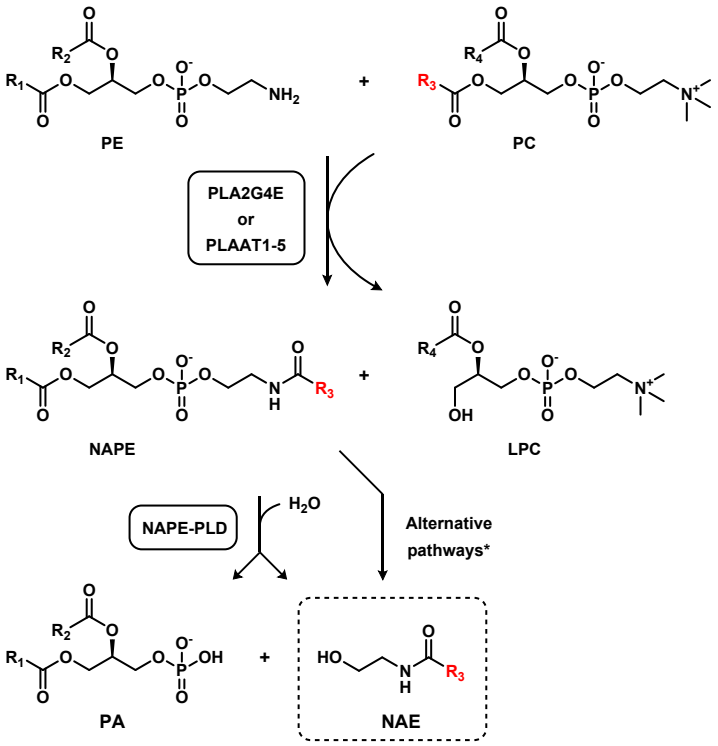
The subfamily of phospholipase A and acyltransferases (PLAATs) consists of five members with reported roles in tumor suppression and phospholipid metabolism.[1,2] They belong to the lecithin retinol acyltransferase (LRAT) protein family that is part of the NlpC/P60 superfamily of thiol hydrolases. The LRAT family has a conserved catalytic motif of six amino acids (NCEHFV) containing a cysteine residue that acts as the active site nucleophile.[3] A C-terminal hydrophobic tail is shared by PLAAT1-4, serving as a single-pass transmembrane anchoring domain.[4] The physiological functions of the PLAAT family members are only partly understood.[5] PLAATs are multifunctional enzymes that display varying degrees of *N*- and *O*-acyltransferase or phospholipase $A_{1/2}$ activity *in vitro*.[5-11] In addition, PLAAT4 (also known as RARRES3, HRASLS4, TIG3 or RIG1) is involved in protein deacylation of WNT and H-RAS proteins in breast cancer cells, thereby controlling cell growth.[12] Biological functions have been described for PLAAT3 (also known as PLA2G16, HRASLS3, AdPLA or HREV107), which primarily acts as a phospholipase $A_{1/2}$ and regulates lipolysis in adipose tissue.[4,10,13] Notably, *Plaat3* knockout mice were protected against diet-induced obesity.[12] Recently, PLAAT3 was reported to be a host factor for picornaviridae by facilitating the entry of viral RNA into the cytosol from virus-containing endosomes.[14,15] As such, inhibitors of PLAAT3 hold promise as anti-obesity or anti-viral agents.

Less is known about the other PLAAT enzymes. Ueda and co-workers reported that *in vitro,* PLAAT2 (also known as HRASLS2) displays the highest *N*-acyltransferase activity of all PLAATs, followed at some distance by PLAAT5 (also known as iNAT or HRASLS5).[16] This reaction involves the transfer of an acyl group from phosphatidylcholine (PC) to phosphatidylethanolamine (PE), generating *N*-acylphosphatidylethanolamine (NAPE) and lyso-phosphatidylcholine (LPC) (Scheme 1). Firstly, the PLAAT enzyme forms an acyl thioester intermediate using its Cys-His-His catalytic triad, thereby expelling LPC. This is followed by nucleophilic attack of the PE-amino group, producing the NAPE product and liberating the catalytic cysteine. NAPEs are an underexplored class of triacylated phospholipids that serve as precursors for *N*-acylethanolamines (NAEs), an important family of signaling molecules that includes the endocannabinoid anandamide (*N*-arachidonoylethanolamine, AEA).[17] Through activation of the cannabinoid CB_1 receptor, anandamide is involved in physiological processes such as appetite, pain sensation, memory formation, stress and anxiety.[18-20] The canonical enzyme responsible for NAPE biosynthesis in the brain is a Ca^{2+}-dependent *N*-acyltransferase (Ca-NAT), recently identified as PLA2G4E.[21] NAPEs are in turn converted to NAEs in one step by NAPE-

phospholipase D (NAPE-PLD) as well as other multistep pathways.[5] In contrast, the PLAAT family members operate via a calcium-independent mechanism, providing an alternative pathway through which NAPEs and NAEs are biosynthesized.[16] PLAAT2 was reported to preferably transfer the sn-1 over the sn-2 acyl group of PC, which suggests that it mostly generates saturated or mono-unsaturated NAEs.[16] Furthermore, HEK293 cells stably overexpressing PLAAT2 exhibited highly increased NAPE and NAE levels. Gene expression of *PLAAT2* was found in the lung, liver, kidney, small intestine, colon, testis and trachea.[9,22] NAEs have well-established signaling roles in the gastrointestinal system.[23] For instance, *N*-oleoylethanolamide (OEA) was found to act as a satiety factor via activation of peroxisome proliferator-activated receptor (PPAR)-α.[24] This raises the possibility that PLAAT2 is involved in NAE biosynthesis in the gut. Notably, rodents lack the gene that encodes PLAAT2, thereby hindering the development of genetic models.[9]

As of yet, no physiological functions are known for PLAAT5. Of all PLAAT family members, PLAAT5 does not have a reported tumor suppressing role.[5] High gene expression levels were found in testis of mice, rats and humans as well as in human pancreas.[8,25] PLAAT5 activity was mainly localized to the cytosol fraction, while an inactive form of the enzyme was found to be membrane associated.[8] *In vitro*, PLAAT5 displayed higher *N*-acyltransferase than phospholipase $A_{1/2}$ activity.[16] Importantly, compared to PLAAT2, PLAAT5 showed no preference for the sn-1 or sn-2 acyl group of PC, suggesting that it could be involved in *N*-arachidonoyl-PE and thus anandamide biosynthesis.[6,8] PLAAT inhibitors would be valuable pharmacological tools to study the biological role of PLAAT2 and PLAAT5.

Recently, we described the discovery of α-ketoamide **LEI-110** as the first pan-active PLAAT inhibitor using a gel-based competitive activity-based protein profiling (ABPP) assay.[26] **LEI-110** was able to reduce arachidonic acid levels in PLAAT3-overexpressing cells and in HepG2 cells. Here, we report the structure-activity relationship of a library of α-ketoamides on the PLAAT family members. Next to **LEI-110**, we also identified **LEI-301** as a nanomolar potent PLAAT2 inhibitor with similar potency for other members of the PLAAT family, which was selective over the proteins of the endocannabinoid system (ECS). **LEI-301** reduced NAE levels in PLAAT2- and PLAAT5-overexpressing cells, but not in control cells. These findings show that **LEI-301** is a new pharmacological tool to study the biological role of PLAATs in cellular systems.



Scheme 1. Biosynthesis of *N*-acylphosphatidylethanolamines (NAPEs) and *N*-acylethanolamines (NAEs). The sn-1 acyl group of phosphatidylcholine (PC) is transferred to the amine of phosphatidylethanolamine (PE) by the acyltransferase activity of PLA2G4E or PLAAT1-5 forming *N*-acyl-PE (NAPE) and lyso-PC (LPC). NAPE-PLD hydrolyzes the phosphodiester bond of NAPE to form NAE and phosphatidic acid (PA). R_1, R_2 and R_3 denote saturated, mono- or polyunsaturated fatty acids. * For the alternative pathways see ref. 5.

Results & Discussion

Screening for PLAAT inhibitors using competitive ABPP.

A focused in-house library of lipase inhibitors was screened for PLAAT inhibition using gel-based competitive ABPP.[26] This method uses an activity-based probe (ABP) containing an electrophilic group that forms a covalent bond with the catalytic nucleophile of an enzyme.[27] ABPs are also equipped with a reporter group such as a fluorophore or biotin, which allows visualization of enzymatic activity in a native biological setting. In a competitive ABPP experiment, potential inhibitors are pre-treated with a cell lysate that contains the protein of interest followed by incubation with an ABP. After resolving the proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and in-gel fluorescence scanning, the residual enzymatic activity can be determined by measuring the probe labeling intensity. Previously, MB064[28], which incorporates a β-

lactone electrophilic group, was validated as an effective ABP for the PLAAT enzyme family.[26] Here, cytosol fractions of human embryonic kidney HEK293T cells that overexpressed PLAAT2-5 were treated with potential inhibitors (10 μM, 30 min), followed by incubation with MB064 (250 nM, 20 min) (Figure 1A). The proteins were then resolved by SDS-PAGE and PLAAT activity was quantified by in-gel fluorescence scanning. A compound that showed residual protein labeling of ≤ 50% was considered to be active. For these active compounds an IC_{50} curve was generated using a dose-response ABPP experiment (Figure 1B). Data for PLAAT2, PLAAT3, PLAAT4 and PLAAT5 is reported in Table 1 as pIC_{50} ± SEM (n = 3). Unfortunately, PLAAT1 could not be tested due to a lack of protein expression in HEK293T cells. α-Ketoamides **1** and **2** were identified as submicromolar hits, showing similar potency for all tested PLAAT members. An early structure-activity relationship emerged from the structurally similar keto- and hydroxyamides (**3**-**22**) present in this focused screening library. The α-position of the ketone next to the amide was essential for binding (compare α-ketoamides **1** and **2** with β-ketoamides **5-8**). β-Hydroxyamides (**3** and **4**) were inactive. Removing the alkyl spacer (**11**) was also detrimental for activity. Furthermore, the phenethylamine of **1** was preferred over benzylamine (**9**) and ethylamine (**10**). *N*-methylation resulted in complete loss of activity (**12**), which suggested that the N-H is potentially involved in hydrogen bond formation or that the methyl group has a steric clash with the protein. Similarly, secondary amides incorporating either (hetero)cyclic (**13**, **15**, **16**, **18-21**) or acyclic (**14**, **17**, **22**) motifs, did not show any activity.

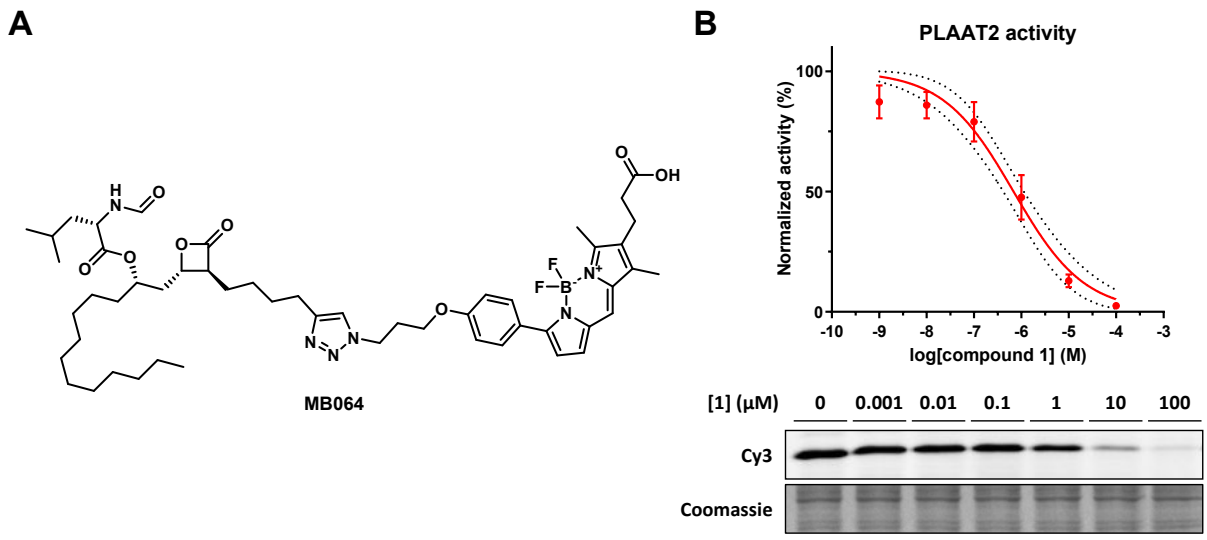
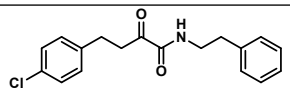
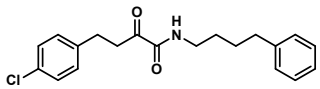
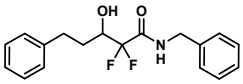
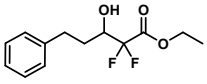
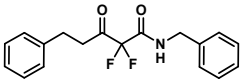
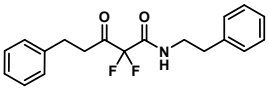
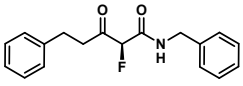
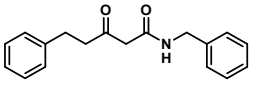
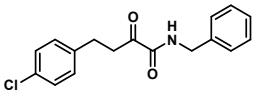
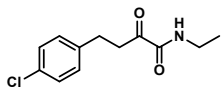
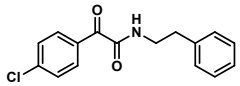
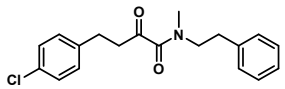
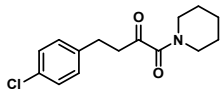
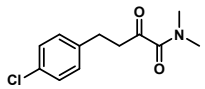
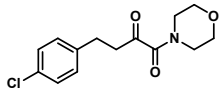
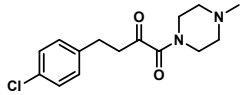
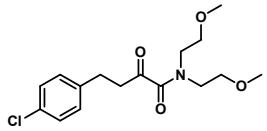
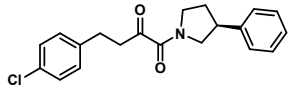
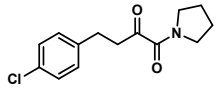
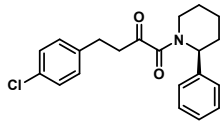
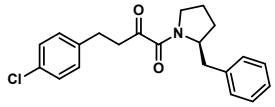
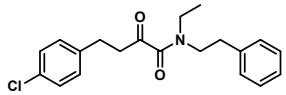


Figure 1. Evaluating PLAAT activity using competitive activity-based protein profiling (ABPP). A) Structure of broad-spectrum lipase probe MB064. **B**) Representative gel and apparent IC_{50} curve of a competitive ABPP experiment for PLAAT2. Labeling by MB064 and dose-dependent inhibition by **1** (apparent pIC_{50} = 6.2 ± 0.1, dotted lines show 95% confidence interval). Data represent mean values ± SEM (n = 3). Coomassie staining was used as a protein loading control.

Table 1. Structure-activity relationship (SAR)-analysis of keto- and hydroxyamides **1**-**22**.

ID	Structure	apparent pIC_{50} ± SEM			
		PLAAT2	PLAAT3	PLAAT4	PLAAT5
1		6.2 ± 0.1	6.0 ± 0.1	5.9 ± 0.1	6.4 ± 0.1
2		6.2 ± 0.1	6.0 ± 0.1	5.8 ± 0.1	6.7 ± 0.1
3		< 5	< 5	< 5	< 5
4		< 5	< 5	< 5	< 5
5		< 5	< 5	< 5	< 5
6		< 5	< 5	< 5	< 5
7		< 5	< 5	< 5	< 5
8		< 5	< 5	< 5	< 5
9		5.6 ± 0.1	5.8 ± 0.1	5.6 ± 0.1	6.6 ± 0.1

10		5.6 ± 0.1	5.1 ± 0.1	5.4 ± 0.1	6.0 ± 0.1
11		< 5	< 5	< 5	< 5
12		< 5	< 5	< 5	< 5
13		< 5	< 5	< 5	< 5
14		< 5	< 5	< 5	< 5
15		< 5	< 5	< 5	< 5
16		< 5	< 5	< 5	< 5
17		< 5	< 5	< 5	< 5
18		< 5	< 5	< 5	< 5
19		< 5	< 5	< 5	< 5
20		< 5	< 5	< 5	< 5
21		< 5	< 5	< 5	< 5
22		< 5	< 5	< 5	< 5

Evaluation of an α-ketoamide inhibitor library delivers nanomolar hit LEI-301.

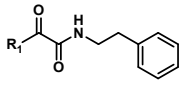
α-Ketoamide **1** exhibited the smallest molecular weight (MW = 316) and highest overall potency for the PLAAT enzymes, therefore this compound was resynthesized and its activity was confirmed on all PLAAT members with a pIC_{50} ranging from 6.0-6.4 (Table 2). It was envisioned that the electrophilic ketone of **1** could bind with the PLAAT active site cysteine through a reversible covalent mechanism forming a hemithioketal adduct, similar to other reported α-ketoamide inhibitors.[26,29] To test this hypothesis, compound **23** was

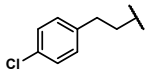
prepared in which the ketone was replaced by an alcohol. This compound showed no activity at 10 μM (Table 2), which is in line with the hypothesis.

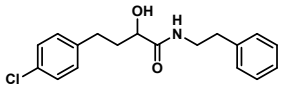
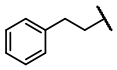
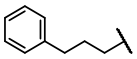
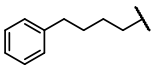
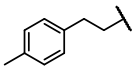
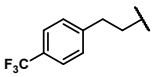
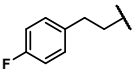
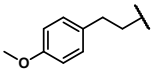
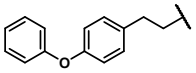
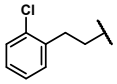
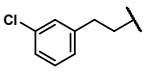
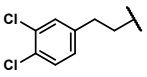
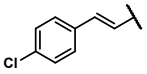
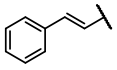
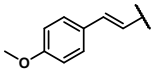
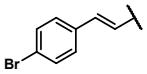
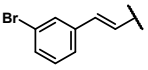
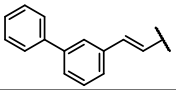
To systematically investigate the SAR and improve the potency of **1**, R_1-ketone and R_2-phenethyl analogues were synthesized (compounds **24**-**56**) (Table 2 and 3). First, the effect of various substitutions on the R_1-group of **1** was evaluated with derivatives **24**-**36** (Table 2). Removal of the chlorine (**24**) was detrimental for the activity for PLAAT2-4, but not PLAAT5. The length of the alkyl chain was studied in analogues **24**-**27**, showing that propylene derivative **25** was optimal, which had increased potency for PLAAT2, PLAAT3 and PLAAT5. The 4-chloro substituent on the phenyl ring seemed to be optimal based on the inhibitory activity of compounds **29**-**33**. Electron-donating groups such as 4-methyl (**29**) and 4-methoxy (**32**) substituents decreased potency, but a lipophilic electron withdrawing group (*e.g.* 4-trifluoromethyl, **30**) was tolerated for PLAAT5. A small (4-fluoro, **31**) substituent lowered the activity, while a large group (4-phenoxy, **33**) provided a selectivity window for PLAAT5 over the other PLAATs of 10- to 30-fold. Furthermore, substitution of the 4-chloro to the *ortho* or *meta* position, did not result in improved potency (compounds **34** and **35**). The 3,4-dichloro derivative **36**, however, presented increased activity for PLAAT3 and PLAAT5. Taken together, these compound series point towards the presence of a small lipophilic pocket, restricted in size, which is occupied by the alkylphenyl group.

Next, β,γ-unsaturated α-ketoamides **37**-**42** were evaluated to test whether conformational restriction of the alkyl linker would lead to a gain in activity (Table 2). Although unsaturation was tolerated in the alkyl chain, no or little improvement in potency was observed for these derivatives (compare compounds **1**, **24**, **32** and **37**-**39**). Also 4-bromo, 3-bromo or 3-phenyl substitutions (**40**-**42**), did not provide the desired inhibitory activity increase. Overall, this suggests that the R_1 group is positioned towards a shallow pocket.

Table 2. Structure-activity relationship (SAR)-analysis of α-ketoamide analogues **1, 23-42**.



ID	R_1:	cLogP[a]	apparent pIC_{50} ± SEM			
			PLAAT2	PLAAT3	PLAAT4	PLAAT5
1		4.37	6.2 ± 0.1	6.0 ± 0.1	6.2 ± 0.1	6.4 ± 0.1

23		3.83	< 5	< 5	< 5	< 5
24		3.66	< 5	< 5	5.5 ± 0.1	6.0 ± 0.1
25		4.04	6.3 ± 0.1	6.4 ± 0.1	6.2 ± 0.1	7.0 ± 0.1
26		4.57	5.5 ± 0.1	6.0 ± 0.1	5.8 ± 0.1	6.8 ± 0.1
27		3.04	< 5	< 5	< 5	< 5
28		1.56	< 5	< 5	< 5	< 5
29		4.16	5.3 ± 0.1	5.7 ± 0.1	5.8 ± 0.1	5.6 ± 0.1
30		4.54	5.7 ± 0.1	5.1 ± 0.1	5.6 ± 0.1	6.5 ± 0.1
31		3.80	5.1 ± 0.1	5.3 ± 0.1	5.0 ± 0.1	5.8 ± 0.1
32		3.58	5.5 ± 0.1	5.5 ± 0.1	5.9 ± 0.1	5.9 ± 0.1
33		5.76	5.4 ± 0.1	5.4 ± 0.1	5.9 ± 0.1	6.9 ± 0.1
34		4.37	5.9 ± 0.1	5.7 ± 0.1	5.6 ± 0.1	5.8 ± 0.1
35		4.37	5.6 ± 0.1	5.8 ± 0.1	5.9 ± 0.1	5.9 ± 0.1
36		4.97	5.7 ± 0.2	7.0 ± 0.2	6.0 ± 0.1	7.0 ± 0.1
37		4.37	5.8 ± 0.1	5.6 ± 0.1	5.6 ± 0.1	6.2 ± 0.1
38		3.66	5.1 ± 0.1	5.8 ± 0.1	5.0 ± 0.1	5.2 ± 0.2
39		3.58	5.8 ± 0.1	5.3 ± 0.1	5.6 ± 0.1	6.0 ± 0.1
40		4.52	5.3 ± 0.1	5.6 ± 0.1	5.2 ± 0.1	6.2 ± 0.1
41		4.52	< 5	< 5	5.1 ± 0.1	5.5 ± 0.1
42		5.55	5.6 ± 0.1	5.5 ± 0.1	5.6 ± 0.1	6.0 ± 0.1

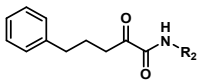
[a] cLogP was calculated using Chemdraw 15.

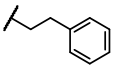
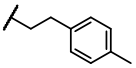
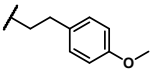
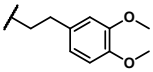
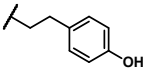
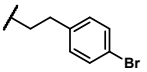
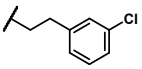
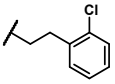
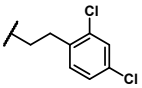
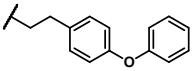
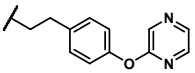
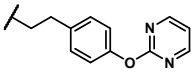
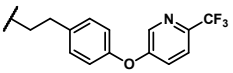
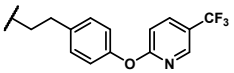
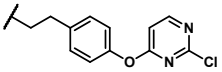
Next, the focus was shifted towards the optimization of the R_2-phenethyl moiety. Analogues **43**-**56** incorporating substituted phenethylamines were prepared in combination with the 2-oxo-5-phenylpentanoyl motif of compound **25**, which demonstrated the highest PLAAT2 activity (Table 3). The compounds showed a

comparable SAR on PLAAT2 and PLAAT4, as substitutions on the *para* position were unfavorable for methyl (**43**), methoxy (**44**) and hydroxyl (**46**). Substitutions on the *meta* and *ortho* position (**48**, **49**) or 2,4-disubstituted methoxy (**45**) or chloro (**50**) also did not afford an improvement in potency. Increasing the lipophilicity gave a 2- to 3-fold increase in activity for 4-bromo analog **47**. Further expansion with a 4-phenoxy moiety (**51**) improved the potency both for PLAAT2 and PLAAT4 with 10-fold compared to **25**. Introduction of a 4-methyl group in compound **43** enhanced the inhibitory activity for PLAAT3 and PLAAT5. Other lipophilic groups (4-bromo (**47**) and 4-phenoxy (**51**)) gave even higher activities for PLAAT5 especially.

Addition of the phenoxy group raised the cLogP of **51** with two log units to 6.14 (calculated with Chemdraw 15), therefore more polar heteroaryl rings were investigated to lower the lipophilicity (**52-56**) (Table 3). Compound **55** (**LEI-110**) was identified as the most potent inhibitor of PLAAT3 and its biological characterization has previously been described in detail.[26] With regard to PLAAT2, a decrease in activity was observed for compounds **52-56** compared to **51**. Therefore, being the most potent inhibitor of PLAAT2, **51** (termed **LEI-301**) was selected for further characterization.

Table 3. SAR-analysis of phenethyl analogues **25**, **43-56**.



ID	R_2:	cLogP[a]	apparent pIC_{50} ± SEM			
			PLAAT2	PLAAT3	PLAAT4	PLAAT5
25		4.04	6.3 ± 0.1	6.4 ± 0.1	6.2 ± 0.1	7.0 ± 0.1
43		4.54	5.7 ± 0.1	6.9 ± 0.1	5.9 ± 0.1	7.4 ± 0.1
44		3.96	5.8 ± 0.1	6.1 ± 0.1	5.8 ± 0.1	7.1 ± 0.1
45		3.70	5.3 ± 0.1	6.0 ± 0.2	5.2 ± 0.1	6.5 ± 0.1
46		3.37	5.9 ± 0.1	5.8 ± 0.1	5.6 ± 0.1	6.8 ± 0.1
47		4.90	6.6 ± 0.1	6.8 ± 0.1	6.7 ± 0.1	8.0 ± 0.1
48		4.75	6.1 ± 0.1	6.7 ± 0.1	6.0 ± 0.1	7.3 ± 0.1
49		4.75	5.2 ± 0.1	5.9 ± 0.1	5.6 ± 0.1	6.1 ± 0.2
50		5.47	5.9 ± 0.1	6.6 ± 0.1	6.1 ± 0.1	7.2 ± 0.1
51 (LEI-301)		6.14	7.3 ± 0.1	6.6 ± 0.1	7.3 ± 0.2	7.4 ± 0.1
52		3.68	6.3 ± 0.1	6.6 ± 0.1	6.2 ± 0.1	7.2 ± 0.1
53		3.68	5.7 ± 0.1	5.7 ± 0.1	5.9 ± 0.1	6.6 ± 0.1
54		5.67	6.7 ± 0.1	6.8 ± 0.1	7.1 ± 0.1	7.1 ± 0.1
55 (LEI-110)		5.67	6.8 ± 0.1	7.0 ± 0.1	6.2 ± 0.1	7.5 ± 0.1
56		4.44	6.2 ± 0.1	6.6 ± 0.1	6.7 ± 0.1	7.1 ± 0.1

[a] cLogP was calculated using Chemdraw 15.

***In silico* modeling of α-ketoamides inhibitors.**

To explain the binding mode of the α-ketoamides inhibitors in PLAAT2, **LEI-301** and **1** were docked in a PLAAT2 crystal structure (PDB: 4DPZ).[4] Residues 39-52 and 105-111 were absent from this structure, therefore a homology model was prepared using the closely related PLAAT3 crystal structure (PDB: 4DOT)[4] from which the shape of the loop for residues 105-111 could be adopted. A second loop comprising residues 39-52 was modeled based on sequence, as it is not present in both crystal structures. Since our data suggested that the electrophilic ketone of α-ketoamides could engage with the active site cysteine through a reversible covalent mechanism[29], **LEI-301** and **1** were covalently docked to Cys113 in the enzyme (Figure 2). Both compounds revealed a hydrogen bonding network between the oxy-anion and amide carbonyl with His23 and the Trp24 backbone amide N-H, while the backbone carbonyl of Leu108 formed a H-bond with the amide of the inhibitors. Introduction of the 4-phenoxy group in **LEI-301** suggested that an additional π-π stacking interaction with Tyr21 would be possible. This offered a potential reason for the observed activity increase of **LEI-301**.

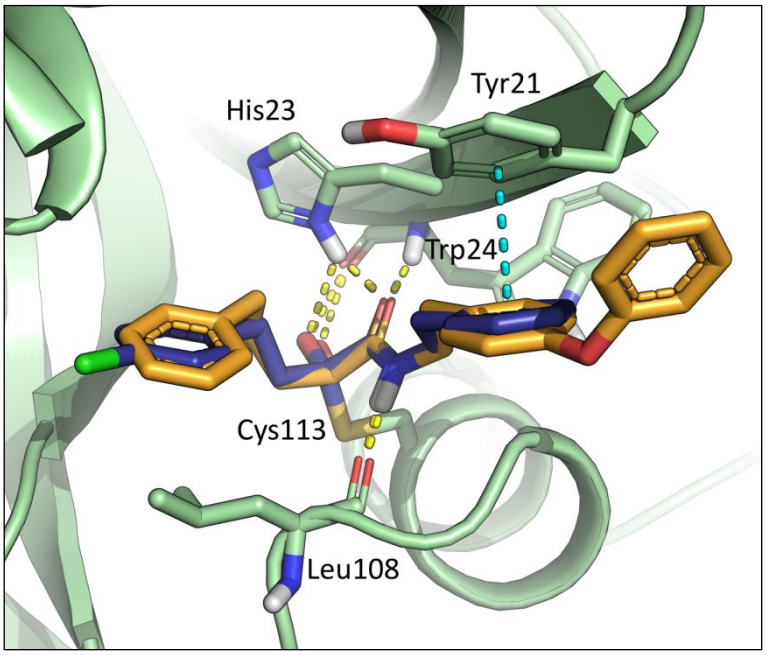


Figure 2. Docking pose of LEI-301 and 1 with PLAAT2. Compounds **1** (blue) and **LEI-301** (orange) in complex with PLAAT2, covalently bound to Cys113. Yellow dotted lines represent a hydrogen bond, cyan represent π-interactions.

Selectivity profile of LEI-301 for the endocannabinoid system.

The affinity or activity of **LEI-301** for the receptors and metabolic enzymes of the endocannabinoid system (ECS) was determined to assess its selectivity profile. Minimal affinity (< 50 %) was observed at 10 μM for the cannabinoid receptors type 1 and 2 (CB_1/CB_2) (Table 4). The enzymes involved in NAE biosynthesis (PLA2G4E, NAPE-PLD) and degradation (FAAH) were also not inhibited at this concentration (Table 5). Enzymes involved in the metabolism of the other endocannabinoid 2-arachidonoylglycerol (2-AG), such as diacylglycerol lipase α and β (DAGLα/β), monoacylglycerol lipase (MAGL) and α,β-hydrolase domain containing 6 (ABHD6) were not inhibited.

Table 4. Affinity of **LEI-301** for cannabinoid receptors CB_1 and CB_2.

Radioligand displacement at 10 μM LEI-301 (% ± SD; N = 2, n = 2)	
hCB_1	hCB_2
49 ± 8	32 ± 4

Table 5. Inhibitory activities of **LEI-301** for metabolic enzymes of the endocannabinoid system (ECS).

Remaining enzyme activity at 10 μM LEI-301 (% ± SD; n = 3)						
hNAPE-PLD	hPLA2G4E	mDAGLα	mDAGLβ	hMAGL	mFAAH	mABHD6
92 ± 8	95 ± 5	97 ± 10	83 ± 1	105 ± 19	108 ± 4	92 ± 5

Activities were obtained from surrogate (hNAPE-PLD) or natural (hMAGL) substrate assays. hPLA2G4E, mDAGLα/β, mFAAH and mABHD6 were determined by gel-based ABBP.

Targeted lipidomics shows that LEI-301 reduces NAEs in PLAAT2-overexpressing cells.

Having established that **LEI-301** is a potent inhibitor of PLAAT2 and selective over the other enzymes of the ECS, it was investigated whether **LEI-301** is active in a cellular setting. To this end, NAE levels, which are downstream metabolites of NAPEs generated by NAPE-PLD, were measured in living cells. Human U2OS osteosarcoma cells were therefore transiently transfected with a pcDNA3.1 plasmid containing the gene for PLAAT2, PLAAT5 or an empty (mock) vector. Of note, mRNA of NAPE-PLD was detected by quantitative PCR (qPCR) in this cell line, suggesting that NAPEs can be converted to NAEs (*NAPEPLD*: quantification cycle C_q ± SEM = 27.3 ± 0.05, *RPS18* (housekeeping gene): C_q ± SEM = 17.8 ± 0.01, n = 3; the presence of reference gene mRNA in combination with a $C_q \leq 29$ for the targeted mRNA is considered sufficient[30]). Targeted lipidomics on the lipid extracts of the transfected cells allowed the quantification of eight different NAEs and ten free fatty

acids (FFAs) by liquid chromatography-mass spectrometry (LC-MS). A striking increase of 9- to 99-fold for all NAE species was observed for the PLAAT2-overexpressing cells compared to control, including anandamide (AEA, 54-fold) (Figure 3A, Table 6). This result confirms previous findings.[16] Notably, PLAAT2 overexpression did not elevate most fatty acid species except for arachidonic acid (fold change ± SD = 1.81 ± 0.45, P = 0.04), while levels of γ-linolenic (18:3-ω6) and linoleic acid (18:2-ω6) were significantly decreased (Figure 3A, Table S1). In PLAAT5-transfected cells also a significant increase of NAE content was observed, although smaller in magnitude compared to PLAAT2 (Figure 3B, Table 6). Interestingly, *N*-docosahexaenoylethanolamine (DHEA) levels were unaffected by overexpression of PLAAT5. Furthermore, fatty acid levels including arachidonic acid, were not elevated for this enzyme (Figure 3B, Table S1). Next, the PLAAT2/PLAAT5 or mock-transfected cells were incubated with 10 μM **LEI-301** for 4 h. A significant 2-fold reduction of anandamide was apparent in the PLAAT2 and PLAAT5 cells, which was absent in the control samples (for PLAAT2: P = 0.006; for PLAAT5: P < 0.0001) (Figure 3C,F). Other saturated, mono- and polyunsaturated NAEs also showed significant reductions upon treatment with **LEI-301** in the PLAAT2 and PLAAT5 overexpressing cells but not in the mock cells (Figure 3F, Figure S1). **LEI-301** did reduce arachidonic acid levels in PLAAT2-transfected cells, however this did not meet significance (P = 0.06) (Figure 3E). In the case of PLAAT5, DHEA as well as arachidonic acid levels were not affected by treatment with **LEI-301** (Figure 3E,F). Notably, **LEI-301** did not alter levels of the other endocannabinoid 2-AG in all tested conditions (Figure 3D). Previously, α-ketoamides (also termed 2-oxo-amides) were reported as inhibitors of various PLA_2 enzymes.[29,31-34] To test the selectivity of **LEI-301** in U2OS cells, we performed competitive ABPP experiments in membrane and cytosol fractions with broad-spectrum lipase probes FP-TAMRA and MB064. No inhibitory activity was observed for **LEI-301** at 10 μM for any of the probe-labelled enzymes (Figure S2A). In addition, members of the PLA2G4 family were also not inhibited by **LEI-301** (Figure S2B-C). Taken together, these results indicate that **LEI-301** can be used to study the biological role of PLAAT2 and PLAAT5 in cellular systems.

Table 6. PLAAT2 and PLAAT5 overexpression greatly increases NAE content in U2OS cells. Data represent mean values ± SD for 4 biological replicates. *P*-values were determined by one-way ANOVA.

NAE	Absolute NAE levels (pmol/10^6 cells ± SD)			Fold change ± SD		*P*-value	
	mock	PLAAT2	PLAAT5	PLAAT2/ mock	PLAAT5/ mock	PLAAT2	PLAAT5
PEA (16:0)	0.196 ± 0.05	18.62 ± 5.97	0.569 ± 0.10	95 ± 30	2.9 ± 0.5	0.0008	0.0005
POEA (16:1)	0.031 ± 0.01	2.247 ± 0.78	0.093 ± 0.01	73 ± 25	3.0 ± 0.4	0.0013	0.0001
SEA (18:0)	0.516 ± 0.12	47.39 ± 13.1	2.916 ± 0.42	92 ± 25	5.7 ± 0.8	0.0004	< 0.0001
OEA (18:1)	0.190 ± 0.05	18.83 ± 5.02	1.027 ± 0.17	99 ± 26	5.4 ± 0.9	0.0003	0.0001
LEA (18:2)	0.047 ± 0.01	1.569 ± 0.50	0.121 ± 0.01	33 ± 10	2.6 ± 0.3	0.0009	0.0002
AEA (20:4)	0.040 ± 0.01	2.194 ± 0.79	0.337 ± 0.09	54 ± 20	8.4 ± 2.2	0.0016	0.0005
EPEA (20:5)	0.010 ± 0.01	0.555 ± 0.15	0.059 ± 0.01	53 ± 14	5.7 ± 0.8	0.0004	< 0.0001
DHEA (22:6)	0.032 ± 0.01	0.286 ± 0.11	0.027 ± 0.01	8.9 ± 3.4	0.84 ± 0.1	0.0034	0.1886

Abbreviations: PEA = *N*-palmitoylethanolamine, POEA = *N*-palmitoleoylethanolamine, SEA = *N*-stearoylethanolamine, OEA = *N*-oleoylethanolamine, LEA = *N*-linoleoylethanolamine, AEA = *N*-arachidonoylethanolamine, EPEA = *N*-eicosapentaenoylethanolamine, DHEA = *N*-docosahexaenoylethanolamine.

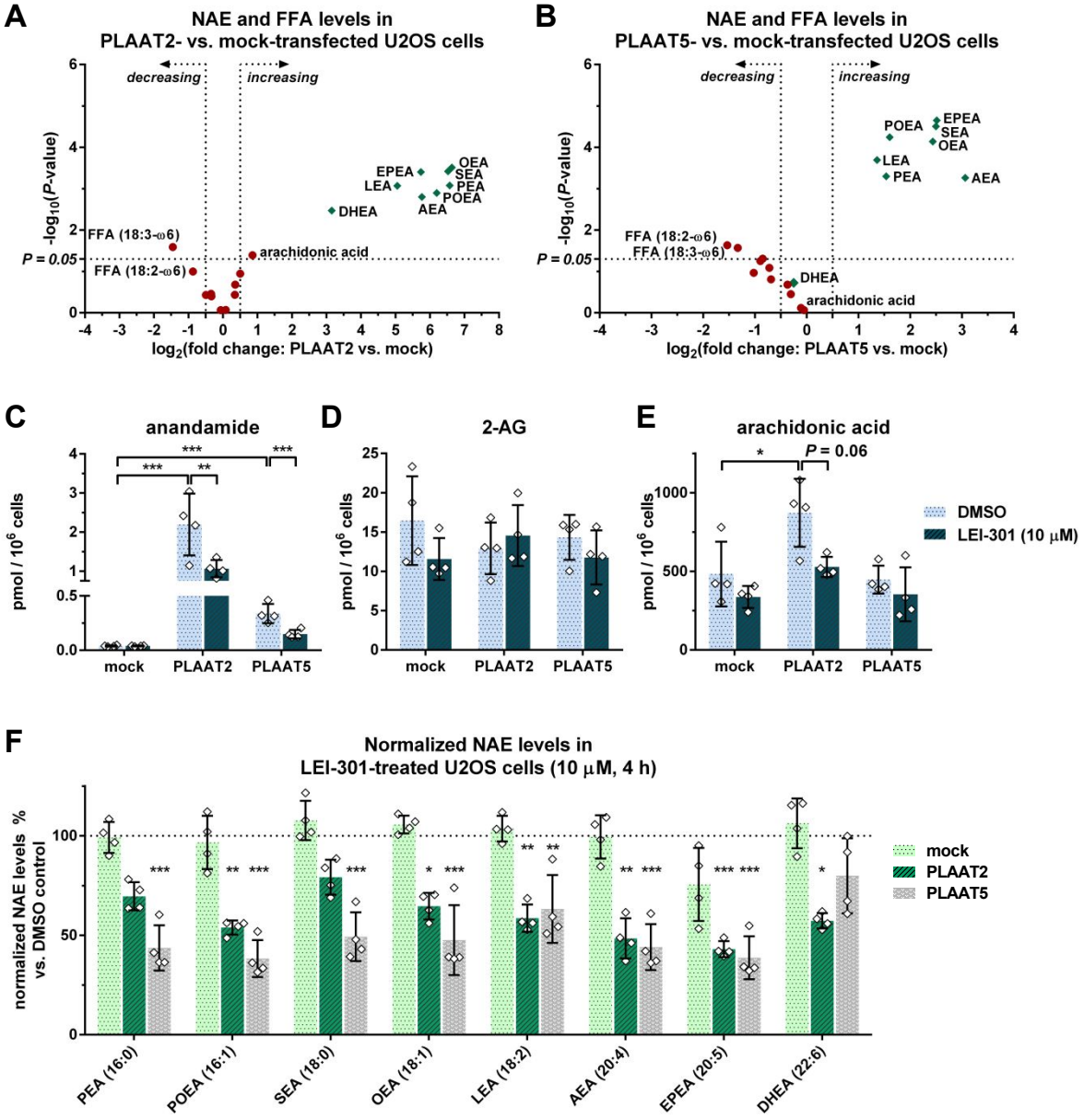


Figure 3. **U2OS cells transfected with PLAAT2 or PLAAT5 exhibit highly increased NAE levels and LEI-301 can inhibit NAE formation. A,B**) Volcano plots depicting the $\log_2$(fold change) vs. $-\log_{10}$(*P*-value) of NAEs (green diamonds) and free fatty acids (FFAs, red circles) in (**A**) PLAAT2- or (**B**) PLAAT5- vs. mock-transfected U2OS cells. **C-E**) Absolute levels of (**C**) anandamide (AEA), (**D**) 2-AG and (**E**) arachidonic acid in mock-, PLAAT2- or PLAAT5-transfected cells treated with vehicle (DMSO) or **LEI-301** (10 μM, 4 h). **F**) Normalized NAE levels of mock-, PLAAT2- or PLAAT5-transfected cells treated with **LEI-301** (10 μM, 4 h) represented as effect %. Data were normalized against mock-, PLAAT2- or PLAAT5-transfected cells treated with vehicle (DMSO). Absolute values are depicted in Figure S1. Data represent mean values ± SD for 4 biological replicates. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$ by one-way ANOVA.

Conclusion

In summary, we have described the discovery and optimization of an α-ketoamide inhibitor library for the PLAAT enzyme subfamily. The SAR of the R_1-ketone moiety proved to be narrow with little room for expansion (Figure 4). The R_2-phenethylamine side did allow introduction of a large hydrophobic *para*-phenoxy group, which led to the identification of **LEI-301** as a potent inhibitor for PLAAT2-5, having a 10-fold higher potency for PLAAT2 and PLAAT5 than our initial hit. Covalent docking in the PLAAT2 crystal structure provided a possible binding mode of **LEI-301**. Overexpression of PLAAT2 in U2OS cells resulted in a large increase of all measured NAE species, including the endocannabinoid anandamide, while no significant elevations of fatty acids were observed except for arachidonic acid. Also PLAAT5 was able to increase NAE content upon transient transfection, although this was smaller in magnitude compared to PLAAT2. These findings support the notion that PLAAT2 and PLAAT5 are involved in the biosynthetic pathways of the NAEs. Furthermore, treatment of overexpressing PLAAT2 and PLAAT5 cells with **LEI-301** gave a 2-fold reduction of anandamide levels, which was absent in control cells. This validates **LEI-301** as a promising tool compound to study PLAAT2 and PLAAT5 function in biological systems. **LEI-301** allows acute blockade of these enzymes, which can be beneficial compared to genetic knockout models, where long-term compensatory effects can occur. In addition, *Plaat2* is not present in the rodent genome, which hampers the study of its biological function. Currently, it is unknown if the Ca^{2+}-independent PLAAT enzymes contribute to physiological NAPE and thus NAE biosynthesis. In contrast to the Ca^{2+}-dependent NAPE production by PLA2G4E, PLAATs may continuously produce NAPEs from their abundant PE and PC substrates. So far, PLA2G4E activity has been reported in heart, brain and skeletal muscle.[21] Peripheral organs such as kidney, small intestine and testis have well-established NAE signaling roles and reported PLAAT2 or PLAAT5 expression.[5,18] Furthermore, these tissues show low Ca^{2+}-dependent PLA2G4E activity.[21] Therefore, these organs are prime candidates to assess the contribution of PLAAT enzymes with regard to NAPE and NAE formation using the inhibitors here disclosed.

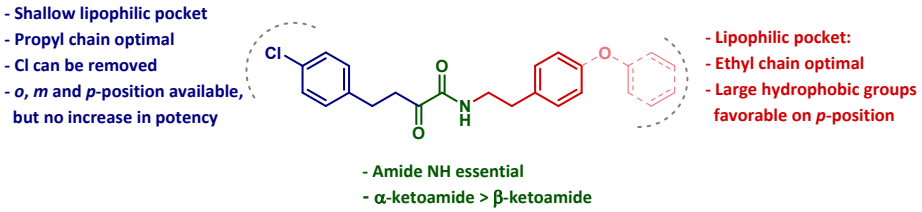
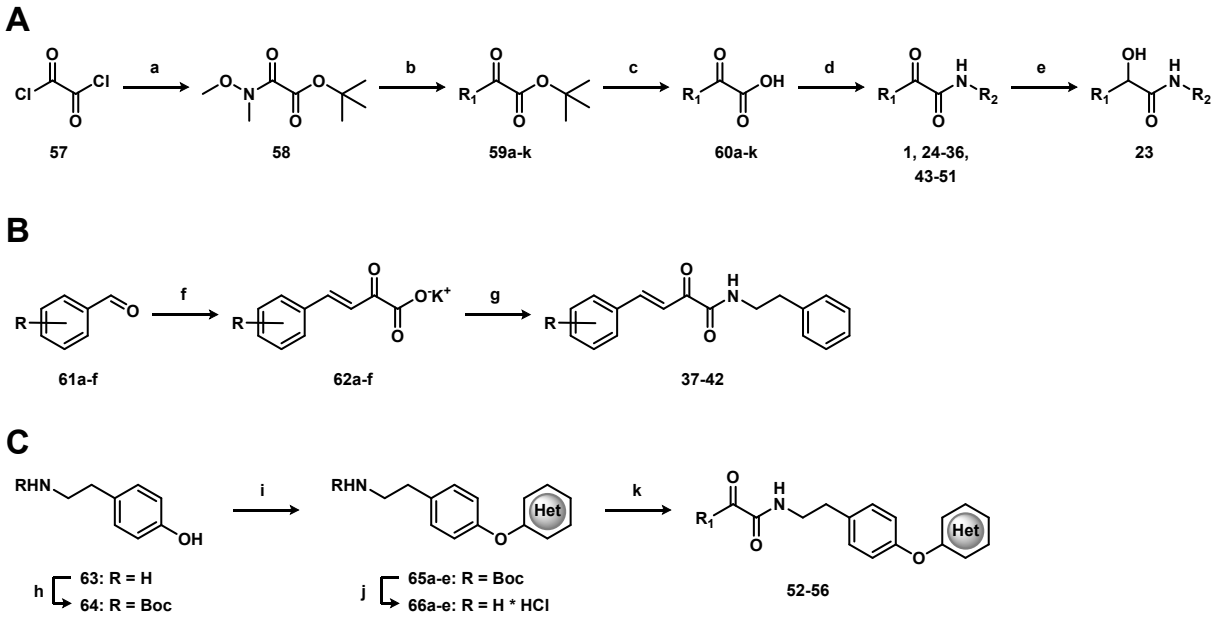


Figure 4. Structure-activity map for the PLAAT α-ketoamide inhibitor library.

Chemistry



Scheme 2. General synthetic routes for **A**) α-ketoamide **1** analogues, **B**) β,γ-unsaturated α-ketoamides and **C**) *O*-heteroaryl phenethylamine derivatives. Reagents and conditions: a) *i. t*-BuOH, THF, 0 °C; *ii. N*,*O*-dimethylhydroxylamine·HCl, Et_3N, 0 °C, 75%; b) *i.* Mg, alkylbromide, Et_2O, reflux; *ii.* Weinreb amide, -78 °C, 21% – 83%; c) TFA, DCM, rt, 99%; d) HATU or HCTU, DiPEA, amine, DMF, rt, 22% – 80%; e) $NaBH_4$, THF, rt, 72%. f) pyruvic acid or sodium pyruvate, KOH, MeOH, 0 °C to rt; g) *i.* oxalyl chloride, DCM, 0 °C to rt; *ii.* phenethylamine, DCM, 0 °C to rt, 14% – 35% over two steps. h) Boc_2O, $NaHCO_3$, THF, H_2O, rt, 85%; i) heteroaryl halide, K_2CO_3, DMSO or DMF, rt or 85 °C, 63% - 92%; j) HCl, dioxane, rt, 99%; k) EDC·HCl, HOBt, ketoacid, NMM, DCM, 0 °C to rt, 15% – 30%.

Oxalyl chloride (**57**) was reacted with *tert*-butanol and *N*,*O*-dimethylhydroxylamine·HCl giving Weinreb amide **58**. Treatment with an *in situ* formed Grignard reagent from 4-chlorophenethyl bromide followed by *tert*-butyl deprotection gave ketoacid **60a**. Finally, amide coupling using HCTU afforded α-ketoamide **1**.

R_1-derivatives **24-36** and R_2-analogues **43-51** were synthesized via the general route (Scheme 2A). β,γ-Unsaturated α-ketoamides (**37**-**42**) were prepared using a two-step procedure (Scheme 2B): condensation of a benzaldehyde (**61a-f**) with pyruvic acid, which afforded the β,γ-unsaturated α-ketoacid as the potassium salt (**62a-f**), followed by acid chloride formation and coupling with phenethylamine. *O*-Arylated 4-hydroxyphenethylamine derivatives **52-56** were synthesized via Scheme 2C. Tyramine (**63**) was Boc-protected,

followed by nucleophilic aromatic substitution (S_NAr) with a heteroaryl halide. Boc deprotection and subsequent amide coupling provided the α-ketoamides **52-56**.

Experimental section

Biological procedures

Plasmids

Full-length cDNA of human PLAAT1-5 (obtained from Natsuo Ueda[8]) was cloned into mammalian expression vector pcDNA3.1 with a C-terminal FLAG-tag and containing genes for ampicillin and neomycin resistance. Plasmids were isolated from transformed XL10-Gold competent cells (prepared using E. coli transformation buffer set; Zymo Research) using plasmid isolation kits following the supplier's protocol (Qiagen). All sequences were analyzed by Sanger sequencing (Macrogen) and verified (CLC Main Workbench).

Cell culture

HEK293T and U2OS cells (ATCC) were cultured at 37 °C and 7% CO_2 in DMEM (Sigma Aldrich, D6546) with GlutaMax (2 mM), penicillin (100 μg/ml, Duchefa), streptomycin (100 μg/ml, Duchefa) and 10% (v/v) newborn calf serum (Seradigm). Medium was refreshed every 2-3 days and cells were passaged twice a week at 80-90% confluence. Cells were passaged twice a week by thorough pipetting (HEK293T) or trypsinization (U2OS) to appropriate confluence.

Transient transfection

Transient transfection was performed as described previously.[1] In brief, 10^7 HEK293T cells were seeded in 15 cm petri dishes one day before transfection. Two hours before transfection the medium was refreshed with 13 mL medium. Transfection was performed with polyethyleneimine (PEI, 60 μg per dish) in a ratio of 3:1 with plasmid DNA (20 μg per dish). PEI and plasmid DNA were incubated in serum-free medium (2 mL per dish) at rt for 15 min, followed by dropwise addition to the cells. Transfection with the empty pcDNA3.1 vector was used to generate control (mock) samples. The medium was refreshed after 24 hours and cells were harvested after 48 or 72 hours in cold PBS. Cells were pelleted by centrifugation (5 min, 1,000 *g*) and the pellet was washed with PBS. The supernatant was removed and cell pellets were flash frozen in liquid N_2 and stored at -80 °C.

Cell lysate preparation

Cell pellets were thawed on ice, and resuspended in cold lysis buffer (50 mM Tris-HCl pH 8, 2 mM DTT, 1 mM $MgCl_2$, 2.5 U/mL benzonase) and incubated on ice for 30 minutes. The cytosolic fraction (supernatant) was separated from the membranes by ultra-centrifugation (100,000 *g*, 45 min, 4 °C, Beckman Coulter, Ti 70.1 rotor). The pellet (membrane fraction) was resuspended in cold storage buffer (50 mM Tris-HCl pH 8, 2 mM DTT) and homogenized by thorough pipetting and passage through an insulin needle (29G). Protein concentrations were determined by a Quick Start™ Bradford protein assay (Bio-Rad) or Qubit™ protein assay (Invitrogen). Samples were flash frozen in liquid N_2 and stored at -80 °C.

Mouse brain lysate preparation

Mouse brains were thawed on ice, dounce homogenized in cold lysis buffer (20 mM HEPES pH 7.2, 2 mM DTT, 1 mM MgCl2, 2.5 U/mL benzonase) and incubated on ice for 30 minutes. The membrane and cytosolic fractions of cell or tissue lysates were separated by ultra-centrifugation (100,000 *g*, 45 min, 4 °C). The supernatant was collected (cytosolic fraction) and the membrane pellet was resuspended in cold storage buffer (20 mM HEPES pH 7.2, 2 mM DTT) and homogenized by thorough pipetting and passage through an insulin needle (29G).

Protein concentrations were determined using a Bradford assay (Bio-Rad). Samples were flash frozen in liquid N_2 and stored at -80 °C.

Activity-based protein profiling on PLAAT2-5 transfected HEK293T cell lysate.

Gel-based activity based protein profiling (ABPP) was performed with minor changes as described previously.[28] For ABPP assays on HEK293T cells overexpressing PLAAT2, the cytosol proteome (0.25 μg/μL, 20 μL) was pre-incubated with vehicle (DMSO) or inhibitor (0.5 μL in DMSO, 30 min, rt) followed by incubation with MB064 (final concentration: 250 nM, 20 min, rt). For PLAAT3, PLAAT4 and PLAAT5 the protocols differed for the protein concentrations (0.5 μg/μL, 1 μg/μL and 1 μg/μL, respectively) and MB064 concentration (250 nM, 500 nM and 500 nM, respectively). Final concentrations for the inhibitors are indicated in the main text and figure legends. For the dose-response experiments only cytosol proteome was used. Proteins were denatured with 4x Laemmli buffer (5 μL, stock concentration: 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 μl sample per reaction was resolved on a 10% acrylamide SDS-PAGE gel (180 V, 70 min). Gels were scanned using Cy3 and Cy5 multichannel settings (605/50 and 695/55 filters, respectively) on a ChemiDoc™ Imaging System (Bio-Rad). Fluorescence was normalized to Coomassie staining and quantified with Image Lab (Bio-Rad). Experiments were performed in triplicate. Dose-response IC_{50} curves were generated with Graphpad Prism 6.

qPCR

For primer sequences used see Table S2. *RNA isolation and cDNA synthesis*: Total RNA from U2OS cells was extracted using a NucleoSpin® RNA kit (Macherey-Nagel) according to the manufacturer's instructions. Subsequently, cDNA synthesis was carried out with a SuperScript™ First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions. *qPCR analysis:* 2.5 ng of input cDNA was analyzed using SYBRGreen qPCR master mix (Thermo-Fisher) on a CFX96 optical thermal cycler (Bio-Rad). Data analysis was performed using CFX Manager software (Bio-Rad). The housekeeping gene 40S ribosomal protein S18 (*RPS18*) was used as a control. Data are expressed in quantitation cycles (C_q) ± SEM of three technical replicates.

Radioligand displacement assays CB_1 and CB_2 receptor

[^{3}H]CP55940 displacement assays to determine the affinity for the cannabinoid CB_1 and CB_2 receptors were performed as previously described.[35]

NAPE-PLD surrogate substrate (PED6) activity assay

The human NAPE-PLD activity assay was performed as previously described.[35]

Natural substrate-based fluorescence assay MAGL

The natural substrate assay for human MAGL was performed as reported previously.[36]

Activity-based protein profiling for determining mDAGLα/β, mFAAH, mABHD6 and hPLA2G4E activities

Gel-based activity based protein profiling (ABPP) was performed as previously described.[28] In brief, mouse brain membrane proteome or hPLA2G4E-overexpressing membrane lysate (9.5 μL and 2 μg/μL or 19.5 μL and 1 μg/μL, respectively) was pre-incubated with vehicle or inhibitor (0.5 μL 20x or 0.5 μL 40x inhibitor stock in DMSO, respectively, 30 min, rt) followed by incubation with the activity based probe MB064 (250 nM, 0.5 μL 20x stock in DMSO) or FP-TAMRA (500 nM, 0.5 μL 20x stock in DMSO) for mouse brain lysate (15 min, rt) or FP-TAMRA (50 nM, 0.5 μL 40x stock in DMSO) for PLA2G4E overexpressing lysate (5 min, rt). Final concentrations for the inhibitors are indicated in the main text and figure legends. Proteins were denatured with 4x Laemmli buffer (3.5 μL, stock concentrations: 240 mM Tris-HCl pH 6.8, 8% w/v SDS, 40% v/v glycerol, 5% v/v β-mercaptoethanol, 0.04% v/v bromophenol blue, 30 min, rt). The samples (10 μL per slot) were resolved by SDS-PAGE (respectively, 10% or 8% acrylamide for mouse brain or PLA2G4E lysate, 180 V, 75 min). Gels were scanned using Cy3 and Cy5 multichannel settings (605/50 and 695/55 filters, respectively) on a ChemiDoc™

Imaging System (Bio-Rad). Fluorescence was normalized to Coomassie staining and quantified with Image Lab (Bio-Rad).

Activity-based protein profiling for determining the selectivity profile of LEI-301 in U2OS cell lysate

U2OS cytosol or membrane lysate (19 μL, 2.5 μg/μL) was pre-incubated with vehicle or inhibitor (0.5 μL 40x stock in DMSO, 30 min, rt) followed by incubation with the activity based probe MB064 (2 μM, 0.5 μL 40x stock in DMSO, 15 min, rt) or FP-TAMRA (500 nM, 0.5 μL 40x stock in DMSO, 15 min, rt). Final concentrations for the inhibitors are indicated in the figure legends. Proteins were denatured with 3x Laemmli buffer (10 μL, stock concentrations: 240 mM Tris-HCl pH 6.8, 8% w/v SDS, 40% v/v glycerol, 5% v/v β-mercaptoethanol, 0.04% v/v bromophenol blue, 15 min, rt). The samples (12.5 μL per slot) were resolved by SDS-PAGE (180 V, 75 min). Gels were scanned using Cy3 and Cy5 multichannel settings (605/50 and 695/55 filters, respectively) on a ChemiDoc™ Imaging System (Bio-Rad). Fluorescence was normalized to Coomassie staining and quantified with Image Lab (Bio-Rad).

Activity-based protein profiling for determining PLA2G4B, PLA2G4C and PLA2G4D activities

hPLA2G4B, hPLA2G4C or hPLA2G4D-overexpressing membrane lysate (19 μL, 1 μg/μL) was pre-incubated with vehicle or inhibitor (0.5 μL 40x inhibitor stock in DMSO, 30 min, rt) followed by incubation with the activity based probe FP-TAMRA (500 nM, 0.5 μL 20x stock in DMSO, 20 min, rt). Final concentrations for the inhibitors are indicated in the figure legends. Proteins were denatured with 3x Laemmli buffer (10 μL, stock concentrations: 240 mM Tris-HCl pH 6.8, 8% w/v SDS, 40% v/v glycerol, 5% v/v β-mercaptoethanol, 0.04% v/v bromophenol blue, 30 min, rt). The samples (12.5 μL per slot) were resolved by SDS-PAGE (180 V, 75 min). Gels were scanned using Cy3 and Cy5 multichannel settings (605/50 and 695/55 filters, respectively) on a ChemiDoc™ Imaging System (Bio-Rad). Fluorescence was normalized to Coomassie staining and quantified with Image Lab (Bio-Rad).

Activity-based protein profiling for determining PLA2G4A activity

Recombinant full length human PLA2G4A (0.73 μL, 440 ng/μL, R&D systems, 6659-PL) was diluted with assay buffer (13.3 μL, 50 mM Tris HCl pH 8.0, NaCl 500 mM, $CaCl_2$ 20 mM in MilliQ) and pre-incubated with vehicle or inhibitor (0.5 μL 29x inhibitor stock in DMSO, 30 min, 37 °C). FP-alkyne was used as a positive control.[37] This was followed by incubation with FP-TAMRA (500 nM, 0.5 μL 30x inhibitor stock in DMSO, 20 min, rt). Final concentrations for the inhibitors are indicated in the figure legends. Proteins were denatured with 4x Laemmli buffer (5 μL, stock concentrations: 240 mM Tris-HCl pH 6.8, 8% w/v SDS, 40% v/v glycerol, 5% v/v β-mercaptoethanol, 0.04% v/v bromophenol blue, 30 min, 95 °C). The samples (12.5 μL, ~200 ng per slot) were resolved by SDS-PAGE (180 V, 75 min). Gels were scanned using Cy3 and Cy5 multichannel settings (605/50 and 695/55 filters, respectively) on a ChemiDoc™ Imaging System (Bio-Rad). Fluorescence was normalized to Coomassie staining and quantified with Image Lab (Bio-Rad).

Targeted lipidomics in U2OS cells

The targeted lipidomics experiments are based on previously reported methods with small alterations as specified below.[38]

Sample preparation

$2 \cdot 10^6$ U2OS cells (grown at 37 °C, 7% CO_2) were seeded 1 day before transfection in 6 cm dishes. After 24 h, PLAAT2, PLAAT5 or mock plasmid DNA (2.7 μg/dish) and polyethyleneimine (PEI, 1 μg/μL, 8 μg/dish) were incubated in serum-free culture medium (15 min, rt), and then added dropwise to the cells in a ratio of 1:5 (plasmid/PEI). After 24 h, medium was aspirated and cells were washed once with serum-free medium. New serum-free medium was added with **LEI-301** (final concentration: 10 μM, 0.1% DMSO) or DMSO as a control. After incubating for 4 hours (37 °C, 7% CO_2) the medium was removed and the cells were washed with cold PBS (3x). The cells were harvested in 1.5 mL Eppendorf tubes by trypsinization, followed by centrifugation (10 min,

1,500 rpm). PBS was removed and the cell pellets were flash frozen with liquid N_2 and stored at -80 °C. Live cell count with trypan blue was performed after compound treatment to test for cell viability and for sample normalization after lipid measurements.

Lipid extraction

Lipid extraction was performed on ice. In brief, cell pellets with $2 \cdot 10^6$ cells were spiked with 10 μL each of deuterium labeled internal standard mix for endocannabinoids (*N*-arachidonoylethanolamine (AEA)-d8, *N*-docosahexaenoylethanolamine (DHEA)-d4, 2-arachidonoylglycerol (2-AG)-d8, *N*-stearoylethanolamine (SEA)-d3, *N*-palmitoylethanolamine (PEA)-d4, *N*-linoleoylethanolamine (LEA)-d3 and *N*-oleoylethanolamine (OEA)-d4), and negative polar lipids (fatty acid (FA)17:0-d33), followed by the addition of ammonium acetate buffer (100 μL, 0.1 M, pH 4). After extraction with methyl *tert*-butyl ether (MTBE, 1 mL), the tubes were thoroughly mixed for 4 min using a bullet blender at medium speed (Next Advance Inc., Averill park, NY, USA), followed by a centrifugation step (5,000 *g*, 12 min, 4 °C). Then 925 μL of the upper MTBE layer was transferred into clean 1.5 mL Eppendorf tubes. Samples were dried in a speedvac followed by reconstitution in acetonitrile/water (50 μL, 90 : 10, v/v). The samples were centrifuged (14,000 *g*, 3 min, 4 °C) before transferring into LC-MS vials. Each sample was injected on two different lipidomics platforms: endocannabinoids (5 μL) and negative polar lipids (8 μL).

LC-MS/MS analysis for endocannabinoids

A targeted analysis of endocannabinoids and related NAEs (*N*-acylethanolamines) was measured using an Acquity UPLC I class binary solvent manager pump (Waters, Milford, USA) in conjugation with AB SCIEX 6500 quadrupole ion trap (QTRAP) (AB Sciex, Massachusetts, USA). Separation was performed with an Acquity HSS T3 column (1.2 x 100 mm, 1.8 μm) maintained at 40 °C. The aqueous mobile phase A consisted of 2 mM ammonium formate and 10 mM formic acid, and the organic mobile phase B was acetonitrile. The flow rate was set to 0.4 ml/min; initial gradient conditions were 55% B held for 2 min and linearly ramped to 100% B over 6 minutes and held for 2 min; after 10 s the system returned to initial conditions and held 2 min before next injection. Electrospray ionization-MS was operated in positive mode for measurement of endocannabinoids and NAEs, and a selective Multiple Reaction Mode (sMRM) was used for quantification.

LC-MS/MS analysis for negative polar lipids

This method is measured on an Acquity UPLC binary solvent manager 8 pump (Waters) coupled to an Agilent 6530 electrospray ionization quadrupole time-of-flight (ESI-Q-TOF, Agilent, Jose, CA, USA) high resolution mass spectrometer using reference mass correction. The chromatographic separation was achieved on an Acquity HSS T3 column (1.2 x 100 mm, 1.8 μm) maintained at 40 °C. The negative apolar lipids that constitute free fatty acids were separated with a flow of 0.4 mL/min over 15 min gradient. In negative mode, the aqueous mobile phase A consisted of 5:95 (v/v) acetonitrile:H_2O with 10 mM ammonium formate, and the organic mobile phase B consisted of 99% (v/v) methanol with 10 mM ammonium formate.

Statistical analysis

Absolute values of lipid levels were corrected using the measured live cell count numbers (cell viability was > 90%). Data were tested for significance with GraphPad v6 using one-way ANOVA with Tukey correction for multiple comparisons. *P*-values < 0.05 were considered significant.

Computational Chemistry

Ligand preparation

Molecular structures of **LEI-301** and **1** were drawn with specified chirality and prepared for docking using Ligprep from Schrödinger.[39] Default Ligprep settings were applied: states of heteroatoms were generated using Epik at a pH 7.[40] No tautomers were created by the program, which resulted in one standardized structure per ligand.

Protein preparation

The x-ray structure of PLAAT2 was extracted from the PDB (PDB ID: 4DPZ).[4] The apo protein structure was prepared for docking with the Protein Preparation tool from the Schrödinger 2017-4 suite. Waters were removed and explicit hydrogens were added. Missing side chains and loops were added with homology modeling using Prime[41]: loop 39-53 was modeled based on the protein sequence and loop 105-111 was based on the structure of PLAAT3 (PDB ID: 4DOT).[4]

Docking

The PLAAT2 binding pocket was induced using the binding pose from **1** in PLAAT3 as previously reported.[26,42] The complex of superposed **1** covalently bound to PLAAT2, was optimized using molecular dynamic simulations (10 ns). Compound **1** was removed and the cysteine was restored to its non-bonded state. Subsequently, **LEI-301** and **1** were covalently docked to Cys113 using the Schrödinger 2017-4 suite.[42] The poses with the lowest docking scores were manually examined and one pose per ligand was selected. Selection was based on docking score, frequency of recurring poses, and interactions made between the ligand and the protein.

Synthetic procedures

General

All chemicals (Sigma-Aldrich, Fluka, Acros, Merck) were used as received. All solvents used for reactions were of analytical grade. THF, Et_2O, DMF, CH_3CN and DCM were dried over activated 4 Å molecular sieves, MeOH over 3 Å molecular sieves. Flash chromatography was performed on silica gel (Screening Devices BV, 40-63 µm, 60 Å). The eluent EtOAc was of technical grade and distilled before use. Reactions were monitored by thin layer chromatography (TLC) analysis using Merck aluminium sheets (Silica gel 60, F_{254}). Compounds were visualized by UV-absorption (254 nm) and spraying for general compounds: $KMnO_4$ (20 g/L) and K_2CO_3 (10 g/L) in water, or for amines: ninhydrin (0.75 g/L) and acetic acid (12.5 mL/L) in ethanol, followed by charring at ~150 °C. ^1H and ^{13}C NMR experiments were recorded on a Bruker AV-300 (300/75 MHz), Bruker AV-400 (400/100 MHz) or Bruker DMX-400 (400/101 MHz). Chemical shifts are given in ppm (δ) relative to tetramethylsilane or $CDCl_3$ as internal standards. Multiplicity: s = singlet, br s = broad singlet, d = doublet, dd = doublet of doublet, t = triplet, q = quartet, p = pentet, m = multiplet. Coupling constants (*J*) are given in Hz. LC-MS measurements were performed on a Thermo Finnigan LCQ Advantage MAX ion-trap mass spectrometer (ESI^+) coupled to a Surveyor HPLC system (Thermo Finnigan) equipped with a standard C18 (Gemini, 4.6 mmD x 50 mmL, 5 µm particle size, Phenomenex) analytical column and buffers A: H_2O, B: CH_3CN, C: 0.1% aq. TFA. High resolution mass spectra were recorded on a LTQ Orbitrap (Thermo Finnigan) mass spectrometer or a Synapt G2-Si high definition mass spectrometer (Waters) equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10 mL min-1, capillary temperature 250 °C) with resolution R = 60000 at m/z 400 (mass range m/z = 150-2000) and dioctylphtalate (m/z = 391.28428) as a lock mass. Preparative HPLC was performed on a Waters Acquity Ultra Performance LC with a C18 column (Gemini, 150 x 21.2 mm, Phenomenex). All final compounds were determined to be >95% pure by integrating UV intensity recorded via HPLC.

General procedure A: α-ketoester synthesis. Magnesium turnings were activated by stirring in a 3 M solution of HCl for 5 min. The magnesium was then washed with water and acetone and dried under reduced pressure. A round-bottom flask connected to a reflux condenser was flame dried before addition of activated magnesium turnings (2 eq) under an argon atmosphere. Dry Et_2O (2 mL) and a small piece of iodine were added followed by dropwise addition of a solution of alkyl bromide (1 - 1.5 eq) in dry Et_2O (1 M). The reaction was initiated with a heat gun and refluxed for 1 h. In a separate flask, a solution of the Weinreb amide **58** (1 eq) in dry Et_2O (1 M) was prepared and cooled to -78 °C. The Grignard solution was taken up by syringe and added dropwise to the Weinreb amide solution. After stirring for 2 h at -78 °C the reaction was quenched with sat. aq. NH_4Cl and extracted with Et_2O (2x). The combined organic layers were dried ($MgSO_4$), filtered and concentrated under reduced pressure. The crude residue was purified using silica gel column chromatography (EtOAc/pentane) affording the α-ketoester.

General procedure B: α-ketoester deprotection. A round bottom flask was charged with α-ketoester (1 eq), DCM (0.3 M) and TFA (5-10 eq) and stirred for 1-24 h at rt. The reaction mixture was concentrated under reduced pressure after TLC analysis showed complete consumption of the starting material, followed by coevaporation with toluene (3x). The obtained α-ketoacid was used in the next step without further purification.

General procedure C: α-ketoamide synthesis. A round bottom flask was charged with α-ketoacid (1 eq) and DMF (0.2 M). HATU or HCTU (1-1.2 eq), DiPEA (1-2 eq) or Et_3N (1-2 eq), and amine (1-1.1 eq) were added and the mixture was stirred for 2-24 h at rt. Water was added and the mixture was extracted with DCM (2x). The combined organic layers were washed with 1 M HCl, sat. aq. $NaHCO_3$, brine, dried ($MgSO_4$), filtered and concentrated under reduced pressure. The crude residue was purified by silica gel column chromatography (EtOAc/pentane) affording the α-ketoamide.

General procedure D: α-ketoamide synthesis. A round bottom flask was charged with α-ketoacid (1 eq) and THF or DCM (0.2 M) at 0 °C. EDC·HCl (1-1.5 eq) and HOBt (1-1.5 eq) were added and the mixture was stirred for 30 min, followed by addition of NMM (optional, 4 eq) and the amine (1.2 eq). The mixture was stirred for 1-4 days warming to rt. Work-up involved addition of sat. aq. $NaHCO_3$ and extraction with EtOAc (2 x 25 mL). The combined organic layers were washed with brine (1x), dried ($MgSO_4$), filtered and concentrated under reduced pressure. The crude residue was purified by silica gel column chromatography (EtOAc/pentane) or preparative HPLC affording the α-ketoamide.

General procedure E: *N*-Boc-tyramine *O*-arylation via S_NAr. A microwave vial was charged with *N*-Boc-tyramine **64** (1 eq), heteroaryl halide (1 eq), K_2CO_3 (2 eq) in DMSO or DMF (0.2 – 1 M) and capped. The mixture was stirred for 24 - 42 h at 85°C in an oil bath until TLC showed complete conversion. The mixture was diluted with H_2O and extracted with EtOAc (3x). The combined organic layers were washed with brine (1x), dried ($MgSO_4$), filtered and concentrated under reduced pressure. The crude residue was purified by silica gel column chromatography (EtOAc/pentane) affording the product.

General procedure F: β,γ-unsaturated α-ketoamide synthesis. A round bottom flask was charged with pyruvic acid or sodium pyruvate (1 eq), aldehyde (1 eq) and MeOH (1 M) and cooled to 0 °C. A solution of KOH (2 M, 1.5 eq) in MeOH was added dropwise while keeping the temperature below 25 °C. The reaction was stirred at rt overnight, forming a yellow precipitate. The reaction mixture was filtered, the precipitate was washed with cold MeOH (2x), Et_2O (2x) and dried affording the α-ketoacid as the potassium salt. A new round bottom flask was charged with the potassium salt and DCM (0.5 M) and the suspension was sonicated for 20 min. This was followed by cooling to 0 °C and addition of oxalyl chloride (2 eq). After consumption of the potassium salt, the reaction mixture was concentrated under reduced pressure and coevaporated with toluene (2x). The α-ketoacid chloride was then dissolved in DCM (0.5 M) and cooled to 0 °C, followed by addition of phenethylamine (1 eq) and Et_3N (2 eq). The reaction was stirred for 2 h. Work-up involved addition of H_2O and extraction with EtOAc. The organic layer was then washed with 1 M HCl (2x), sat. aq. $NaHCO_3$ (2x) and brine (1x), dried ($MgSO_4$), filtered and concentrated under reduced pressure. The crude residue was purified by silica gel column chromatography (EtOAc/pentane) affording the α-ketoamide.

4-(4-Chlorophenyl)-2-oxo-*N*-phenethylbutanamide (1). *t-Butyl deprotection **59a:*** the α-ketoacid was prepared according to general procedure B using α-ketoester **59a** (0.85 g, 3.2 mmol, 1 eq) and TFA (2.5 mL, 32 mmol, 10 eq) affording the α-ketoacid **60a** (0.68 g, 3.2 mmol, quant.). *Amide coupling:* the title compound was prepared according to general procedure C using the α-ketoacid **60a** (0.68 g, 3.2 mmol, 1 eq), phenethylamine (0.15 mL, 1.2 mmol, 1.1 eq), HATU (1.2 g, 3.2 mmol, 1 eq) and DiPEA (0.61 mL, 3.5 mmol, 1.1 eq) in DMF, affording the product (0.70 g, 2.2 mmol, 70%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.54 – 6.79 (m, 10H), 3.52 (q, *J* = 6.9 Hz, 2H), 3.21 (t, *J* = 7.4 Hz, 2H), 2.91 – 2.73 (m, 4H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 197.80, 159.82, 138.78, 138.13, 131.88,

129.71, 128.67, 128.60, 128.51, 126.67, 40.44, 38.06, 35.28, 28.35. HRMS $[C_{18}H_{18}NClO_2 + H]^+$: 316.1099 calculated, 316.1099 foun.

4-(4-Chlorophenyl)-2-hydroxy-*N*-phenethylbutanamide (23). A round bottom flask was charged with α-ketoamide **1** (70 mg, 0.22 mmol, 1 eq) and THF (1 mL). $NaBH_4$ (12 mg, 0.33 mmol, 1.5 eq) was added and the mixture was stirred for 15 min. The reaction was quenched with water (10 mL) and extracted with EtOAc (1 x 10 mL). The organic layer was washed with 1 M aq. HCl (2 x 10 mL), brine (1 x 10 mL), dried ($MgSO_4$), filtered and concentrated under reduced pressure. Purification by silica gel column chromatography afforded the product (50 mg, 0.16 mmol, 72%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.34 – 7.25 (m, 2H), 7.25 – 7.14 (m, 5H), 7.08 (d, *J* = 8.4 Hz, 2H), 6.61 (t, *J* = 5.4 Hz, 1H), 4.03 (dd, *J* = 7.9, 3.7 Hz, 1H), 3.66 – 3.40 (m, 2H), 3.24 (br s, 1H), 2.80 (t, *J* = 7.0 Hz, 2H), 2.65 (t, *J* = 7.9 Hz, 2H), 2.11 – 1.97 (m, 1H), 1.90 – 1.80 (m, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.79, 139.74, 138.63, 131.87, 129.93, 128.81, 128.78, 128.65, 126.74, 71.33, 40.33, 36.38, 35.76, 30.57. HRMS $[C_{18}H_{20}NClO_2 + H]^+$: 318.1255 calculated, 318.1252 found.

2-Oxo-*N*-phenethyl-4-phenylbutanamide (24). *t-Butyl deprotection **59b:*** the α-ketoacid was prepared according to general procedure B using α-ketoester **59b** (0.50 g, 2.1 mmol, 1 eq) and TFA (0.80 mL, 32 mmol, 5 eq) affording the α-ketoacid **60b** (0.40 g, 2.1 mmol, quant.). *Amide coupling:* the title compound was prepared according to general procedure C using the α-ketoacid **60b** (0.20 g, 1.2 mmol, 1 eq), phenethylamine (0.15 mL, 1.2 mmol, 1.1 eq), HCTU (0.48 g, 1.15 mmol, 1 eq) and DiPEA (0.22 mL, 1.3 mmol, 1.1 eq) in DMF, affording the product (80 mg, 0.28 mmol, 24%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.40 – 7.07 (m, 10H), 7.07 – 6.88 (m, 1H), 3.55 (q, *J* = 6.9 Hz, 2H), 3.26 (t, *J* = 7.5 Hz, 2H), 2.92 (t, *J* = 7.5 Hz, 2H), 2.83 (t, *J* = 7.1 Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 198.25, 160.05, 140.46, 138.27, 128.85, 128.77, 128.60, 128.46, 126.85, 126.36, 40.58, 38.40, 35.51, 29.22. HRMS $[C_{18}H_{19}NO_2 + H]^+$: 282.1489 calculated, 282.1487 found.

2-Oxo-*N*-phenethyl-5-phenylpentanamide (25). The title compound was prepared according to general procedure C using the α-ketoacid **60c** (0.12 g, 0.63 mmol, 1 eq), phenethylamine (86 μL, 0.69 mmol, 1.1 eq), HCTU (0.26 g, 0.63 mmol, 1 eq) and DiPEA (0.12 mL, 0.70 mmol, 1.1 eq) in DCM, affording the product (70 mg, 0.24 mmol, 38%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.34 – 7.20 (m, 5H), 7.20 – 7.13 (m, 5H), 6.98 (br s, 1H), 3.53 (q, *J* = 7.0 Hz, 2H), 2.92 (t, *J* = 7.3 Hz, 2H), 2.83 (t, *J* = 7.1 Hz, 2H), 2.64 (t, *J* = 7.6 Hz, 2H), 1.92 (p, *J* = 7.4 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 198.97, 160.15, 141.42, 138.30, 128.83, 128.75, 128.57, 128.51, 126.83, 126.12, 40.56, 36.20, 35.52, 35.12, 24.92. HRMS $[C_{19}H_{21}NO_2 + H]^+$: 296.1645 calculated, 296.1646 found.

2-Oxo-*N*-phenethyl-6-phenylhexanamide (26). *t-Butyl deprotection **59d:*** the α-ketoacid was prepared according to general procedure B using α-ketoester **59d** (0.33 g, 1.3 mmol, 1 eq) and TFA (1.9 mL, 25 mmol, 19 eq) affording the α-ketoacid **60d** (0.26 g, 1.3 mmol, quant.). *Amide coupling:* the title compound was prepared according to general procedure C using the α-ketoacid **60d** (0.26 g, 1.3 mmol, 1 eq), phenethylamine (0.22 mL, 1.72 mmol, 1.3 eq), HATU (0.59 g, 1.56 mmol, 1.2 eq) and DiPEA (0.30 mL, 1.72 mmol, 1.3 eq), affording the product (0.34 g, 1.11 mmol, 71%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.43 – 7.08 (m, 10H), 7.09 – 6.89 (m, 1H), 3.52 (q, *J* = 6.9 Hz, 2H), 3.01 – 2.86 (m, 2H), 2.81 (t, *J* = 7.1 Hz, 2H), 2.61 (t, *J* = 7.0 Hz, 2H), 1.62 (p, *J* = 3.5 Hz, 4H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 198.99, 160.11, 142.05, 138.25, 128.74, 128.68, 128.40, 128.34, 126.73, 125.81, 40.50, 36.55, 35.62, 35.42, 30.79, 22.81. HRMS $[C_{20}H_{23}NO_2 + H]^+$: 310.1802 calculated, 310.1801 found.

2-Oxo-*N*-phenethyl-2-phenylacetamide (27). *t-Butyl deprotection **59e:*** the α-ketoacid was prepared according to general procedure B using α-ketoester **59e** (0.68 g, 3.3 mmol, 1 eq) and TFA (2.4 mL, 32 mmol, 10 eq) affording the α-ketoacid **60e** (0.58 g, 3.2 mmol, quant.). *Amide coupling:* the title compound was prepared according to general procedure C using the α-ketoacid **60e** (0.25 g, 1.7 mmol, 1 eq), phenethylamine (0.23 mL, 1.8 mmol, 1.1 eq), HCTU (0.69 g, 1.7 mmol, 1 eq) and DiPEA (0.32 mL, 1.8 mmol, 1.1 eq) in DCM, affording the product (0.22 g, 0.86 mmol, 52%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.33 – 8.19 (m, 2H), 7.62 – 7.52 (m, 1H), 7.47 – 7.38 (m, 2H), 7.34 – 7.26 (m, 2H), 7.26 – 7.14 (m, 4H), 3.62 (q, *J* = 7.0 Hz, 2H), 2.88 (t, *J* = 7.2 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 187.86, 161.98, 138.36, 134.36, 133.30, 131.11, 128.75, 128.72, 128.47, 126.68, 40.60, 35.46. HRMS $[C_{16}H_{15}NO_2 + H]^+$: 254.1176 calculated, 254.1175 found.

2-Oxo-*N*-phenethylpropanamide (28). A round bottom flask was charged with pyruvic acid (0.79 mL, 12 mmol, 1 eq) and cooled to 0 °C. Thionyl chloride (0.93 mL, 13 mmol, 1.1 eq) was added and the mixture was stirred for 3 h at rt. The reaction mixture was concentrated under reduced pressure and coevaporated with toluene (3x). The acid chloride was dissolved in DCM (50 mL) and cooled to 0 °C. Phenethylamine (1.5 mL, 12 mmol, 1 eq) and Et_3N (1.8 mL, 13 mmol, 1.1 eq) were added and the reaction was stirred for 2 h. Work-up involved addition of H_2O and extraction with EtOAc. The organic layer was then washed with 1 M HCl (2x), sat. aq. $NaHCO_3$ (2x) and brine (1x), dried ($MgSO_4$), filtered and concentrated under reduced pressure. The crude residue was purified by silica gel column chromatography (EtOAc/pentane) affording the product (100 mg, 0.52 mmol, 4%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.43 – 7.15 (m, 5H), 7.13 – 6.82 (m, 1H), 3.57 (q, *J* = 7.0 Hz, 2H), 2.87 (t, *J* = 7.1 Hz, 2H), 2.47 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 197.16, 160.18, 138.27, 128.88, 128.78, 126.87, 40.65, 35.54, 24.58. HRMS $[C_{11}H_{13}NO_2 + H]^+$: 192.1019 calculated, 192.1019 found.

2-Oxo-*N*-phenethyl-4-(*p*-tolyl)butanamide (29). *t-Butyl deprotection **59f:*** the α-ketoacid was prepared according to general procedure B using α-ketoester **59f** (0.54 g, 2.2 mmol, 1 eq) and TFA (1.6 mL, 22 mmol, 10 eq) affording the α-ketoacid **60f** (0.42 g, 2.2 mmol, quant.). *Amide coupling:* the title compound was prepared according to general procedure C using the α-ketoacid **60f** (0.42 g, 2.2 mmol, 1 eq), phenethylamine (0.30 mL, 2.4 mmol, 1.1 eq), HATU (0.83 g, 2.2 mmol, 1 eq) and DiPEA (0.42 mL, 2.4 mmol, 1.1 eq) in DCM, affording the product (0.48 g, 1.6 mmol, 74%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.32 – 7.23 (m, 2H), 7.23 – 7.18 (m, 1H), 7.18 – 7.11 (m, 2H), 7.11 – 6.97 (m, 5H), 3.51 (q, *J* = 6.9 Hz, 2H), 3.21 (t, *J* = 7.5 Hz, 2H), 2.97 – 2.69 (m, 4H), 2.28 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 198.18, 159.96, 138.21, 137.24, 135.61, 129.12, 128.66, 128.62, 128.18, 126.64, 40.44, 38.37, 35.33, 28.64, 20.97. HRMS $[C_{19}H_{21}NO_2 + H]^+$: 296.1645 calculated, 296.1643 found.

2-Oxo-*N*-phenethyl-4-(4-(trifluoromethyl)phenyl)butanamide (30). *t-Butyl deprotection **59g:*** the α-ketoacid was prepared according to general procedure B using α-ketoester **59g** (0.25 g, 0.83 mmol, 1 eq) and TFA (0.62 mL, 8.3 mmol, 10 eq) affording the α-ketoacid **60g** (0.20 g, 0.83 mmol, quant.). *Amide coupling:* The title compound was prepared according to general procedure C using the α-ketoacid **60g** (0.20 g, 0.80 mmol, 1 eq), phenethylamine (0.11 mL, 0.88 mmol, 1.1 eq), HATU (0.38 g, 0.80 mmol, 1 eq) and DiPEA (0.15 mL, 0.80 mmol, 1.1 eq) in DMF. Column chromatography (20% -> 60% EtOAc in pentane) afforded the product (0.22 g, 0.64 mmol, 80%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.57 (d, *J* = 8.1 Hz, 2H), 7.39 – 7.31 (m, 4H), 7.31 – 7.25 (m, 1H), 7.25 – 7.19 (m, 2H), 7.12 – 7.00 (m, 1H), 3.60 (q, *J* = 7.0 Hz, 2H), 3.32 (t, *J* = 7.4 Hz, 2H), 3.01 (t, *J* = 7.4 Hz, 2H), 2.88 (t, *J* = 7.1 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 197.70, 159.88, 144.56 (q, *J* = 1.3 Hz), 138.19, 128.78, 128.70, 128.48, 126.80, 125.46 (q, *J* = 3.8 Hz), 124.31 (q, *J* = 271.8 Hz), 40.55, 37.89, 35.39, 28.89. HRMS $[C_{19}H_{18}F_3NO_2 + H]^+$: 350.1362 calculated, 350.1362 found.

4-(4-Fluorophenyl)-2-oxo-*N*-phenethylbutanamide (31). *t-Butyl deprotection **59h:*** the α-ketoacid was prepared according to general procedure B using α-ketoester **59h** (0.15 g, 0.59 mmol, 1 eq) and TFA (0.44 mL, 5.9 mmol, 10 eq) affording the α-ketoacid **60h** (0.12 g, 0.59 mmol, quant.). *Amide coupling:* the title compound was prepared according to general procedure C using the α-ketoacid **60h** (0.12 g, 0.63 mmol, 1 eq), phenethylamine (80 µL, 0.63 mmol, 1 eq), HCTU (0.26 g, 0.63 mmol, 1 eq) and DiPEA (0.12 mL, 0.69 mmol, 1.1 eq) in DMF, affording the product (0.12 g, 0.39 mmol, 62%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.35 – 7.21 (m, 3H), 7.21 – 7.08 (m, 4H), 7.08 – 6.99 (m, 1H), 6.99 – 6.88 (m, 2H), 3.54 (q, *J* = 6.9 Hz, 2H), 3.22 (t, *J* = 7.4 Hz, 2H), 2.99 – 2.64 (m, 4H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 198.05, 163.13, 159.97, 138.22, 136.05 (d, *J* = 3.2 Hz), 129.85 (d, *J* = 7.9 Hz), 128.77 (d, *J* = 6.8 Hz), 126.82, 115.32 (d, *J* = 21.2 Hz), 40.55, 38.44, 35.44, 28.39. HRMS $[C_{18}H_{18}FNO_2 + H]^+$: 300.1394 calculated, 300.1393 found.

4-(4-Methoxyphenyl)-2-oxo-*N*-phenethylbutanamide (32). A round bottom flask was charged with unsaturated α-ketoamide **39** (0.10 g, 0.32 mmol, 1 eq) and MeOH (1 mL) and flushed with N_2. Pd/C (10 wt. %, 10 mg, 9.4 µmol, 3 mol%) was added and the flask was purged again with N_2, followed by H_2 and the reaction was stirred overnight under a H_2 atmosphere (balloon). The reaction was filtered over Celite, which was washed with MeOH and the filtrate was concentrated under reduced pressure. The crude residue was purified by silica gel column chromatography (EtOAc/pentane) affording the product (50 mg, 0.16 mmol, 50%). ^{1}H NMR

(400 MHz, $CDCl_3$) δ 7.36 – 7.27 (m, 2H), 7.27 – 7.21 (m, 1H), 7.18 (d, *J* = 7.0 Hz, 2H), 7.11 (d, *J* = 8.6 Hz, 2H), 7.05 – 6.90 (m, 1H), 6.88 – 6.71 (m, 2H), 3.77 (s, 3H), 3.55 (q, *J* = 7.0 Hz, 2H), 3.22 (t, *J* = 7.5 Hz, 2H), 2.95 – 2.73 (m, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 198.37, 160.05, 158.13, 138.26, 132.48, 129.41, 128.86, 128.77, 126.85, 113.99, 55.35, 40.57, 38.67, 35.51, 28.38. HRMS $[C_{19}H_{21}NO_3 + H]^+$: 312.1594 calculated, 312.1593 found.

2-Oxo-*N*-phenethyl-4-(4-phenoxyphenyl)butanamide (33). A round bottom flask was charged with unsaturated α-ketoamide **67** (0.12 g, 0.33 mmol, 1 eq) and MeOH (1 mL) and flushed with N_2. Pd/C (10 wt. %, 10 mg, 9.4 μmol, 3 mol%) was added and the flask was purged again with N_2, followed by H_2 and the reaction was stirred overnight under a H_2 atmosphere (balloon). The reaction was filtered over Celite, which was washed with MeOH and the filtrate was concentrated under reduced pressure. The ketone was overreduced to the alcohol according to NMR analysis, therefore it was reoxidized. A round bottom flask was charged with the alcohol, Dess-Martin periodinane (0.21 g, 0.49 mmol, 1.5 eq) and DCM (5 mL) and stirred at rt. Work-up involved addition of H_2O and extraction with EtOAc. The organic layer was washed with 1 M HCl (2x), sat. aq. $NaHCO_3$ (2x) and brine (1x), dried ($MgSO_4$), filtered and concentrated under reduced pressure. The crude residue was purified by silica gel column chromatography (EtOAc/pentane) affording the product (80 mg, 0.23 mmol, 65%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36 – 7.27 (m, 4H), 7.27 – 7.20 (m, 1H), 7.20 – 7.11 (m, 4H), 7.11 – 7.04 (m, 1H), 7.04 – 6.95 (m, 3H), 6.95 – 6.88 (m, 2H), 3.55 (q, *J* = 7.0 Hz, 2H), 3.25 (t, *J* = 7.5 Hz, 2H), 2.90 (t, *J* = 7.5 Hz, 2H), 2.84 (t, *J* = 7.1 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 198.20, 160.01, 157.51, 155.62, 138.23, 135.36, 129.79, 129.71, 128.85, 128.76, 126.85, 123.16, 119.14, 118.74, 40.58, 38.55, 35.49, 28.49. HRMS $[C_{24}H_{23}NO_3 + H]^+$: 374.1751 calculated, 374.1748 found.

4-(2-Chlorophenyl)-2-oxo-*N*-phenethylbutanamide (34). *t-Butyl deprotection **59i:*** the α-ketoacid was prepared according to general procedure B using α-ketoester **59i** (0.23 g, 0.87 mmol, 1 eq) and TFA (0.94 mL, 13 mmol, 15 eq) affording the α-ketoacid **60i** (0.18 g, 0.87 mmol, quant.). *Amide coupling:* the title compound was prepared according to general procedure C using the α-ketoacid **60i** (0.18 g, 0.87 mmol, 1 eq), phenethylamine (0.12 mL, 0.95 mmol, 1.1 eq), HATU (0.33 g, 0.87 mmol, 1 eq) and DiPEA (0.16 mL, 0.95 mmol, 1.1 eq) in DCM, affording the product (0.13 g, 0.42 mmol, 48%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.50 – 7.08 (m, 9H), 7.08 – 6.89 (m, 1H), 3.55 (q, *J* = 6.9 Hz, 2H), 3.27 (t, *J* = 7.5 Hz, 2H), 3.01 (t, *J* = 7.4 Hz, 2H), 2.84 (t, *J* = 7.1 Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 197.96, 159.93, 138.24, 138.01, 134.04, 130.52, 129.65, 128.82, 128.74, 127.92, 126.96, 126.82, 40.56, 36.78, 35.48, 27.23. HRMS $[C_{18}H_{18}ClNO_2 + H]^+$: 316.1099 calculated, 316.1100 found.

4-(3-Chlorophenyl)-2-oxo-*N*-phenethylbutanamide (35). *t-Butyl deprotection **59j:*** the α-ketoacid was prepared according to general procedure B using α-ketoester **59j** (0.55 g, 2.0 mmol, 1 eq) and TFA (2 mL, 26 mmol, 13 eq) affording the α-ketoacid **60j** (0.47 g, 2.0 mmol, quant.). *Amide coupling:* The title compound was prepared according to general procedure C using the α-ketoacid **60j** (0.47 g, 2.0 mmol, 1 eq), phenethylamine (0.31 mL, 2.4 mmol, 1.2 eq), HATU (0.84 mg, 2.2 mmol, 1.1 eq) and DiPEA (0.42 mL, 2.4 mmol, 1.2 eq), affording the product (0.37 g, 1.2 mmol, 53%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.73 – 6.73 (m, 10H), 3.58 (q, *J* = 6.9 Hz, 2H), 3.28 (t, *J* = 7.4 Hz, 2H), 3.07 – 2.74 (m, 4H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 197.71, 159.83, 142.40, 138.16, 134.13, 129.72, 128.69, 128.62, 128.53, 126.69, 126.55, 126.42, 40.47, 37.95, 35.32, 28.65. HRMS $[C_{18}H_{18}ClNO_2 + H]^+$: 316.1099 calculated, 316.1098 found.

4-(3,4-Dichlorophenyl)-2-oxo-*N*-phenethylbutanamide (36). *t-Butyl deprotection **59k:*** the α-ketoacid was prepared according to general procedure B using α-ketoester **59k** (90 mg, 0.30 mmol, 1 eq) and TFA (0.25 mL, 32 mmol, 10 eq) affording the α-ketoacid **60k** (74 mg, 0.30 mmol, quant.). *Amide coupling:* the title compound was prepared according to general procedure C using α-ketoacid **60k** (66 mg, 0.27 mmol, 1 eq), phenethylamine (36 μL, 0.29 mmol, 1.1 eq), HATU (110 mg, 0.29 mmol, 1.1 eq) and DiPEA (92 μL, 0.53 mmol, 2 eq) in DCM, affording the product (42 mg, 0.12 mmol, 44%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.41 – 7.32 (m, 4H), 7.32 – 7.26 (m, 1H), 7.22 (d, *J* = 7.0 Hz, 2H), 7.07 (dd, *J* = 8.2, 2.0 Hz, 1H), 7.05 – 6.97 (m, 1H), 3.60 (q, *J* = 7.0 Hz, 2H), 3.28 (t, *J* = 7.4 Hz, 2H), 3.03 – 2.79 (m, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 197.63, 159.85, 140.68, 138.17, 132.46, 130.53, 130.50, 130.39, 128.88, 128.76, 127.98, 126.90, 40.61, 37.94, 35.49, 28.29. HRMS $[C_{18}H_{17}Cl_2NO_2 + H]^+$: 350.0709 calculated, 350.0708 found.

(*E*)-4-(4-Chlorophenyl)-2-oxo-*N*-phenethylbut-3-enamide (37). *α-Ketoacid formation:* the α-ketoacid salt was prepared according to general procedure F using pyruvic acid (1.7 mL, 18 mmol, 1 eq), 4-chlorobenzaldehyde (2.2 mL, 19 mmol, 1 eq), KOH (2.1 g, 38 mmol, 2 eq) in MeOH affording potassium 4-(4-chlorophenyl)-2-oxobut-3-enoate **62a** (2.0 g, 8.0 mmol, 42%). *Amide coupling:* the title compound was prepared according to general procedure F using potassium salt **62a** (2.0 g, 8.0 mmol, 1 eq), oxalyl chloride (1.4 mL, 16 mmol, 2 eq), phenethylamine (1.1 mL, 8.8 mmol, 1.1 eq) and Et_3N (2.2 mL, 16 mmol, 2 eq) in DCM, affording the product (0.30 g, 0.96 mmol, 12%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.86 (d, *J* = 16.2 Hz, 1H), 7.73 (d, *J* = 16.1 Hz, 1H), 7.59 (d, *J* = 8.5 Hz, 2H), 7.43 – 7.36 (m, 2H), 7.36 – 7.28 (m, 2H), 7.28 – 7.17 (m, 4H), 3.63 (q, *J* = 7.0 Hz, 2H), 2.89 (t, *J* = 7.1 Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 185.32, 161.24, 146.51, 138.34, 137.61, 132.94, 130.37, 129.48, 128.87, 128.81, 126.86, 119.09, 40.77, 35.58. HRMS $[C_{18}H_{16}ClNO_2 + H]^+$: 314.0942 calculated, 314.0939 found.

(*E*)-2-Oxo-*N*-phenethyl-4-phenylbut-3-enamide (38). *α-Ketoacid formation:* the α-ketoacid salt was prepared according to general procedure F using pyruvic acid (0.79 mL, 11 mmol, 1 eq), benzaldehyde (1.2 g, 11 mmol, 1 eq), KOH (0.98 g, 17 mmol, 1.5 eq) in MeOH affording potassium 2-oxo-4-phenylbut-3-enoate **62b** (0.85 g, 3.9 mmol, 36%). *Amide coupling:* the title compound was prepared according to general procedure C using potassium salt **62b** (0.20 g, 0.93 mmol, 1 eq), phenethylamine (0.12 mL, 0.93 mmol, 1 eq), HCTU (0.39 g, 0.93 mmol, 1 eq) and DiPEA (0.32 mL, 1.86 mmol, 2 eq) in DMF (5 mL) affording the product (70 mg, 0.25 mmol, 27%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.93 (d, *J* = 16.2 Hz, 1H), 7.77 (d, *J* = 16.1 Hz, 1H), 7.71 – 7.62 (m, 2H), 7.49 – 7.37 (m, 3H), 7.36 – 7.16 (m, 6H), 3.63 (q, *J* = 7.0 Hz, 2H), 2.89 (t, *J* = 7.1 Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 185.49, 161.38, 148.12, 138.40, 134.48, 131.60, 129.28, 129.15, 128.85, 128.82, 126.83, 118.67, 40.76, 35.61. HRMS $[C_{18}H_{17}NO_2 + H]^+$: 280.1332 calculated, 280.1331 found.

(*E*)-4-(4-Methoxyphenyl)-2-oxo-*N*-phenethylbut-3-enamide (39). *α-Ketoacid formation:* the α-ketoacid salt was prepared according to general procedure F using sodium pyruvate (3.0 g, 27 mmol, 1 eq), 4-methoxybenzaldehyde (3.3 mL, 27 mmol, 1 eq), KOH (2.3 g, 41 mmol, 1.5 eq) in MeOH affording potassium 4-(4-methoxyphenyl)-2-oxobut-3-enoate **62c** (6.5 g, 27 mmol, 98%). *Amide coupling:* the title compound was prepared according to general procedure F using potassium salt **62c** (1.0 g, 4.1 mmol, 1 eq), oxalyl chloride (0.87 mL, 8.2 mmol, 2 eq), phenethylamine (0.52 mL, 4.1 mmol, 1 eq) and Et_3N (1.1 mL, 8.2 mmol, 2 eq) in DCM, affording the product (1.2 g, 3.8 mmol, 93%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.89 (d, *J* = 16.0 Hz, 1H), 7.68 – 7.57 (m, 3H), 7.37 – 7.27 (m, 3H), 7.26 – 7.18 (m, 3H), 6.95 – 6.88 (m, 2H), 3.83 (s, 3H), 3.61 (q, *J* = 7.0 Hz, 2H), 2.88 (t, *J* = 7.2 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 185.15, 162.54, 161.66, 147.93, 138.44, 131.23, 128.76, 127.27, 126.72, 116.20, 114.59, 55.50, 40.69, 35.56. HRMS $[C_{19}H_{19}NO_3 + H]^+$: 310.1438 calculated, 310.1435 found.

(*E*)-4-(4-Bromophenyl)-2-oxo-*N*-phenethylbut-3-enamide (40). *α-Ketoacid formation:* the α-ketoacid salt was prepared according to general procedure F using pyruvic acid (1.4 mL, 16 mmol, 1 eq), 4-bromobenzaldehyde (1.6 mL, 16 mmol, 1 eq), KOH (1.8 g, 32 mmol, 2 eq) in MeOH affording potassium 4-(4-bromophenyl)-2-oxobut-3-enoate **62d** (2.0 g, 6.8 mmol, 42%). *Amide coupling:* the title compound was prepared according to general procedure F using potassium salt **62d** (2.0 g, 6.8 mmol, 1 eq), oxalyl chloride (1.2 mL, 14 mmol, 2 eq), phenethylamine (0.94 mL, 7.5 mmol, 1.1 eq) and Et_3N (1.90 mL, 14 mmol, 2 eq) in DCM, affording the product (0.84 g, 2.3 mmol, 34%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.84 (d, *J* = 16.2 Hz, 1H), 7.74 (d, *J* = 16.1 Hz, 1H), 7.64 – 7.42 (m, 4H), 7.38 – 7.15 (m, 6H), 3.62 (q, *J* = 7.0 Hz, 2H), 2.89 (t, *J* = 7.1 Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 185.37, 161.22, 146.55, 138.35, 133.37, 132.45, 130.52, 128.87, 128.81, 126.86, 126.08, 119.23, 40.77, 35.60. HRMS $[C_{18}H_{16}BrNO_2 + H]^+$: 358.0437 calculated, 358.0437 found.

(*E*)-4-(3-Bromophenyl)-2-oxo-*N*-phenethylbut-3-enamide (41). *α-Ketoacid formation:* the α-ketoacid salt was prepared according to general procedure F using sodium pyruvate (1.0 g, 11 mmol, 1 eq), 3-bromobenzaldehyde (1.3 mL, 11 mmol, 1 eq), KOH (0.96 g, 17 mmol, 1.5 eq) in MeOH affording potassium 4-(3-bromophenyl)-2-oxobut-3-enoate **62e** (0.40 g, 1.4 mmol, 12%). *Amide coupling:* the title compound was prepared according to general procedure F using potassium salt **62e** (0.40 g, 1.4 mmol, 1 eq), oxalyl chloride (0.24 mL, 2.7 mmol, 2 eq), phenethylamine (0.19 mL, 1.5 mmol, 1.1 eq) and Et_3N (0.38 mL, 2.7 mmol, 2 eq) in

DCM, affording the product (0.31 g, 0.87 mmol, 62%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.86 – 7.78 (m, 2H), 7.74 (d, *J* = 16.2 Hz, 1H), 7.62 – 7.51 (m, 2H), 7.37 – 7.28 (m, 3H), 7.28 – 7.19 (m, 4H), 3.63 (q, *J* = 7.0 Hz, 2H), 2.89 (t, *J* = 7.1 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 185.33, 161.12, 146.13, 138.34, 136.52, 134.25, 131.84, 130.63, 128.88, 128.82, 127.72, 126.88, 123.27, 119.96, 40.78, 35.59. HRMS $[C_{18}H_{16}BrNO_2 + H]^+$: 358.0437 calculated, 358.0437 found.

(*E*)-4-([1,1'-Biphenyl]-3-yl)-2-oxo-*N*-phenethylbut-3-enamide (42). A round bottom flask was charged with aryl bromide **41** (0.20 g, 0.56 mmol, 1 eq) and toluene/EtOH (4:1, 3 mL) and degassed for 20 min with sonication. $Pd(PPh_3)_4$ (13 mg, 0.01 mmol, 2 mol%), phenylboronic acid (0.10 g, 0.84 mmol, 1.5 eq) and K_2CO_3 (0.46 g, 3.4 mmol, 6 eq) were added and the reaction was stirred for 16 h at 80 °C. The reaction mixture was filtered over a pad of Celite and concentrated under reduced pressure. The crude residue was purified by silica gel column chromatography (EtOAc/pentane) affording the product (0.15 g, 0.42 mmol, 75%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.03 (d, *J* = 16.2 Hz, 1H), 7.94 – 7.78 (m, 2H), 7.73 – 7.18 (m, 14H), 3.67 (q, *J* = 6.9 Hz, 2H), 2.93 (t, *J* = 7.1 Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 185.46, 161.38, 148.00, 142.21, 140.23, 138.39, 134.95, 130.35, 129.57, 129.02, 128.85, 128.81, 128.04, 127.94, 127.91, 127.24, 126.82, 118.96, 40.76, 35.59. HRMS $[C_{24}H_{21}NO_2 + H]^+$: 356.1645 calculated, 356.1641 found.

***N*-(4-Methylphenethyl)-2-oxo-5-phenylpentanamide (43).** The title compound was prepared according to general procedure D using α-ketoacid **60c** (57 mg, 0.29 mmol, 1 eq), 2-(*p*-tolyl)ethan-1-amine (47 μL, 0.32 mmol, 1.1 eq), EDC·HCl (85 mg, 0.44 mmol, 1.5 eq), HOBt (60 mg, 0.44 mmol, 1.5 eq) in DCM. Column chromatography (2.5% -> 20% EtOAc in pentane) afforded the product (18 mg, 58 μmol, 20%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39 – 7.22 (m, 3H), 7.22 – 7.14 (m, 3H), 7.12 (d, *J* = 7.9 Hz, 2H), 7.07 (d, *J* = 8.0 Hz, 2H), 7.01 – 6.86 (m, 1H), 3.52 (q, *J* = 7.0 Hz, 2H), 2.93 (t, *J* = 7.3 Hz, 2H), 2.80 (t, *J* = 7.1 Hz, 2H), 2.65 (t, *J* = 7.6 Hz, 2H), 2.32 (s, 3H), 1.93 (p, *J* = 7.5 Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 199.02, 160.11, 141.47, 136.44, 135.16, 129.56, 128.65, 128.61, 128.56, 126.21, 126.16, 40.68, 36.24, 35.13, 24.94, 21.18. HRMS $[C_{20}H_{23}NO_2 + H]^+$: 310.1802 calculated, 310.1803 found.

***N*-(4-Methoxyphenethyl)-2-oxo-5-phenylpentanamide (44).** The title compound was prepared according to general procedure D using α-ketoacid **60c** (89 mg, 0.46 mmol, 1 eq), 2-(4-methoxyphenyl)ethan-1-amine (75 μl, 0.51 mmol, 1.1 eq), EDC·HCl (0.13 g, 0.69 mmol, 1.5 eq), HOBt (94 mg, 0.69 mmol, 1.5 eq) in DCM. Column chromatography (2.5% -> 40% EtOAc in pentane) afforded the product (13 mg, 40 μmol, 9%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.32 – 7.24 (m, 2H), 7.23 – 7.14 (m, 3H), 7.10 (d, *J* = 8.6 Hz, 2H), 7.04 – 6.90 (m, 1H), 6.85 (d, *J* = 8.6 Hz, 2H), 3.79 (s, 3H), 3.50 (q, *J* = 6.9 Hz, 2H), 2.94 (t, *J* = 7.3 Hz, 2H), 2.78 (t, *J* = 7.1 Hz, 2H), 2.65 (t, *J* = 7.6 Hz, 2H), 1.93 (p, *J* = 7.5 Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 199.03, 160.10, 158.52, 141.45, 130.25, 129.75, 128.60, 128.54, 126.15, 114.27, 55.39, 40.77, 36.23, 35.14, 34.65, 24.94. HRMS $[C_{20}H_{23}NO_3 + H]^+$: 326.1751 calculated, 326.1752 found.

***N*-(3,4-Dimethoxyphenethyl)-2-oxo-5-phenylpentanamide (45).** The title compound was prepared according to general procedure D using α-ketoacid **60c** (57 mg, 0.30 mmol, 1 eq), 2-(3,4-dimethoxyphenyl)ethan-1-amine (56 μL, 0.33 mmol, 1.1 eq), EDC·HCl (86 mg, 0.45 mmol, 1.5 eq), HOBt (61 mg, 0.45 mmol, 1.5 eq) in DCM. Column chromatography (2.5% -> 40% EtOAc in pentane) afforded the product (6 mg, 17 μmol, 6%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.31 – 7.26 (m, 2H), 7.22 – 7.11 (m, 3H), 7.03 – 6.91 (m, 1H), 6.81 (d, *J* = 8.1 Hz, 1H), 6.77 – 6.66 (m, 2H), 3.87 (s, 3H), 3.86 (s, 3H), 3.52 (q, *J* = 7.0 Hz, 2H), 2.94 (t, *J* = 7.3 Hz, 2H), 2.78 (t, *J* = 7.1 Hz, 2H), 2.65 (t, *J* = 7.6 Hz, 2H), 1.93 (p, *J* = 7.5 Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 199.04, 160.13, 149.01, 147.96, 141.44, 130.77, 128.56, 126.17, 120.73, 111.86, 111.53, 56.02, 40.69, 36.25, 35.16, 24.95. HRMS $[C_{21}H_{25}NO_4 + H]^+$: 356.1856 calculated, 356.1858 found.

***N*-(4-Hydroxyphenethyl)-2-oxo-5-phenylpentanamide (46).** The title compound was prepared according to general procedure D using α-ketoacid **60c** (98 mg, 0.51 mmol, 1 eq), 4-(2-aminoethyl)phenol (77 mg, 0.56 mmol, 1.1 eq), EDC·HCl (0.15 g, 0.77 mmol, 1.5 eq), HOBt (0.10 g, 0.77 mmol, 1.5 eq) in DCM. Column chromatography (10% -> 60% EtOAc in pentane) afforded the product (18 mg, 58 μmol, 20%). ^{1}H NMR (400

MHz, $CDCl_3$) δ 7.32 – 7.23 (m, 2H), 7.23 – 7.12 (m, 3H), 7.04 (d, *J* = 8.5 Hz, 2H), 7.01 – 6.90 (m, 1H), 6.83 – 6.72 (m, 2H), 5.26 (br s, 1H), 3.50 (q, *J* = 7.0 Hz, 2H), 2.93 (t, *J* = 7.3 Hz, 2H), 2.76 (t, *J* = 7.1 Hz, 2H), 2.64 (t, *J* = 7.6 Hz, 2H), 1.93 (p, *J* = 7.5 Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 198.97, 160.18, 154.71, 141.43, 130.14, 129.92, 128.61, 128.55, 126.16, 115.75, 40.84, 36.27, 35.13, 34.65, 24.95. HRMS $[C_{19}H_{21}NO_3 + H]^+$: 312.1594 calculated, 312.1595 found.

***N*-(4-Bromophenethyl)-2-oxo-5-phenylpentanamide (47).** The title compound was prepared according to general procedure D using α-ketoacid **60c** (0.96 g, 5.0 mmol, 1 eq), 2-(4-bromophenyl)ethan-1-amine (0.85 mL, 5.5 mmol, 1.1 eq), EDC·HCl (1.5 g, 7.5 mmol, 1.5 eq), HOBt (1.2 g, 7.5 mmol, 1.5 eq) and Et_3N (1.4 mL, 10 mmol, 2.0 eq) in DCM. Column chromatography (2.5% -> 20% EtOAc in pentane) afforded the product (0.28 g, 0.74 mmol, 15%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.41 (d, *J* = 7.6 Hz, 2H), 7.32 – 7.22 (m, 2H), 7.22 – 7.12 (m, 3H), 7.04 (d, *J* = 7.8 Hz, 2H), 7.02 – 6.95 (m, 1H), 3.50 (q, *J* = 6.8 Hz, 2H), 2.92 (t, *J* = 7.2 Hz, 2H), 2.78 (t, *J* = 7.1 Hz, 2H), 2.64 (t, *J* = 7.5 Hz, 2H), 1.92 (p, *J* = 7.3 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 198.86, 160.13, 141.35, 137.23, 131.87, 130.47, 128.55, 128.51, 126.12, 120.68, 40.31, 36.18, 35.07, 34.91, 24.87. HRMS $[C_{19}H_{20}NBrO_2 + H]^+$: 374.0750 calculated, 374.0751 found.

***N*-(3-Chlorophenethyl)-2-oxo-5-phenylpentanamide (48).** The title compound was prepared according to general procedure D using α-ketoacid **60c** (72 mg, 0.37 mmol, 1 eq), 2-(3-chlorophenyl)ethan-1-amine (57 μl, 0.41 mmol, 1.1 eq), EDC·HCl (0.11 g, 0.56 mmol, 1.5 eq), HOBt (86 mg, 0.56 mmol, 1.5 eq) in DCM. Column chromatography (2.5% -> 40% EtOAc in pentane) afforded the product (29 mg, 87 μmol, 24%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.33 – 7.13 (m, 8H), 7.11 – 7.02 (m, 1H), 7.02 – 6.90 (m, 1H), 3.53 (q, *J* = 6.8 Hz, 2H), 2.93 (t, *J* = 7.3 Hz, 2H), 2.82 (t, *J* = 7.2 Hz, 2H), 2.65 (t, *J* = 7.6 Hz, 2H), 1.94 (p, *J* = 7.5 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 198.91, 165.51, 140.31, 130.11, 128.94, 128.61, 128.56, 127.12, 126.97, 126.17, 40.35, 36.23, 35.24, 35.15, 24.95. HRMS $[C_{19}H_{20}ClNO_2 + H]^+$: 330.1255 calculated, 330.1256 found.

***N*-(2-Chlorophenethyl)-2-oxo-5-phenylpentanamide (49).** The title compound was prepared according to general procedure D using α-ketoacid **60c** (81 mg, 0.42 mmol, 1 eq), 2-(2-chlorophenyl)ethan-1-amine (66 μl, 0.47 mmol, 1.1 eq), EDC·HCl (0.12 g, 0.64 mmol, 1.5 eq) and HOBt (86 mg, 0.64 mmol, 1.5 eq) in DCM. Column chromatography (2.5% -> 20% EtOAc in pentane) afforded the product (11 mg, 32 μmol, 8%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.41 – 7.32 (m, 1H), 7.32 – 7.26 (m, 2H), 7.24 – 7.08 (m, 6H), 7.07 – 6.91 (m, 1H), 3.57 (q, *J* = 6.9 Hz, 2H), 2.99 (t, *J* = 7.1 Hz, 2H), 2.93 (t, *J* = 7.3 Hz, 2H), 2.65 (t, *J* = 7.6 Hz, 2H), 1.93 (p, *J* = 7.4 Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 198.96, 160.23, 141.45, 136.03, 134.27, 131.00, 129.90, 128.63, 128.56, 128.45, 127.23, 126.17, 39.08, 36.23, 35.15, 33.30, 24.96. HRMS $[C_{19}H_{20}ClNO_2 + H]^+$: 330.1255 calculated, 330.1256 found.

***N*-(2,4-Dichlorophenethyl)-2-oxo-5-phenylpentanamide (50).** The title compound was prepared according to general procedure D using α-ketoacid **60c** (95 mg, 0.49 mmol, 1 eq), 2-(2,4-dichlorophenyl)ethan-1-amine (89 μl, 0.54 mmol, 1.1 eq), EDC·HCl (0.14 g, 0.74 mmol, 1.5 eq) and HOBt (0.10 g, 0.74 mmol, 1.5 eq) in DCM. Column chromatography (2.5% -> 40% EtOAc in pentane) afforded the product (14 mg, 38 μmol, 8%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38 (s, 1H), 7.32 – 7.26 (m, 2H), 7.23 – 7.15 (m, 4H), 7.15 – 7.10 (m, 1H), 7.07 – 6.87 (m, 1H), 3.54 (q, *J* = 6.8 Hz, 2H), 2.94 (q, *J* = 7.5 Hz, 4H), 2.65 (t, *J* = 7.6 Hz, 2H), 1.94 (p, *J* = 7.4 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 198.87, 160.25, 141.40, 134.92, 134.63, 133.46, 131.72, 129.68, 128.61, 128.56, 127.50, 126.18, 38.91, 36.21, 35.13, 32.79, 24.93. HRMS $[C_{19}H_{19}Cl_2NO_2 + H]^+$: 364.0866 calculated, 364.0867 found.

2-Oxo-*N*-(4-phenoxyphenethyl)-5-phenylpentanamide (51, LEI-301). The title compound was prepared according to general procedure D using α-ketoacid **60c** (0.19 g, 1.0 mmol, 1 eq), 2-(4-phenoxyphenyl)ethan-1-amine TFA salt (0.36 g, 1.1 mmol, 1.1 eq), EDC·HCl (0.29 g, 1.5 mmol, 1.5 eq), HOBt (0.23 g, 1.5 mmol, 1.5 eq) and Et_3N (0.28 mL, 2.0 mmol, 2.0 eq) in DCM. Column chromatography (2.5% -> 20% EtOAc in pentane) afforded the product (32 mg, 86 μmol, 9%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36 – 7.24 (m, 4H), 7.22 – 7.06 (m, 6H), 7.04 – 6.91 (m, 5H), 3.53 (q, *J* = 6.7 Hz, 2H), 2.94 (t, *J* = 7.2 Hz, 2H), 2.81 (t, *J* = 7.1 Hz, 2H), 2.65 (t, *J* = 7.5 Hz, 2H), 1.94 (p, *J* = 7.4 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 199.01, 160.14, 157.33, 156.18, 141.42, 133.07,

130.04, 129.86, 128.60, 128.55, 126.16, 123.36, 119.24, 118.95, 40.68, 36.23, 35.13, 34.82, 24.93. HRMS $[C_{25}H_{25}NO_3 + H]^+$: 388.1907 calculated, 388.1909 found.

2-Oxo-5-phenyl-*N*-(4-(pyrazin-2-yloxy)phenethyl)pentanamide (52). *Boc-deprotection **65a**:* a round bottom flask was charged with Boc-protected amine **65a** (0.32 g, 1.0 mmol, 1 eq) and HCl (4 M in dioxane, 4.5 mL, 18 mmol, 18 eq). After stirring for 1 h the mixture was concentrated under reduced pressure and coevaporated with toluene (3x), which afforded the deprotected amine **66a** as the HCl salt (0.25 g, 1.0 mmol, quant.). *Amide coupling:* the title compound was prepared according to general procedure D using α-ketoacid **60c** (38 mg, 0.20 mmol, 1 eq), EDC·HCl (46 mg, 0.24 mmol, 1.2 eq), HOBt (32 mg, 0.24 mmol, 1.2 eq), NMM (87 μL, 0.80 mmol, 4 eq) and the amine HCl salt **66a** (60 mg, 0.24 mmol, 1.2 eq). Column chromatography (30% -> 70% EtOAc in pentane) afforded the product (23 mg, 59 μmol, 30%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.43 (d, *J* = 1.3 Hz, 1H), 8.26 (d, *J* = 2.7 Hz, 1H), 8.10 (dd, *J* = 2.7, 1.4 Hz, 1H), 7.33 – 7.22 (m, 4H), 7.22 – 7.15 (m, 3H), 7.14 – 7.08 (m, 2H), 7.07 – 6.98 (m, 1H), 3.57 (q, *J* = 7.0 Hz, 2H), 2.95 (t, *J* = 7.3 Hz, 2H), 2.87 (t, *J* = 7.2 Hz, 2H), 2.66 (t, *J* = 7.6 Hz, 2H), 1.94 (p, *J* = 7.4 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 198.97, 160.17, 151.83, 141.42, 141.17, 138.62, 136.08, 135.48, 130.17, 128.60, 128.54, 126.16, 121.66, 40.54, 36.23, 35.13, 35.01, 24.92. HRMS $[C_{23}H_{23}N_3O_3 + H]^+$: 390.1812 calculated, 390.1823 found.

2-Oxo-5-phenyl-*N*-(4-(pyrimidin-2-yloxy)phenethyl)pentanamide (53). *Boc-deprotection **65b**:* a round bottom flask was charged with Boc-protected amine **65b** (0.30 g, 0.94 mmol, 1 eq) and HCl (4 M in dioxane, 4.5 mL, 18 mmol, 19 eq). After stirring for 1 h the mixture was concentrated under reduced pressure and coevaporated with toluene (3x), which afforded the deprotected amine **66b** as the HCl salt (0.24 g, 0.94 mmol, quant.). *Amide coupling:* the title compound was prepared according to general procedure D using α-ketoacid **60c** (38 mg, 0.20 mmol, 1 eq), EDC·HCl (46 mg, 0.24 mmol, 1.2 eq), HOBt (32 mg, 0.24 mmol, 1.2 eq), NMM (87 μL, 0.80 mmol, 4 eq) and the amine HCl salt **66b** (76 mg, 0.24 mmol, 1.2 eq). Purification by preparative HPLC (C18 reverse phase, 45% to 55% ACN/H_2O + 0.2% TFA, RT 10.86 min) afforded the product (12 mg, 31 μmol, 15%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.56 (d, *J* = 4.8 Hz, 2H), 7.33 – 7.22 (m, 4H), 7.22 – 7.12 (m, 5H), 7.04 (t, *J* = 4.8 Hz, 2H), 3.57 (q, *J* = 6.9 Hz, 2H), 2.95 (t, *J* = 7.3 Hz, 2H), 2.87 (t, *J* = 7.2 Hz, 2H), 2.66 (t, *J* = 7.6 Hz, 2H), 1.95 (p, *J* = 7.4 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 198.96, 165.46, 160.19, 159.86, 151.70, 141.43, 135.50, 130.02, 128.61, 128.54, 126.15, 122.03, 116.32, 40.54, 36.24, 35.13, 35.04, 24.92. HRMS $[C_{23}H_{23}N_3O_3 + H]^+$: 390.1812 calculated, 390.1824 found.

2-Oxo-5-phenyl-*N*-(4-((6-(trifluoromethyl)pyridin-3-yl)oxy) phenethyl)pentanamide (54). *Boc-deprotection **65c**:* a round bottom flask was charged with Boc-protected amine **65c** (0.18 g, 0.48 mmol, 1 eq) and HCl (4 M in dioxane, 3 mL, 12 mmol, 25 eq). After stirring for 1 h the mixture was concentrated under reduced pressure and coevaporated with toluene (3x), which afforded the deprotected amine **66c** as the HCl salt (0.15 g, 0.48 mmol, quant.). *Amide coupling:* the title compound was prepared according to general procedure D using α-ketoacid **60c** (38 mg, 0.20 mmol, 1 eq), EDC·HCl (46 mg, 0.24 mmol, 1.2 eq), HOBt (32 mg, 0.24 mmol, 1.2 eq), NMM (87 μL, 0.80 mmol, 4 eq) and the amine HCl salt **66c** (68 mg, 0.24 mmol, 1.2 eq). Purification by preparative HPLC (C18 reverse phase, 65% to 75% ACN/H_2O + 0.2% TFA, RT 8.55 min) afforded the product (21 mg, 46 μmol, 23%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.46 (d, *J* = 2.7 Hz, 1H), 7.62 (d, *J* = 8.6 Hz, 1H), 7.34 – 7.22 (m, 5H), 7.21 – 7.15 (m, 3H), 7.07 – 6.95 (m, 3H), 3.56 (q, *J* = 6.9 Hz, 2H), 2.95 (t, *J* = 7.3 Hz, 2H), 2.87 (t, *J* = 7.2 Hz, 2H), 2.69 – 2.62 (m, 2H), 1.94 (p, *J* = 7.5 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 198.97, 160.15, 156.50, 153.74, 142.12 (q, *J* = 35.1 Hz), 141.38, 140.89, 135.45, 130.69, 128.60, 128.56, 126.18, 124.53, 124.08 (q, *J* = 209.2 Hz), 121.63 (q, *J* = 2.7 Hz), 120.28, 40.55, 36.23, 35.12, 34.92, 24.93. HRMS $[C_{25}H_{23}F_3N_2O_3 + H]^+$: 457.1734 calculated, 457.1743 found.

2-Oxo-5-phenyl-*N*-(4-((5-(trifluoromethyl)pyridin-2-yl)oxy) phenethyl)pentanamide (55). *Boc-deprotection **65d**:* a round bottom flask was charged with Boc-protected amine **65d** (0.19 g, 0.50 mmol, 1 eq) and HCl (4 M in dioxane, 4.5 mL, 18 mmol, 36 eq). After stirring for 1 h the mixture was concentrated under reduced pressure and coevaporated with toluene (3x), which afforded the deprotected amine **66d** as the HCl salt (0.16 g, 0.50 mmol, quant.). *Amide coupling:* the title compound was prepared according to general procedure D using α-

ketoacid **60c** (38 mg, 0.20 mmol, 1 eq), EDC·HCl (46 mg, 0.24 mmol, 1.2 eq), HOBt (32 mg, 0.24 mmol, 1.2 eq), NMM (87 μL, 0.80 mmol, 4 eq) and the amine HCl salt **66d** (76 mg, 0.24 mmol, 1.2 eq). Purification by preparative HPLC (C18 reverse phase, 55% to 65% ACN/H_2O + 0.2% TFA, RT 8.74 min) afforded the product (26 mg, 57 μmol, 28%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.51 – 8.37 (m, 1H), 7.90 (dd, *J* = 8.7, 2.3 Hz, 1H), 7.32 – 7.22 (m, 4H), 7.22 – 7.15 (m, 3H), 7.13 – 7.07 (m, 2H), 7.07 – 6.98 (m, 2H), 3.57 (q, *J* = 7.0 Hz, 2H), 2.95 (t, *J* = 7.3 Hz, 2H), 2.87 (t, *J* = 7.2 Hz, 2H), 2.74 – 2.59 (m, 2H), 1.94 (p, *J* = 7.5 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 198.97, 165.88, 160.18, 151.99, 145.56 (q, *J* = 4.3 Hz), 141.41, 136.85 (q, *J* = 3.2 Hz), 135.52, 130.17, 128.60, 128.55, 126.16, 123.79 (q, *J* = 271.4 Hz), 121.86, 121.49, 111.52, 40.54, 36.24, 35.13, 35.01, 24.93. HRMS [$C_{25}H_{23}F_3N_2O_3$ + H]$^+$: 457.1734 calculated, 457.1746 found.

***N*-(4-((4-Chloropyrimidin-2-yl)oxy)phenethyl)-2-oxo-5-phenyl pentanamide (56).** *Boc-deprotection **65e**:* a round bottom flask was charged with Boc-protected amine **65e** (0.18 g, 0.50 mmol, 1 eq) and HCl (4 M in dioxane, 3 mL, 12 mmol, 24 eq). After stirring for 1 h the mixture was concentrated under reduced pressure and coevaporated with toluene (3x), which afforded the deprotected amine **66e** as the HCl salt (0.14 g, 0.50 mmol, quant.). *Amide coupling:* the title compound was prepared according to general procedure D using α-ketoacid **60c** (38 mg, 0.20 mmol, 1 eq), EDC·HCl (46 mg, 0.24 mmol, 1.2 eq), HOBt (32 mg, 0.24 mmol, 1.2 eq), NMM (87 μL, 0.80 mmol, 4 eq) and the amine HCl salt **66e** (60 mg, 0.24 mmol, 1.2 eq). Purification by preparative HPLC (C18 reverse phase, 50% to 60% ACN/H_2O + 0.2% TFA, RT 10.44 min) afforded the product (20 mg, 47 μmol, 24%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.43 (d, *J* = 5.7 Hz, 1H), 7.32 – 7.22 (m, 4H), 7.22 – 7.14 (m, 3H), 7.14 – 7.07 (m, 2H), 7.04 (t, *J* = 5.7 Hz, 1H), 6.78 (d, *J* = 5.7 Hz, 1H), 3.57 (q, *J* = 6.9 Hz, 2H), 2.95 (t, *J* = 7.3 Hz, 2H), 2.88 (t, *J* = 7.2 Hz, 2H), 2.70 – 2.60 (m, 2H), 1.94 (p, *J* = 7.5 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 198.95, 170.46, 160.74, 160.38, 160.18, 150.64, 141.39, 136.40, 130.27, 128.59, 128.54, 126.16, 121.62, 106.77, 40.51, 36.23, 35.12, 35.01, 24.92. HRMS [$C_{23}H_{22}ClN_3O_3$ + Na]$^+$: 446.1242 calculated, 446.1274 found.

***tert*-Butyl 2-(methoxyamino)-2-oxoacetate (58).** Literature procedure[43]: a round bottom flask was charged with oxalyl chloride (13.5 ml, 158 mmol, 1 eq) in dry THF (200 mL) under an inert atmosphere and was cooled to 0 °C. *tert*-Butanol (14.7 ml, 154 mmol, 0.975 eq.) was added in one batch and the mixture was stirred for 1 h at 0 °C. *N,O*-dimethylhydroxylamine hydrochloride (15.4 g, 158 mmol, 1 eq) was added to the reaction mixture followed by Et_3N (66 mL, 472 mmol, 3 eq). The reaction mixture was stirred for 2 h at 0 °C, followed by quenching with H_2O (200 mL). The aqueous layer was extracted with EtOAc (2 x 200 mL). The combined organic layers were washed with sat. aq. $NaHCO_3$ (2 x 200 mL), brine (1 x 200 mL), dried ($MgSO_4$), filtered and concentrated under reduced pressure. The crude residue was purified using silica gel column chromatography (0% -> 20% EtOAc in pentane) affording the product (22.3 g, 117 mmol, 75%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.76 (s, 3H), 3.20 (s, 3H), 1.56 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 161.98, 161.63, 83.89, 61.82, 30.88, 27.62.

***tert*-Butyl 4-(4-chlorophenyl)-2-oxobutanoate (59a).** The title compound was prepared according to general procedure A using magnesium (0.42 gram, 18.3 mmol, 2.0 eq), 1-(2-bromoethyl)-4-chlorobenzene (1.3 mL, 9.1 mmol, 1 eq) and Weinreb amide **58** (1.7 gram, 9.1 mmol, 1.0 eq), affording the product (0.85 g, 3.2 mmol, 35%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.24 (d, *J* = 8.4 Hz, 2H), 7.14 (d, *J* = 8.4 Hz, 2H), 3.10 (t, *J* = 7.3 Hz, 2H), 2.90 (t, *J* = 7.4 Hz, 2H), 1.53 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 194.31, 160.25, 138.78, 132.05, 129.84, 128.63, 84.12, 40.57, 28.42, 27.78.

***tert*-Butyl 2-oxo-4-phenylbutanoate (59b).** The title compound was prepared according to general procedure A using magnesium (72 mg, 3.0 mmol, 0.7 eq), (2-bromoethyl)benzene (0.58 mL, 4.2 mmol, 1 eq) and Weinreb amide **58** (0.80 g, 4.2 mmol, 1 eq), affording the product (0.22 g, 0.95 mmol, 22%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.33 – 7.25 (m, 2H), 7.25 – 7.09 (m, 3H), 3.15 – 3.06 (m, 2H), 2.93 (t, *J* = 7.5 Hz, 2H), 1.53 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 194.74, 160.47, 140.38, 128.65, 128.49, 126.40, 84.11, 40.92, 29.20, 27.88.

***tert*-Butyl 2-oxo-5-phenylpentanoate (59c).** The title compound was prepared according to general procedure A using magnesium (2.3 g, 94 mmol, 2.0 eq), (3-bromopropyl)benzene (11 mL, 71 mmol, 1.5 eq) and Weinreb amide **58** (8.9 g, 47 mmol, 1.0 eq). Column chromatography (0% -> 10% EtOAc in pentane) afforded the product

(7.4 g, 30 mmol, 64%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.34 – 7.21 (m, 2H), 7.16 (t, *J* = 7.1 Hz, 3H), 2.76 (t, *J* = 7.3 Hz, 2H), 2.63 (t, *J* = 7.6 Hz, 2H), 1.92 (p, *J* = 7.4 Hz, 2H), 1.51 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 195.22, 160.57, 141.16, 128.34, 125.95, 83.65, 38.21, 34.75, 27.66, 24.56.

***tert*-Butyl 4-(2-chlorophenyl)-2-oxobutanoate (59d).** The title compound was prepared according to general procedure A using magnesium (0.17 g, 7.0 mmol, 2 eq), (4-bromobutyl)benzene (0.62 mL, 3.5 mmol, 1 eq) and Weinreb amide **58** (0.67 g, 3.5 mmol, 1 eq), affording the product (0.33 g, 1.3 mmol, 36%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.29 – 7.20 (m, 2H), 7.20 – 7.10 (m, 3H), 2.83 – 2.70 (m, 2H), 2.66 – 2.55 (m, 2H), 1.71 – 1.57 (m, 4H), 1.51 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 195.46, 160.77, 141.94, 128.36, 128.31, 125.79, 83.76, 38.89, 35.59, 30.69, 27.77, 22.64.

***tert*-Butyl 2-oxo-2-phenylacetate (59e).** The title compound was prepared according to general procedure A using magnesium (0.21 g, 8.5 mmol, 2 eq), bromobenzene (0.43 mL, 4.2 mmol, 1 eq) and Weinreb amide **58** (0.80 g, 4.2 mmol, 1 eq), affording the product (0.73 g, 3.5 mmol, 83%).^{1}H NMR (400 MHz, MeOD) δ 7.97 – 7.87 (m, 2H), 7.58 (t, *J* = 7.4 Hz, 1H), 7.46 (t, *J* = 7.7 Hz, 2H), 1.55 (s, 9H). ^{13}C NMR (101 MHz, MeOD) δ 187.86, 164.89, 135.68, 133.15, 130.42, 129.84, 85.53, 85.32, 28.22.

***tert*-Butyl 2-oxo-4-(*p*-tolyl)butanoate (59f).** The title compound was prepared according to general procedure A using magnesium (0.24 g, 10 mmol, 2 eq), 1-(2-bromoethyl)-4-methylbenzene (0.77 mL, 5.0 mmol, 1 eq) and Weinreb amide **58** (0.95 g, 5.0 mmol, 1 eq), affording the product (0.54 g, 2.2 mmol, 44%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.09 – 7.03 (m, 4H), 3.10 – 3.01 (m, 2H), 2.86 (t, *J* = 7.5 Hz, 2H), 2.28 (s, 3H), 1.50 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 194.42, 160.24, 137.09, 135.51, 129.06, 128.13, 83.59, 40.81, 28.54, 27.58.

***tert*-Butyl 2-oxo-4-(4-(trifluoromethyl)phenyl)butanoate (59g).** The title compound was prepared according to general procedure A using magnesium (0.18 g, 7.9 mmol, 2 eq), 1-(2-bromoethyl)-4-(trifluoromethyl)benzene (0.67 mL, 4.0 mmol, 1 eq) and Weinreb amide **58** (0.75 g, 4.0 mmol, 1 eq), affording the product (0.21 g, 0.83 mmol, 21%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.53 (d, *J* = 8.1 Hz, 2H), 7.33 (d, *J* = 8.0 Hz, 2H), 3.25 – 3.09 (m, 2H), 2.99 (t, *J* = 7.3 Hz, 2H), 1.53 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 194.12, 160.18, 144.54 (q, *J* = 1.1 Hz), 128.86, 128.67 (q, *J* = 36.7 Hz), 125.45 (q, *J* = 3.8 Hz), 124.30 (q, *J* = 272.0 Hz), 84.21, 40.27, 28.83, 27.70.

***tert*-Butyl 4-(4-fluorophenyl)-2-oxobutanoate (59h).** The title compound was prepared according to general procedure A using magnesium (0.24 g, 10.6 mmol, 2 eq), 1-(2-bromoethyl)-4-fluorobenzene (0.72 mL, 5.3 mmol, 1 eq) and Weinreb amide **59** (1.1 g, 5.3 mmol, 1 eq), affording the product (0.28 g, 1.1 mmol, 21%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.23 – 7.07 (m, 2H), 7.04 – 6.88 (m, 2H), 3.09 (t, *J* = 7.5 Hz, 2H), 2.91 (t, *J* = 7.4 Hz, 2H), 1.53 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 194.55, 162.77, 160.37 (d, *J* = 5.3 Hz), 136.00 (d, *J* = 3.2 Hz), 129.93 (d, *J* = 7.8 Hz), 115.37 (d, *J* = 21.2 Hz), 84.18, 40.90, 28.37, 27.85.

***tert*-Butyl 4-(2-chlorophenyl)-2-oxobutanoate (59i).** The title compound was prepared according to general procedure A using magnesium (0.22 g, 9.1 mmol, 2 eq), 1-(2-bromoethyl)-2-chlorobenzene (0.69 mL, 4.6 mmol, 1 eq) and Weinreb amide **58** (0.86 g, 4.6 mmol, 1 eq), affording the product (0.34 g, 1.3 mmol, 28%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.32 (dd, *J* = 7.4, 1.8 Hz, 1H), 7.25 (dd, *J* = 7.2, 2.1 Hz, 1H), 7.20 – 7.10 (m, 2H), 3.21 – 3.07 (m, 2H), 3.08 – 2.97 (m, 2H), 1.52 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 194.24, 160.12, 137.77, 133.79, 130.64, 129.48, 127.86, 126.93, 83.90, 38.87, 27.68, 27.14.

***tert*-Butyl 4-(3-chlorophenyl)-2-oxobutanoate (59j).** The title compound was prepared according to general procedure A using magnesium (0.22 g, 9.1 mmol, 2 eq), 1-(2-bromoethyl)-3-chlorobenzene (0.67 mL, 4.6 mmol, 1 eq) and Weinreb amide **58** (0.86 g, 4.6 mmol, 1 eq), affording the product (0.51 g, 1.9 mmol, 42%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.24 – 7.11 (m, 3H), 7.11 – 7.03 (m, 1H), 3.10 (t, *J* = 7.5 Hz, 2H), 2.89 (t, *J* = 7.5 Hz, 2H), 1.52 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 194.02, 160.08, 142.29, 134.06, 129.73, 128.45, 126.61, 126.38, 83.92, 40.29, 28.59, 27.64.

***tert*-Butyl 4-(3,4-dichlorophenyl)-2-oxobutanoate (59k).** The title compound was prepared according to general procedure A using magnesium (34 mg, 1.4 mmol, 1.2 eq), 4-(2-bromoethyl)-1,2-dichlorobenzene (0.30 g, 1.2 mmol, 1 eq) and Weinreb amide **58** (0.27 g, 1.4 mmol, 1.2 eq), affording the product (90 mg, 0.30 mmol, 25%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.43 – 7.22 (m, 2H), 7.05 (dd, *J* = 8.2, 1.6 Hz, 1H), 3.10 (t, *J* = 7.4 Hz, 2H), 2.89 (t, *J* = 7.4 Hz, 2H), 1.54 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 194.04, 160.22, 140.61, 132.46, 130.65, 130.53, 130.52, 128.06, 84.36, 40.28, 28.23, 27.86.

2-Oxo-5-phenylpentanoic acid (60c). The title compound was prepared according to general procedure B using α-ketoester **59c** (7.4 g, 30 mmol, 1 eq) and TFA (23 mL, 300 mmol, 10 eq) affording the product (5.8 g, 30 mmol, quant.). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.33 – 7.12 (m, 5H), 2.91 (t, *J* = 7.2 Hz, 2H), 2.66 (t, *J* = 7.5 Hz, 2H), 1.98 (p, *J* = 7.4 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 195.53, 160.50, 140.94, 128.63, 128.56, 126.32, 37.15, 34.86, 24.66. HRMS $[C_{11}H_{12}O_3 + H]^+$: 193.0859 calculated, 193.0859 found.

***N*-Boc-tyramine (64).** A round bottom flask was charged with tyramine (5.0 gram, 36 mmol, 1.0 eq), and dissolved in THF (160 mL). Boc_2O (8.1 gram, 37 mmol, 1.0 eq) and a solution of $NaHCO_3$ (3.4 gram, 40 mmol, 1.1 eq) in water (80 mL) was added and the reaction was stirred vigorously overnight. The mixture was then extracted with Et_2O (3 x 50 mL) and the combined organic layers were sequentially washed with 0.1 M HCl (1 x 100 mL), water (1 x 100 mL) and brine (1 x 100 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure. Purification by silica gel column chromatography (10% -> 40% EtOAc in pentane) afforded (9.1 g, 31 mmol, 85%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.65 (br s, 1H), 6.98 (d, *J* = 7.8 Hz, 2H), 6.80 (d, *J* = 8.0 Hz, 2H), 4.92 – 4.47 (m, 1H), 3.46 – 3.17 (m, 2H), 2.77 – 2.56 (m, 2H), 1.44 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 156.53, 155.06, 130.01, 129.81, 115.61, 79.87, 42.14, 35.22, 28.48. HRMS $[C_{13}H_{19}NO_3 + Na]^+$: 260.1257 calculated, 260.1253 found.

***tert*-Butyl (4-(pyrazin-2-yloxy)phenethyl)carbamate (65a).** The title compound was prepared according to general procedure E using *N*-Boc-tyramine **64** (0.48 g, 2.0 mmol, 1 eq), 2-chloropyrazine (0.18 mL, 2.0 mmol, 1 eq) and K_2CO_3 (0.55 g, 4.0 mmol, 2 eq) in DMSO (2 mL). Column chromatography (20% -> 60% EtOAc/pentane) afforded the product (0.50 g, 1.6 mmol, 79%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.40 (s, 1H), 8.23 (s, 1H), 8.08 (s, 1H), 7.25 (d, *J* = 8.0 Hz, 2H), 7.10 (d, *J* = 8.0 Hz, 2H), 5.14 – 4.70 (m, 1H), 3.58 – 3.20 (m, 2H), 2.82 (t, *J* = 6.8 Hz, 2H), 1.44 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 160.04, 155.80, 151.32, 140.91, 138.28, 136.15, 135.74, 130.02, 121.16, 78.97, 41.62, 35.53, 28.31. HRMS $[C_{17}H_{21}N_3O_3 + Na]^+$: 338.1475 calculated, 338.1469 found.

***tert*-Butyl (4-(pyrimidin-2-yloxy)phenethyl)carbamate (65b).** The title compound was prepared according to general procedure E using *N*-Boc-tyramine **64** (0.48 g, 2 mmol, 1 eq), 2-chloro-pyrimidine (0.23 g, 2 mmol, 1 eq) and K_2CO_3 (0.55 g, 4 mmol, 2 eq) in DMSO (2 mL). Column chromatography (40% -> 70% EtOAc/pentane) afforded the product (0.52 g, 1.7 mmol, 83%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.56 (d, *J* = 4.8 Hz, 2H), 7.31 – 7.19 (m, 2H), 7.14 (d, *J* = 8.4 Hz, 2H), 7.04 (t, *J* = 4.8 Hz, 1H), 4.84 – 4.29 (m, 1H), 3.40 (q, *J* = 6.3 Hz, 2H), 2.82 (t, *J* = 6.9 Hz, 2H), 1.44 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.60, 159.82, 155.95, 151.48, 136.25, 130.10, 121.77, 116.23, 79.26, 41.74, 35.75, 28.51. HRMS $[C_{17}H_{21}N_3O_3 + H]^+$: 316.1656 calculated, 316.1653 found.

***tert*-Butyl (4-((6-(trifluoromethyl)pyridin-3-yl)oxy)phenethyl) carbamate (65c).** The title compound was prepared according to general procedure E using *N*-Boc-tyramine **64** (0.25 g, 1.05 mmol, 1.05 eq), 2-trifluoromethyl-5-fluoropyridine (0.12 mL, 1 mmol, 1 eq) and K_2CO_3 (0.21 g, 1.5 mmol, 1.5 eq) in DMF (5 mL). Column chromatography (10% -> 40% EtOAc/pentane) afforded the product (0.28 g, 0.73 mmol, 73%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.46 (d, *J* = 2.6 Hz, 1H), 7.61 (d, *J* = 8.7 Hz, 1H), 7.45 – 7.15 (m, 3H), 7.02 (d, *J* = 8.5 Hz, 2H), 4.89 – 4.35 (m, 1H), 3.39 (q, *J* = 6.6 Hz, 2H), 2.82 (t, *J* = 7.1 Hz, 2H), 1.44 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 156.60, 155.96, 153.40, 140.77, 136.36, 130.76, 124.35, 121.54, 120.10, 79.38, 41.89, 35.75, 28.48. HRMS $[C_{19}H_{21}F_3N_2O_3 + H]^+$: 383.1577 calculated, 383.1576 found.

***tert*-Butyl(4-((5-(trifluoromethyl)pyridin-2-yl)oxy)phenethyl) carbamate (65d).** The title compound was prepared according to general procedure E using *N*-Boc-tyramine **64** (0.24 g, 1.0 mmol, 1 eq), 2-chloro-5-

(trifluoromethyl)pyridine (0.18 g, 1.0 mmol, 1 eq) and K_2CO_3 (0.28 g, 2.0 mmol, 2 eq). Column chromatography (20% -> 60% EtOAc/pentane) afforded the product (0.35 g, 0.92 mmol, 92%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.44 (s, 1H), 8.03 – 7.76 (m, 1H), 7.26 (d, *J* = 7.7 Hz, 2H), 7.09 (d, *J* = 8.2 Hz, 2H), 7.01 (d, *J* = 8.6 Hz, 1H), 4.80 – 4.21 (m, 1H), 3.40 (q, *J* = 6.0 Hz, 2H), 2.82 (t, *J* = 6.9 Hz, 2H), 1.45 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.95, 155.99, 151.80, 145.56, 136.77, 136.36, 130.28, 121.63, 111.48, 41.85, 35.81, 28.54. HRMS $[C_{19}H_{21}F_3N_2O_3 + H]^+$: 383.1577 calculated, 383.1575 found.

***tert*-Butyl (4-((2-chloropyrimidin-4-yl)oxy)phenethyl)carbamate (65e).** The title compound was prepared according to general procedure E using *N*-Boc-tyramine **64** (0.25 g, 1.05 mmol, 1.05 eq), 2,4-dichloropyrimidine (0.15 g, 1 mmol, 1 eq) and K_2CO_3 (0.21 g, 1.5 mmol, 1.5 eq) in DMF (5 mL). The reaction was stirred for 19 h at rt. Column chromatography (20% -> 80% EtOAc/pentane) afforded the product (0.22 g, 0.63 mmol, 63%).^{1}H NMR (300 MHz, $CDCl_3$) δ 8.42 (d, *J* = 5.7 Hz, 1H), 7.26 (d, *J* = 8.5 Hz, 2H), 7.13 – 7.03 (m, 2H), 6.78 (d, *J* = 5.7 Hz, 1H), 4.93 – 4.42 (m, 1H), 3.40 (q, *J* = 6.5 Hz, 2H), 2.83 (t, *J* = 7.0 Hz, 2H), 1.44 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.49, 160.66, 160.31, 155.91, 150.36, 137.23, 130.32, 121.30, 106.64, 79.32, 41.77, 35.77, 28.47. HRMS $[C_{17}H_{20}ClN_3O_3 + Na]^+$: 372.1085 calculated, 372.1080 found.

(*E*)-2-Oxo-*N*-phenethyl-4-(4-phenoxyphenyl)but-3-enamide (67). *α-Ketoacid formation:* the α-ketoacid salt was prepared according to general procedure F using sodium pyruvate (0.44 g, 5.1 mmol, 1 eq), 4-phenoxybenzaldehyde (1.0 g, 5.1 mmol, 1 eq), KOH (0.42 g, 7.6 mmol, 1.5 eq) in MeOH affording potassium 4-(4-phenoxyphenyl)-2-oxobut-3-enoate **62f** (0.51 g, 1.9 mmol, 38%). *Amide coupling:* the title compound was prepared according to general procedure C using potassium salt **62f** (0.20 g, 0.93 mmol, 1 eq), phenethylamine (0.26 mL, 2.1 mmol, 1.1 eq), HCTU (0.87 g, 2.1 mmol, 1.1 eq) and DiPEA (0.66 mL, 3.8 mmol, 2 eq) in DMF affording the product (0.31 g, 0.82 mmol, 88%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.93 (d, *J* = 15.9 Hz, 1H), 7.82 – 7.53 (m, 3H), 7.50 – 7.16 (m, 9H), 7.16 – 6.91 (m, 4H), 3.76 – 3.48 (m, 2H), 2.91 (t, *J* = 6.8 Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 185.23, 161.51, 160.85, 149.08, 147.45, 138.41, 131.18, 130.11, 129.07, 128.81, 126.78, 124.56, 120.11, 118.27, 117.19, 40.73, 35.59.

Associated Content

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:

Table S1, listing fatty acid changes in mock-, PLAAT2- or PLAAT5-transfected U2OS cells. Table S2, containing primer sequences used for qPCR experiments. Figure S1, showing absolute NAE levels of the lipidomics experiments as performed in Figure 3. Figure S2, displaying **LEI-301** selectivity on serine hydrolases and PLA_2 enzymes as measured by gel-based ABPP. HPLC-traces of **1** and **LEI-301**. Molecular formula strings (CSV)

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Notes

The authors declare no competing financial interest

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Abbreviations Used

PLAAT, phospholipase and acyltransferase; NAPE-PLD, *N*-acylphosphatidylethanolamine phospholipase D; NAPE, *N*-acylphosphatidylethanolamine; NAE, *N*-acylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; LPC, lyso-phosphatidylcholine; PA, phosphatidic acid; FFA, free fatty acid; ECS, endocannabinoid system; PEA = *N*-palmitoylethanolamine, POEA = *N*-palmitoleoylethanolamine, SEA = *N*-stearoylethanolamine, OEA = *N*-oleoylethanolamine, LEA = *N*-linoleoylethanolamine, AEA = *N*-arachidonoylethanolamine, EPEA = *N*-eicosapentaenoylethanolamine, DHEA = *N*-docosahexaenoylethanolamine; 2-AG, 2-arachidonoylglycerol; PLA2G4E, phospholipase A_2 group IV E; $CB_{1/2}$, cannabinoid receptor 1/2; DAGL, diacylglycerol lipase; FAAH, fatty acid amide hydrolase; MAGL, monoacylglycerol lipase; ABHD, α/β-hydrolase domain containing protein; ABPP, activity-based protein profiling; ABP, activity-based probe; FP-TAMRA, fluorophosphonate-carboxytetramethylrhodamine; DiPEA, *N*,*N*-diisopropylethylamine; EDC, *N*-(3-(dimethylamino)propyl)-N'-ethylcarbodiimide; HOBt, 1-hydroxybenzotriazole; HCTU, *O*-(6-Chlorobenzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium

hexafluorophosphate; HATU, 1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide hexafluorophosphate.

References

(1) Ueda, N.; Tsuboi, K.; Uyama, T. Metabolism of endocannabinoids and related *N*-acylethanolamines: canonical and alternative pathways. *FEBS J.* **2013,** 280, 1874-1894.

(2) Mardian, E. B.; Bradley, R. M.; Duncan, R. E. The HRASLS (PLA/AT) subfamily of enzymes. *J. Biomed. Sci.* **2015,** 22, 99.

(3) Kiser, P. D.; Golczak, M.; Palczewski, K. Chemistry of the retinoid (visual) cycle. *Chem. Rev.* **2014,** 114, 194-232.

(4) Golczak, M.; Kiser, P. D.; Sears, A. E.; Lodowski, D. T.; Blaner, W. S.; Palczewski, K. Structural basis for the acyltransferase activity of lecithin:retinol acyltransferase-like proteins. *J. Biol. Chem.* **2012,** 287, 23790-23807.

(5) Hussain, Z.; Uyama, T.; Tsuboi, K.; Ueda, N. Mammalian enzymes responsible for the biosynthesis of *N*-acylethanolamines. *Biochim. Biophys. Acta* **2017,** 1862, 1546-1561.

(6) Jin, X.-H.; Okamoto, Y.; Morishita, J.; Tsuboi, K.; Tonai, T.; Ueda, N. Discovery and characterization of a Ca^{2+}-independent phosphatidylethanolamine *N*-acyltransferase generating the anandamide precursor and its congeners. *J. Biol. Chem.* **2007,** 282, 3614-3623.

(7) Duncan, R. E.; Sarkadi-Nagy, E.; Jaworski, K.; Ahmadian, M.; Sul, H. S. Identification and functional characterization of adipose-specific phospholipase A2 (AdPLA). *J. Biol. Chem.* **2008,** 283, 25428-25436.

(8) Jin, X.-H.; Uyama, T.; Wang, J.; Okamoto, Y.; Tonai, T.; Ueda, N. cDNA cloning and characterization of human and mouse Ca^{2+}-independent phosphatidylethanolamine *N*-acyltransferases. *Biochim. Biophys. Acta* **2009,** 1791, 32-38.

(9) Uyama, T.; Jin, X.-H.; Tsuboi, K.; Tonai, T.; Ueda, N. Characterization of the human tumor suppressors TIG3 and HRASLS2 as phospholipid-metabolizing enzymes. *Biochim. Biophys. Acta* **2009,** 1791, 1114-1124.

(10) Uyama, T.; Morishita, J.; Jin, X.-H.; Okamoto, Y.; Tsuboi, K.; Ueda, N. The tumor suppressor gene H-Rev107 functions as a novel Ca^{2+}-independent cytosolic phospholipase $A_{1/2}$ of the thiol hydrolase type. *J. Lipid Res.* **2009,** 50, 685-693.

(11) Shinohara, N.; Uyama, T.; Jin, X.-H.; Tsuboi, K.; Tonai, T.; Houchi, H.; Ueda, N. Enzymological analysis of the tumor suppressor A-C1 reveals a novel group of phospholipid-metabolizing enzymes. *J. Lipid Res.* **2011,** 52, 1927-1935.

(12) Hsu, T. H.; Jiang, S. Y.; Chan, W. L.; Eckert, R. L.; Scharadin, T. M.; Chang, T. C. Involvement of RARRES3 in the regulation of Wnt proteins acylation and signaling activities in human breast cancer cells. *Cell Death Differ.* **2014,** 22, 801.

(13) Pang, X.-Y.; Cao, J.; Addington, L.; Lovell, S.; Battaile, K. P.; Zhang, N.; Rao, J. L. U. M.; Dennis, E. A.; Moise, A. R. Structure/function relationships of adipose phospholipase A_2 containing a Cys-His-His catalytic triad. *J. Biol. Chem.* **2012,** 287, 35260-35274.

(14) Elling, U.; Wimmer, R. A.; Leibbrandt, A.; Burkard, T.; Michlits, G.; Leopoldi, A.; Micheler, T.; Abdeen, D.; Zhuk, S.; Aspalter, I. M.; Handl, C.; Liebergesell, J.; Hubmann, M.; Husa, A.-M.; Kinzer, M.; Schuller, N.; Wetzel, E.; van de Loo, N.; Martinez, J. A. Z.; Estoppey, D.; Riedl, R.; Yang, F.; Fu, B.; Dechat, T.; Ivics, Z.; Agu, C. A.; Bell, O.; Blaas, D.; Gerhardt, H.; Hoepfner, D.; Stark, A.; Penninger, J. M. A reversible haploid mouse embryonic stem cell biobank resource for functional genomics. *Nature* **2017,** 550, 114–118.

(15) Staring, J.; von Castelmur, E.; Blomen, V. A.; van den Hengel, L. G.; Brockmann, M.; Baggen, J.; Thibaut, H. J.; Nieuwenhuis, J.; Janssen, H.; van Kuppeveld, F. J. M.; Perrakis, A.; Carette, J. E.; Brummelkamp, T. R. PLA2G16 represents a switch between entry and clearance of Picornaviridae. *Nature* **2017,** 541, 412–416.

(16) Uyama, T.; Ikematsu, N.; Inoue, M.; Shinohara, N.; Jin, X.-H.; Tsuboi, K.; Tonai, T.; Tokumura, A.; Ueda, N. Generation of *N*-acylphosphatidylethanolamine by members of the phospholipase A/acyltransferase (PLA/AT) family. *J. Biol. Chem.* **2012,** 287, 31905-31919.

(17) Wellner, N.; Diep, T. A.; Janfelt, C.; Hansen, H. S. *N*-acylation of phosphatidylethanolamine and its biological functions in mammals. *Biochim. Biophys. Acta* **2013,** 1831, 652-662.

(18) Maccarrone, M.; Bab, I.; Bíró, T.; Cabral, G. A.; Dey, S. K.; Di Marzo, V.; Konje, J. C.; Kunos, G.; Mechoulam, R.; Pacher, P.; Sharkey, K. A.; Zimmer, A. Endocannabinoid signaling at the periphery: 50 years after THC. *Trends Pharmacol. Sci.* **2015,** 36, 277-296.

(19) van Eenige, R.; van der Stelt, M.; Rensen, P. C. N.; Kooijman, S. Regulation of adipose tissue metabolism by the endocannabinoid system. *Trends Endrocrinol. Metab.* **2018,** 29, 326-337.

(20) Lutz, B.; Marsicano, G.; Maldonado, R.; Hillard, C. J. The endocannabinoid system in guarding against fear, anxiety and stress. *Nat. Rev. Neurosci.* **2015,** 16, 705–718.

(21) Ogura, Y.; Parsons, W. H.; Kamat, S. S.; Cravatt, B. F. A calcium-dependent acyltransferase that produces *N*-acyl phosphatidylethanolamines. *Nat. Chem. Biol.* **2016,** 12, 669–671.

(22) Shyu, R.-Y.; Hsieh, Y.-C.; Tsai, F.-M.; Wu, C.-C.; Jiang, S.-Y. Cloning and functional characterization of the HRASLS2 gene. *Amino Acids* **2008,** 35, 129-137.

(23) Hansen, H. S.; Vana, V. Non-endocannabinoid *N*-acylethanolamines and 2-monoacylglycerols in the intestine. *Br. J. Pharmacol.* **2019,** 176, 1443-1454.

(24) Fu, J.; Gaetani, S.; Oveisi, F.; Lo Verme, J.; Serrano, A.; Rodríguez de Fonseca, F.; Rosengarth, A.; Luecke, H.; Di Giacomo, B.; Tarzia, G.; Piomelli, D. Oleylethanolamide regulates feeding and body weight through activation of the nuclear receptor PPAR-α. *Nature* **2003,** 425, 90–93.

(25) Yamano, Y.; Asano, A.; Ohyama, K.; Ohta, M.; Nishio, R.; Morishima, I. Expression of the Ha-ras suppressor family member 5 gene in the maturing rat testis. *Biosci. Biotechnol. Biochem.* **2008,** 72, 1360-1363.

(26) Zhou, J.; Mock, E. D.; Martella, A.; Kantae, V.; Di, X.; Burggraaff, L.; Baggelaar, M. P.; Al-Ayed, K.; Bakker, A.; Florea, B. I.; Grimm, S. H.; den Dulk, H.; Li, C. T.; Mulder, L.; Overkleeft, H. S.; Hankemeier, T.; van Westen, G. J. P.; van der Stelt, M. Activity-based protein profiling identifies α-ketoamides as inhibitors for phospholipase A_2 group XVI. *ACS Chem. Biol.* **2019,** 14, 164-169.

(27) Niphakis, M. J.; Cravatt, B. F. Enzyme inhibitor discovery by activity-based protein profiling. *Annu. Rev. Biochem.* **2014,** 83, 341-377.

(28) Baggelaar, M. P.; Janssen, F. J.; van Esbroeck, A. C. M.; den Dulk, H.; Allara, M.; Hoogendoorn, S.; McGuire, R.; Florea, B. I.; Meeuwenoord, N.; van den Elst, H.; van der Marel, G. A.; Brouwer, J.; Di Marzo, V.; Overkleeft, H. S.; van der Stelt, M. Development of an activity-based probe and in silico design reveal highly selective inhibitors for diacylglycerol lipase-alpha in brain. *Angew. Chem., Int. Edit.* **2013,** 52, 12081-12085.

(29) De Risi, C.; Pollini, G. P.; Zanirato, V. Recent developments in general methodologies for the synthesis of α-ketoamides. *Chem. Rev.* **2016,** 116, 3241-3305.

(30) Taylor, S. C.; Nadeau, K.; Abbasi, M.; Lachance, C.; Nguyen, M.; Fenrich, J. The ultimate qPCR experiment: producing publication quality, reproducible data the first time. *Trends Biotechnol.* **2019,** 37, 761-774.

(31) Kokotos, G.; Six, D. A.; Loukas, V.; Smith, T.; Constantinou-Kokotou, V.; Hadjipavlou-Litina, D.; Kotsovolou, S.; Chiou, A.; Beltzner, C. C.; Dennis, E. A. Inhibition of group IVA cytosolic phospholipase A_2 by novel 2-oxoamides in vitro, in cells, and in vivo. *J. Med. Chem.* **2004,** 47, 3615-3628.

(32) Stephens, D.; Barbayianni, E.; Constantinou-Kokotou, V.; Peristeraki, A.; Six, D. A.; Cooper, J.; Harkewicz, R.; Deems, R. A.; Dennis, E. A.; Kokotos, G. Differential inhibition of group IVA and group VIA phospholipases A_2 by 2-oxoamides. *J. Med. Chem.* **2006,** 49, 2821-2828.

(33) Six, D. A.; Barbayianni, E.; Loukas, V.; Constantinou-Kokotou, V.; Hadjipavlou-Litina, D.; Stephens, D.; Wong, A. C.; Magrioti, V.; Moutevelis-Minakakis, P.; Baker, S. F.; Dennis, E. A.; Kokotos, G. Structure–activity relationship of 2-oxoamide Inhibition of group IVA cytosolic phospholipase A_2 and group V secreted phospholipase A_2. *J. Med. Chem.* **2007,** 50, 4222-4235.

(34) Vasilakaki, S.; Barbayianni, E.; Leonis, G.; Papadopoulos, M. G.; Mavromoustakos, T.; Gelb, M. H.; Kokotos, G. Development of a potent 2-oxoamide inhibitor of secreted phospholipase A_2 guided by molecular docking calculations and molecular dynamics simulations. *Bioorg. Med. Chem.* **2016,** 24, 1683-1695.

(35) Soethoudt, M.; Grether, U.; Fingerle, J.; Grim, T. W.; Fezza, F.; de Petrocellis, L.; Ullmer, C.; Rothenhäusler, B.; Perret, C.; van Gils, N.; Finlay, D.; MacDonald, C.; Chicca, A.; Gens, M. D.; Stuart, J.; de Vries, H.; Mastrangelo, N.; Xia, L.; Alachouzos, G.; Baggelaar, M. P.; Martella, A.; Mock, E. D.; Deng, H.; Heitman, L. H.; Connor, M.; Di Marzo, V.; Gertsch, J.; Lichtman, A. H.; Maccarrone, M.; Pacher, P.; Glass, M.; van der Stelt, M. Cannabinoid CB_2 receptor ligand profiling reveals biased signalling and off-target activity. *Nat. Commun.* **2017,** 8, 13958.

(36) Navia-Paldanius, D.; Savinainen, J. R.; Laitinen, J. T. Biochemical and pharmacological characterization of human α/β-hydrolase domain containing 6 (ABHD6) and 12 (ABHD12). *J. Lipid Res.* **2012,** 53, 2413-2424.

(37) Janssen, A. P. A.; van der Vliet, D.; Bakker, A. T.; Jiang, M.; Grimm, S. H.; Campiani, G.; Butini, S.; van der Stelt, M. Development of a multiplexed activity-based protein profiling assay to evaluate activity of endocannabinoid hydrolase inhibitors. *ACS Chem. Biol.* **2018,** 13, 2406-2413.

(38) van Esbroeck, A. C. M.; Janssen, A. P. A.; Cognetta, A. B.; Ogasawara, D.; Shpak, G.; van der Kroeg, M.; Kantae, V.; Baggelaar, M. P.; de Vrij, F. M. S.; Deng, H.; Allarà, M.; Fezza, F.; Lin, Z.; van der Wel, T.; Soethoudt,

M.; Mock, E. D.; den Dulk, H.; Baak, I. L.; Florea, B. I.; Hendriks, G.; De Petrocellis, L.; Overkleeft, H. S.; Hankemeier, T.; De Zeeuw, C. I.; Di Marzo, V.; Maccarrone, M.; Cravatt, B. F.; Kushner, S. A.; van der Stelt, M. Activity-based protein profiling reveals off-target proteins of the FAAH inhibitor BIA 10-2474. *Science* **2017,** 356, 1084-1087.

(39) *Schrödinger*, release 2017-4, LigPrep, Schrödinger, LLC, New York, NY, 2017.

(40) *Schrödinger*, release 2017-4, Epik, Schrödinger, LLC, New York, NY, 2017.

(41) *Schrödinger*, Small-Molecule Drug Discovery Suite 2017-4, Schrödinger, LLC, New York, NY, 2017.

(42) *Schrödinger*, release 2017-4: Desmond Molecular Dynamics System, D. E. Shaw Research, New York, NY, 2017. *Maestro-Desmond Interoperability Tools*, Schrödinger, New York, NY, 2017.

(43) Nakamura, A.; Lectard, S.; Hashizume, D.; Hamashima, Y.; Sodeoka, M. Diastereo- and enantioselective conjugate addition of α-ketoesters to nitroalkenes catalyzed by a chiral $Ni(OAc)_2$ complex under mild conditions. *J. Am. Chem. Soc.* **2010,** 132, 4036-4037.

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