Illuminating N-acylethanolamine biosynthesis with new chemical tools

Mock, E.D.

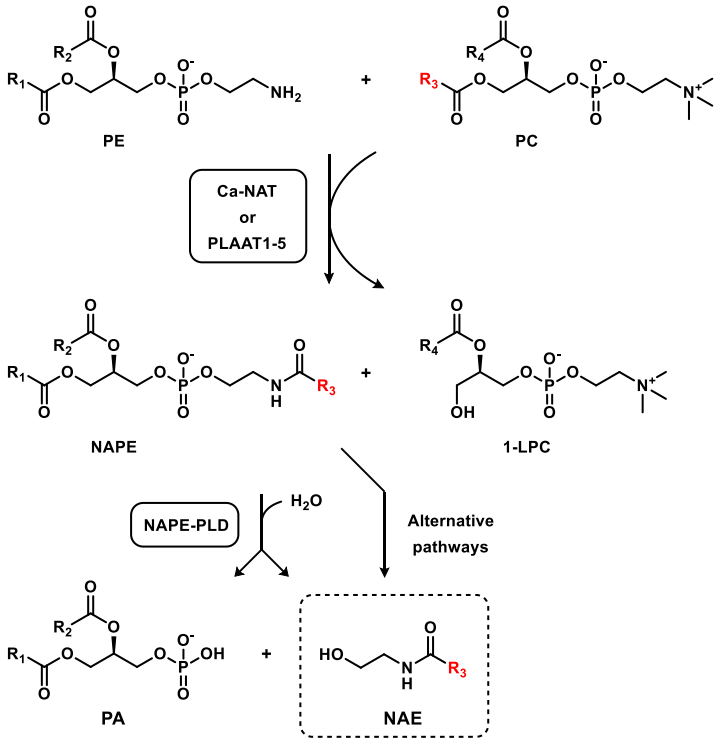
Chapter 6

Discovery and optimization of α-ketoamide inhibitors for the *N*-acyltransferase PLAAT2

6.1 Introduction

The subfamily of phospholipase A and acyltransferases (PLAAT) consists of five members that are part of the lecithin retinol acyltransferase (LRAT) protein family.[1,2] The LRAT protein family itself is part of the NlpC/P60 superfamily of thiol hydrolases. They share a conserved catalytic motif of six amino acids (NCEHFV) containing a cysteine residue that acts as the active site nucleophile.[3] Though initially described as tumor suppressors named HRASLS1-5 (Harvey Rat Sarcoma viral oncogene Like Suppressor 1-5), it was shown that PLAAT1-5 are in fact enzymes involved in phospholipid metabolism.[4-9] The PLAAT family members exhibit differing levels of *N*- and *O*-acyltransferase or phospholipase $A_{1/2}$ activity *in vitro*, which may lead to the production of *N*-acylphosphatidylethanolamines (NAPEs) and lyso-phosphatidylcholine (LPC), phosphatidylcholine (PC) and LPC or fatty acids.[10] NAPEs are an underexplored class of triacylated phospholipids that function as precursor for the *N*-acylethanolamines (NAEs), an important family of signaling molecules that includes the endocannabinoid anandamide.[11] The canonical enzyme responsible for

NAPE biosynthesis in the brain is a Ca^{2+}-dependent *N*-acyltransferase (Ca-NAT), recently identified as PLA2G4E (Scheme 1).[12] The NAPEs are in turn converted to the NAEs in one step by NAPE- phospholipase D, although other multistep pathways have also been reported.[13] In contrast, the PLAAT family members operate via a calcium-independent mechanism, providing a new pathway through which NAEs are biosynthesized.[10]



Scheme 1. Biosynthesis of *N*-acylethanolamines (NAEs). The *sn*-1-acyl 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 1-lyso-PC (1-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.

Ueda and co-workers reported that PLAAT2 in particular displays high *N*-acyltransferase activity.[10] HEK293 cells stably overexpressing PLAAT2 exhibited a remarkable increase in NAPE and NAE content. The enzyme showed a preference for the transfer of the *sn*-1 acyl group over the *sn*-2 acyl from phosphatidylcholine (PC) to the amine of phosphatidylethanolamine (PE) (Scheme 1). Utilizing a Cys-His-His catalytic triad, the enzymatic mechanism involves the formation of a thioester followed by nucleophilic attack of the amine acceptor, which generates the NAPE product and releases the catalytic cysteine. Expression of PLAAT2 was found to be high in the liver, kidney, small

intestine, colon, testis and trachea.[7,14] NAEs have well-established signaling roles in the gastrointestinal system.[15] For instance, *N*-oleoylethanolamide (OEA) was found to act as a satiety factor via activation of peroxisome proliferator-activated receptor (PPAR)-α.[16] This raises the possibility that PLAAT2 is involved in NAE biosynthesis in the gut. To validate the role of PLAAT2 in NAE signaling, pharmacological inhibitors would be valuable tools to confirm its *N*-acyltransferase activity. However, so far no inhibitors have been described for PLAAT2.

In this chapter, the discovery and optimization of a library of α-ketoamide PLAAT2 inhibitors is described. A previously reported activity-based protein profiling (ABPP) assay[17] for PLAAT2 was used to screen a focused library of lipase inhibitors for activity. This furnished hit compound **1**, which was modified through structure-activity relationship (SAR) analysis to deliver nanomolar potent inhibitor **LEI-301**. **LEI-301** showed similar potency for the other members of the PLAAT family and was selective over the proteins of the endocannabinoid system. NAE levels including anandamide were found to be highly increased in U2OS cells overexpressing PLAAT2. Importantly, **LEI-301** could partially reverse the NAE elevation in PLAAT2 transfected cells, but not in control cells. These findings suggest that PLAAT2 is involved in NAE biosynthesis and provide **LEI-301** as a new pharmacological tool to investigate its role in biological systems.

6.2 Results

6.2.1 Screening for PLAAT2 inhibitors using competitive ABPP.

A focused library of lipase inhibitors was screened for PLAAT2 inhibition using gel-based competitive ABPP.[17] Cytosol fractions of HEK293T cells overexpressing PLAAT2 were treated with inhibitors at 10 μM, followed by incubation with the broad-spectrum lipase probe MB064[18] (Figure 1A). The proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which allowed visualization of PLAAT2 activity by in-gel fluorescence scanning. A leftover protein activity of ≤ 50% was considered to be a hit, upon which an IC_{50} curve was generated using a dose-response ABPP experiment (Figure 1B). Data are reported in Table 1 as pIC_{50} ± SEM (n = 3). α-Ketoamides **1** and **2** were identified as submicromolar hits (for both: pIC_{50} = 6.2 ± 0.1). A structure-activity relationship emerged from the structurally similar keto- and hydroxyamides (**3**-**22**) present in this library. The position of the ketone relative to the amide was essential for binding (compare α-ketoamides **1** and **2** with β-ketoamides **5-8**). Furthermore, the phenethylamine of **1** was preferred over benzylamine (**10**) and ethylamine (**11**). *N*-methylation resulted in complete loss of activity (**12**), which suggested that the N-H is potentially involved in hydrogen bond formation. Similarly, secondary amides **13-22** did not show any activity.

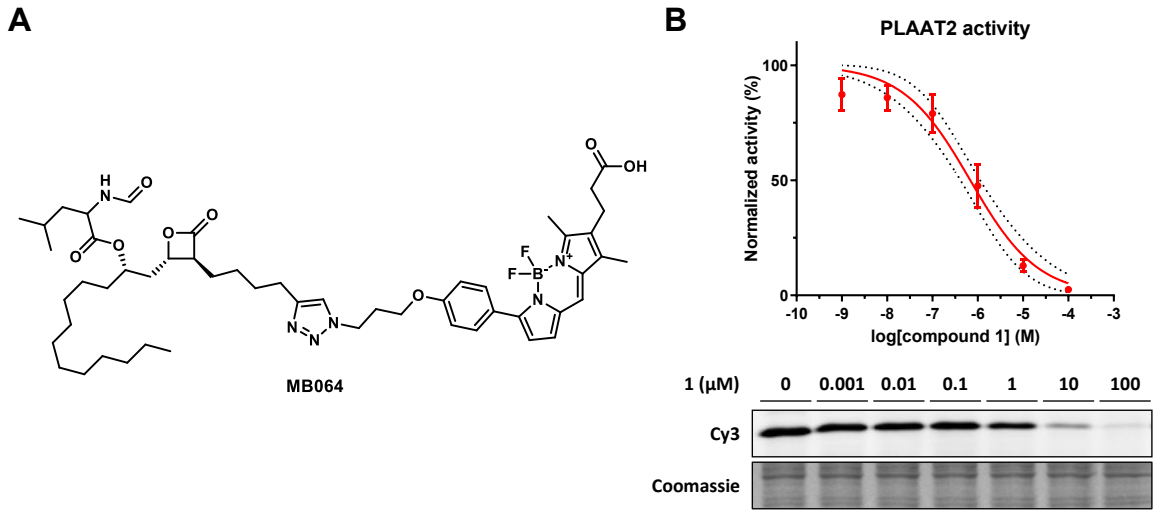
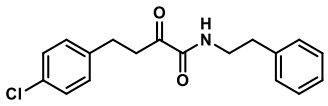
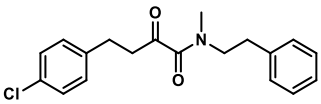
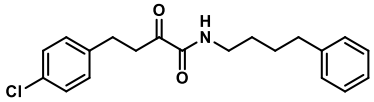
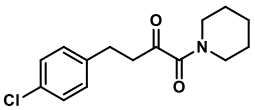
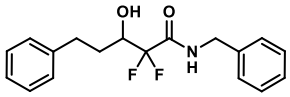
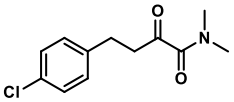
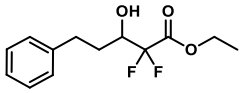
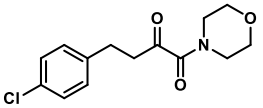
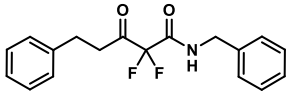
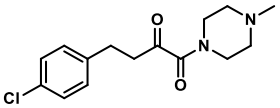
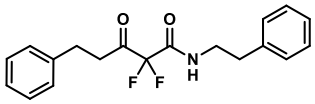
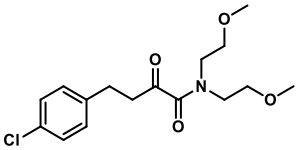
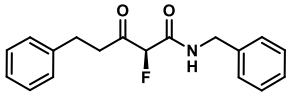
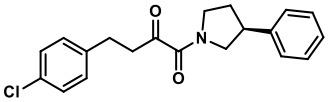
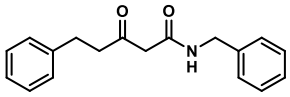
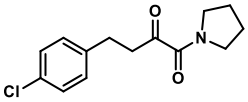
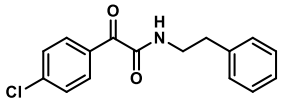
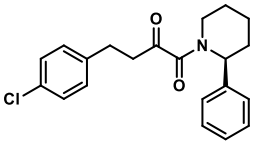
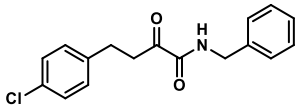
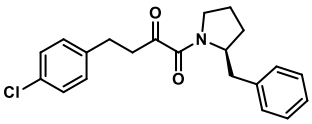
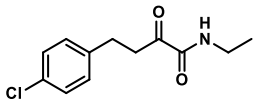
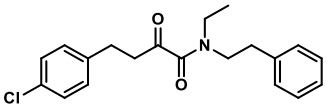


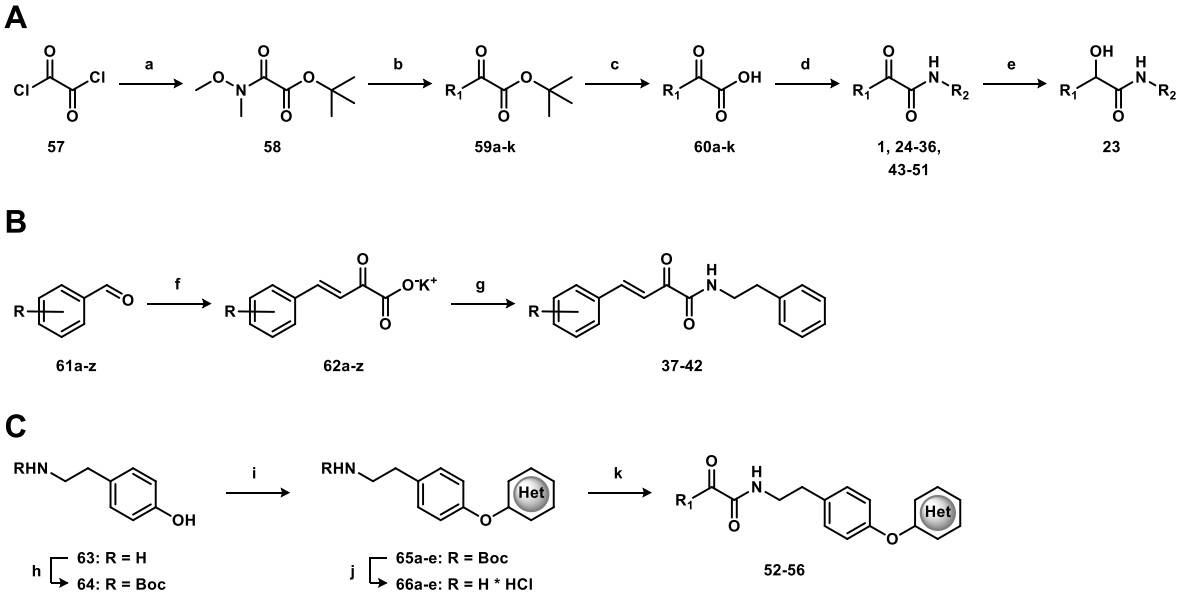
Figure 1. A) Structure of broad-spectrum lipase probe MB064. **B**) Representative gel and IC_{50} curve of a competitive ABPP experiment. Labeling of PLAAT2 by MB064 and dose-dependent inhibition by **1** (pIC_{50} = 6.2 ± 0.1, dotted lines show 95% confidence interval). Data represent mean values ±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

Table 1. PLAAT2 activity of the lipase inhibitor library (**1-22**).

ID	Structure	pIC_{50} ± SEM	ID	Structure	pIC_{50} ± SEM
1		6.2 ± 0.1	**12**		< 5
2		6.2 ± 0.1	**13**		< 5
3		< 5	**14**		< 5
4		< 5	**15**		< 5
5		< 5	**16**		< 5
6		< 5	**17**		< 5
7		< 5	**18**		< 5
8		< 5	**19**		< 5
9		< 5	**20**		< 5
10		5.6 ± 0.1	**21**		< 5
11		5.6 ± 0.1	**22**		< 5

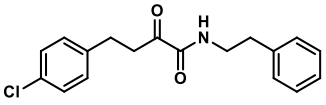
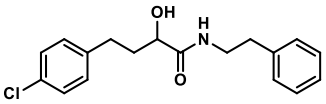
6.2.2 Evaluation of an α-ketoamide inhibitor library delivers nanomolar hit LEI-301.

α-Ketoamide **1** exhibited optimal PLAAT2 activity in this library, therefore this compound was resynthesized utilizing a general route depicted in Scheme 1A. 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 **59a**. Finally, amide coupling using HCTU afforded α-ketoamide **1**. After retesting in the ABPP assay, compound **1** was confirmed as an active hit (pIC_{50} = 6.2 ± 0.1, n = 3) (Table 2). It was envisioned that the electrophilic ketone of **1** could bind with the PLAAT2 active site cysteine through a reversible covalent mechanism forming a hemithioacetal adduct, similar to other reported α-ketoamide inhibitors.[19] To test this hypothesis, α-hydroxyamide **23** was prepared by reduction of **1** using sodium borohydride (Scheme 1A) and tested, showing no activity at 10 μM (Table 2).



Scheme 1. 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, 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%.

Table 2. Structure and PLAAT2 activity of α-ketoamide **1** and α-hydroxyamide **23**.

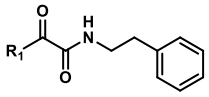
ID	Structure	pIC_{50} ± SEM
1		6.2 ± 0.1
23		< 5

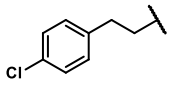
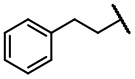
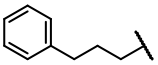
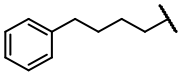
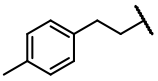
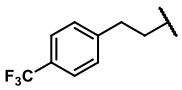
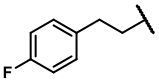
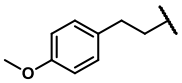
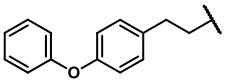
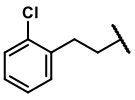
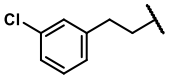
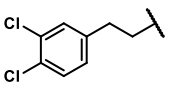
To improve the potency of **1**, R_1-ketone and R_2-phenethylamine analogues were systematically synthesized (compounds **24-56**). R_1-derivatives **24-36** and R_2-analogues **44-52** were synthesized via the general route (Scheme 1A). β,γ-Unsaturated α-ketoamides (**37**-**43**) were prepared using a two-step procedure (Scheme 1B): condensation of a benzaldehyde (**61a-z**) with pyruvic acid, which afforded the β,γ-unsaturated α-ketoacid as the potassium salt (**62a-z**), followed by acid chloride formation and coupling with phenethylamine. *O*-Arylated 4-hydroxyphenethylamine derivatives **53-57** were synthesized via Scheme 1C. 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**.

First, the effect of various substitutions on the R_1-group of **1** was evaluated with derivatives **24**-**36** (Table 3). Removal of the chloride was detrimental for the activity (**24**). The length of the alkyl chain was investigated (**24**-**27**), showing that propyl derivative **25** was optimal, which had similar potency as **1**, but lower lipophilicity (cLogP). The 4-chloro on the phenyl ring seemed to be optimal (**29**-**33**). Electron-donating groups such as 4-methyl (**29**) and 4-methoxy (**32**) substituents decreased potency as well as a lipophilic electron withdrawing group (*e.g.* 4-trifluoromethyl, **30**). Small (4-fluoro, **31**) and large (4-phenoxy, **33**) substituents lowered the activity. Furthermore, substitution of the 4-chloro to the *ortho* or *meta* position, did not result in improved potency (compounds **34-36**). This indicated the presence of a small lipophilic pocket restricted in size, which is occupied by the 4-chlorophenyl 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 4). Although unsaturation was tolerated in the alkyl chain (compare compounds **1** and **37**), no activity improvement was observed for these derivatives. Overall, this indicates that the R_1 group is positioned towards a shallow pocket.

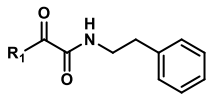
Table 3. Structure-activity relationship analysis of α-ketoamide analogues **24-36**.

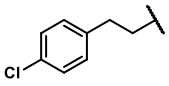
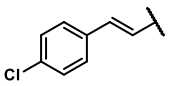
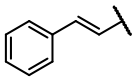
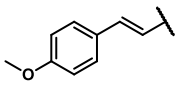
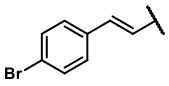
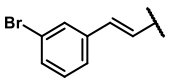
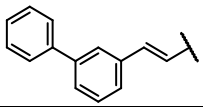


ID	R_1:	pIC_{50} ± SEM	cLogP[a]
1		6.2 ± 0.1	4.37
24		< 5	3.66
25		6.3 ± 0.1	4.04
26		5.5 ± 0.1	4.57
27		< 5	3.04
28		< 5	1.56
29		5.3 ± 0.1	4.16
30		5.7 ± 0.1	4.54
31		5.1 ± 0.1	3.80
32		5.5 ± 0.1	3.58
33		5.4 ± 0.1	5.76
34		5.9 ± 0.1	4.37
35		5.6 ± 0.1	4.37
36		5.7 ± 0.2	4.97

[a] cLogP was calculated using Chemdraw 15.

Table 4. Structure-activity relationship analysis of α-ketoamide analogues **37-43**.

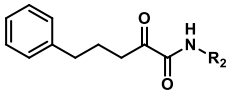


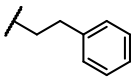
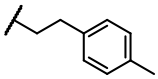
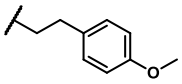
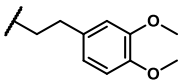
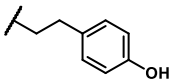
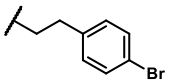
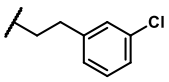
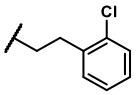
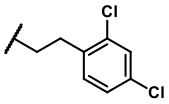
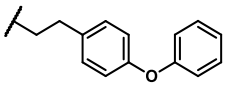
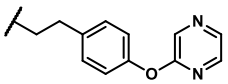
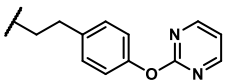
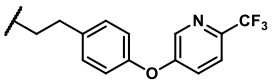
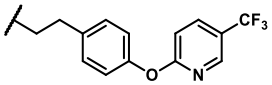
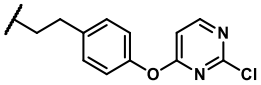
ID	R_1:	pIC_{50} ± SEM	cLogP[a]
1		6.2 ± 0.1	4.37
37		5.8 ± 0.1	4.37
38		5.1 ± 0.1	3.66
39		5.8 ± 0.1	3.58
40		5.3 ± 0.1	4.52
41		< 5	4.52
42		5.6 ± 0.1	5.55

[a] cLogP was calculated using Chemdraw 15.

Focus was shifted towards the optimization of the phenethylamine moiety. Analogues **43**-**56** incorporating substituted phenethylamines, were prepared in combination with the optimal 2-oxo-5-phenylpentanoyl motif of compound **25** (Table 5). Substitutions on the *para* position were unfavorable for methyl (**43**), methoxy (**44**) and hydroxyl (**46**). The *meta* and *ortho* positions (**48**, **49**) also did not afford an improvement in potency. Increasing the lipophilicity gave a 2-fold increase in activity for 4-bromo analog **47**, while further expansion with a 4-phenoxy moiety (**51**) improved the potency 10-fold compared to **25**. Addition of the phenoxy group raised the cLogP of **51** with two log units to 6.14, therefore more polar heteroaryl rings were introduced to lower the lipophilicity (**52-56**). After testing for activity, a decrease in potency was observed for all these inhibitors compared to **51**. Therefore, being the most potent inhibitor of PLAAT2, **51** (termed **LEI-301**) was selected for further characterization.

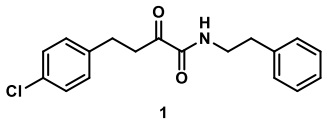
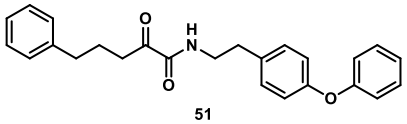
Table 5. Structure-activity relationship analysis of phenethylamine analogues **43-56**.



ID	R_2:	pIC_{50} ± SEM	cLogP[a]
3		6.3 ± 0.1	4.04
43		5.7 ± 0.1	4.54
44		5.8 ± 0.1	3.96
45		5.3 ± 0.1	3.70
46		5.9 ± 0.1	3.37
47		6.6 ± 0.1	4.90
48		6.1 ± 0.1	4.75
49		5.2 ± 0.1	4.75
50		5.9 ± 0.1	5.47
51 (LEI-301)		7.3 ± 0.1	6.14
52		6.3 ± 0.1	3.68
53		5.7 ± 0.1	3.68
54		6.7 ± 0.1	5.67
55		6.8 ± 0.1	5.67
56		6.2 ± 0.1	4.44

[a] cLogP was calculated using Chemdraw 15.

Table 6. PLAAT2 activity data and physicochemical parameters of **1** and **LEI-301**.

Compound	pIC_{50} ± SEM	cLogP[a]	LipE[b]	MW[c] (Da)	tPSA[a] (Å^2)	HBD[d]	HBA[e]	RB[f]
 **1**	6.2 ± 0.1	4.4	1.8	316	46.2	1	2	8
 **51 (LEI-301)**	7.3 ± 0.1	6.1	1.2	387	55.4	1	3	11

[a] cLogP and topological polar surface area (tPSA) were calculated using Chemdraw 15; [b] Lipophilic efficiency (LipE) = pIC_{50} – cLogP; [c] MW: molecular weight; [d] HBD: H-bond donors; [e] HBA: H-bond acceptors; [f] RB: rotatable bonds.

A summary of the activity data and physicochemical parameters of **1** and **LEI-301** is shown in Table 6. **LEI-301** is a nanomolar potent PLAAT2 inhibitor (IC_{50} = 50 nM), displaying a 13-fold increase in activity compared to **1**. Due to a 50-fold increase in lipophilicity, the lipophilic efficiency (LipE) is too low to regard **LEI-301** as a suitable candidate for *in vivo* experiments. Nevertheless, **LEI-301** shows favorable physicochemical parameters such as low molecular weight (MW < 500), topological polar surface area (tPSA < 90 Å^2), hydrogen bond donors (HBD < 5) and acceptors (HBA < 10). This highlights **LEI-301** as a promising starting point to obtain *in vivo* active PLAAT2 inhibitors.

6.2.3 *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).[20] 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)[20] 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, since it is not present in both crystal structures.

It has been proposed that the electrophilic ketone of α-ketoamides could engage with the active site cysteine through a reversible covalent mechanism[19], therefore **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. Hydrophobic interactions were apparent for the R_1-ketone substituent of **LEI-301** and **1** with the Leu108 sidechain. The modelled loop of residue 39-52 enclosed the R_1-phenyl ring, thereby providing an explanation why expansion on this side of the molecule was unfavored. Introduction of the 4-phenoxy group in **LEI-301** enabled an additional π-π stacking interaction with Tyr21. This offered a possible reason for the observed activity increase of **LEI-301**.

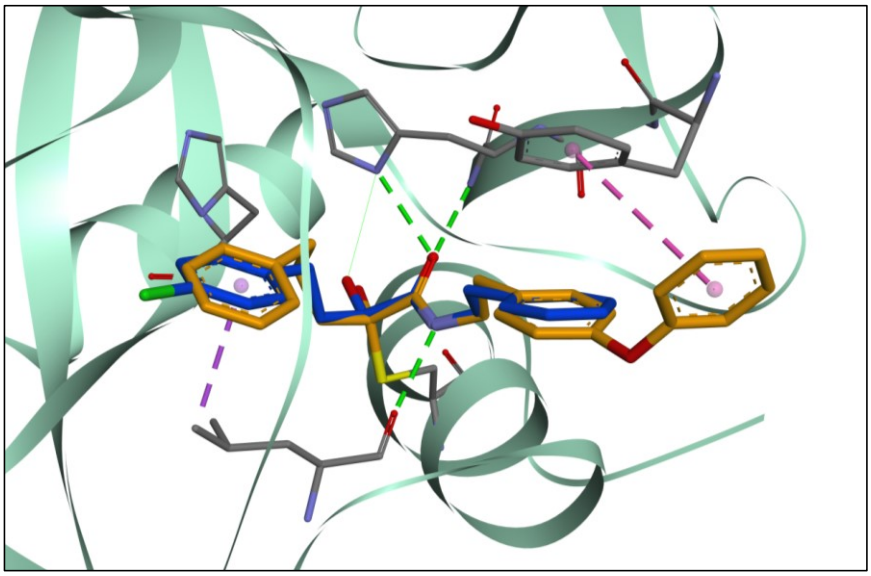


Figure 2. Compounds **1** (blue) and **LEI-301** (orange) in complex with PLAAT2, covalently bound to Cys113. Green dotted lines represent a hydrogen bond, pink and purple represent π-interactions.

6.2.4 Off-target profile of LEI-301 for the PLAAT family and endocannabinoid system.

The activity of **LEI-301** for the other members of the PLAAT family was assessed via ABPP with **MB064** using cytosolic fractions of HEK293T cells overexpressing the corresponding PLAAT enzymes. **LEI-301** was found to be equally potent towards PLAAT4 and PLAAT5, but less active for PLAAT3 (Table 7). Unfortunately, PLAAT1 could not be expressed and could therefore not be tested. Next, the activity of **LEI-301** for the receptors and metabolic enzymes of the endocannabinoid system (ECS) was determined. No inhibitory activity was observed at 10 μM for the cannabinoid receptors type 1 and 2 (CB_1/CB_2) (Table 7). The enzymes involved in NAE biosynthesis (PLA2G4E, NAPE-PLD) and degradation (FAAH) were also not inhibited at this concentration (Table 8). Enzymes involved in the metabolism of the other endocannabinoid 2-arachidonoylglycerol (2-AG), such as diacylglycerol lipase α and β (DAGL-α/β), monoacylglycerol lipase (MAGL) and α,β-hydrolase domain containing 6 (ABHD6) were not inhibited.

Table 7. **LEI-301** is equally potent for PLAAT4 and PLAAT5, but less active towards PLAAT3.

pIC_{50} ± SEM (n = 3)		
PLAAT3	PLAAT4	PLAAT5
6.6 ± 0.1	7.3 ± 0.2	7.4 ± 0.1

Table 8. **LEI-301** shows no significant inhibitory activity for the cannabinoid receptors.

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

> 50% is considered a target

Table 9. **LEI-301** shows no inhibitory activity for metabolic enzymes of the 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, mDAGLβ) or natural (hMAGL) substrate assays. hPLA2G4E, mDAGLα/β, mFAAH and mABHD6 were determined by gel-based ABBP. < 50% is considered a target.

6.2.5 Targeted lipidomics shows that LEI-301 inhibits PLAAT2 in 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** can decrease anandamide levels in living cells. Human U2OS osteosarcoma cells were transiently transfected with a pcDNA3.1 plasmid containing *PLAAT2* or an empty (mock) vector. To ensure that the NAPEs generated by PLAAT2 could be converted to NAEs, the expression of NAPE-PLD in this cell line was confirmed by quantitative PCR (qPCR) (*NAPEPLD*: C_q ± SEM = 27.30 ± 0.050, *RPS18* (housekeeping gene): C_q ± SEM = 17.76 ± 0.011). Targeted lipidomics on the lipid extracts of the transfected cells allowed the quantification of eight different NAEs and ten 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, fold change ± SD = 54 ± 20, P = 0.0016) (Table 10, Figure 3A-B). This is in contrast to a previous report, where only a small elevation of anandamide levels was detected.[10] Notably, PLAAT2 transfection did not

produce significant elevations of fatty acid species, except for arachidonic acid (fold change ± SD = 1.81 ± 0.45, *P* = 0.040) while a decrease was measured for γ-linolenic (18:3-ω6) and α-linoleic acid (18:2-ω3) (Figure 3A, Supplementary Table 1). Next, **LEI-301** was incubated at 10 μM for 4 hours with the PLAAT2-transfected or control cells. A significant 2-fold reduction of anandamide was apparent in the PLAAT2 cells, which was absent in the control samples (Figure 3B and E). Other mono- and polyunsaturated NAE also showed significant reductions upon treatment with **LEI-301** in the PLAAT2 overexpressing cells but not in the mock cells (Figure 3E). Of note, the saturated *N*-palmitoylethanolamine (PEA) and *N*-stearoylethanolamine (SEA) did not reach statistical significance (*P* = 0.085 and *P* = 0.25). Furthermore, **LEI-301** reduced arachidonic acid levels, but not significantly (*P* = 0.06) (Figure 3E). No changes were observed for 2-AG (Figure 3D).

Table 10. PLAAT2 overexpression greatly increases NAE levels 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 PLAAT2 vs. mock	*P*-value
	mock	PLAAT2		
PEA (16:0)	0.196 ± 0.05	18.62 ± 5.97	95 ± 30	0.0008
POEA (16:1)	0.031 ± 0.01	2.247 ± 0.78	73 ± 25	0.0013
SEA (18:0)	0.516 ± 0.12	47.39 ± 13.1	92 ± 25	0.0004
OEA (18:1)	0.190 ± 0.05	18.83 ± 5.02	99 ± 26	0.0003
LEA (18:2)	0.047 ± 0.01	1.569 ± 0.50	33 ± 10	0.0009
AEA (20:4)	0.040 ± 0.01	2.194 ± 0.79	54 ± 20	0.0016
EPEA (20:5)	0.010 ± 0.01	0.555 ± 0.15	53 ± 14	0.0004
DHEA (22:6)	0.032 ± 0.01	0.286 ± 0.11	8.9 ± 3.4	0.0034

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.

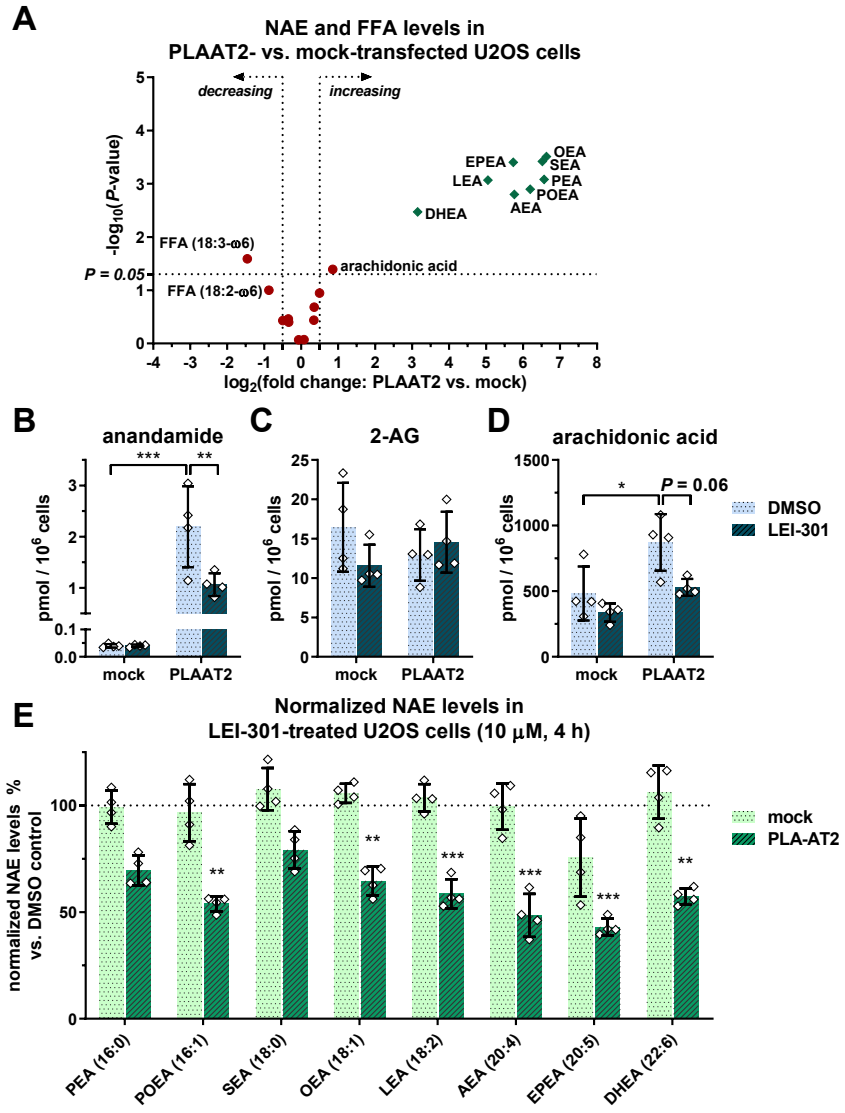


Figure 3. U2OS cells transiently transfected with PLAAT2 exhibit highly increased NAE levels and **LEI-301** can inhibit NAE formation. **A**) Volcano plot depicting the $\log_2$(fold change) vs. $-\log_{10}$(*P*-value) of NAEs (green diamonds) and free fatty acids (FFAs, red circles) in PLAAT2 vs. mock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

6.3 Conclusion

In this chapter the discovery and optimization of an α-ketoamide PLAAT2 inhibitor library is described. A map displaying an overview of the SAR is presented in Figure 4. Extension of the ketone alkyl chain to three methylenes and removal of the chloride on the left phenyl group gave a similar potency with reduced lipophilicity. The phenethylamine was expanded with a *para*-phenoxy moiety affording the nanomolar potent inhibitor **LEI-301**, having a 13-fold higher activity for PLAAT2 compared to the initial hit. Attempts to decrease the high lipophilicity of **LEI-301** while retaining the activity, were not yet successful. Covalent docking in the PLAAT2 crystal structure provided a possible binding mode of **LEI-301** and a potential explanation for the observed potency increase. Subsequent selectivity profiling revealed that **LEI-301** is also a potent inhibitor for PLAAT3, PLAAT4 and PLAAT5. Further inhibitor optimization is desired to obtain selectivity over the other PLAAT family members. No off-targets were found for proteins of the endocannabinoid system.

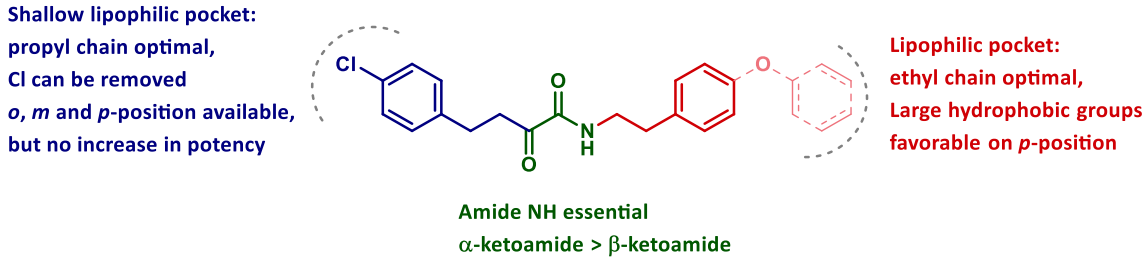


Figure 4. Structure activity map for the PLAAT2 α-ketoamide inhibitor library.

Overexpression of PLAAT2 in U2OS cells gave a dramatic increase of all measured NAE species, while no significant elevations of fatty acids were observed, except for arachidonic acid. These findings provide more evidence that PLAAT2 can produce NAEs and confirms that this enzyme can function as an *N*-acyltransferase. Furthermore, treatment of overexpressing PLAAT2 cells with **LEI-301** decreased NAE levels, including a 2-fold reduction for the endocannabinoid anandamide, which was absent in the control cells. This validates **LEI-301** as a promising tool compound to study PLAAT2 function in biological systems.

Acknowledgements

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6.4 Experimental section

A. Biological procedures

Plasmids

Full-length human cDNA of PLAAT1-5 (obtained from Natsuo Ueda[6]) 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 XL-10 Z-competent cells (Maxi Prep kit: Qiagen) and sequenced at the Leiden Genome Technology Center. Sequences were analyzed and verified (CLC Main Workbench).

Cell culture

HEK293T and U2OS cells (ATCC) were cultured at 37 ^{o}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 (Thermo Fisher). Medium was refreshed every 2-3 days and cells were passaged twice a week at 80-90% confluence. Cells were passaged twice a week to appropriate confluence by thorough pipetting (HEK293T) or trypsinization (U2OS).

Transient transfection

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 ^{o}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 ^{o}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 ^{o}C.

Mouse brain lysate preparation

Mouse brain membrane or cytosol fractions were prepared as described in Chapter 2.

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.[18] 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

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) in combination with 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.

Table S1. Primer sequences of *Napepld* and *Hprt* used for qPCR.

Gene	Forward (5'-3')	Reverse (5'-3')	Accession number
NAPEPLD	CTTTAGCTCTCGTGCTTCACC	CGCATCTATTGGAGGGAGTTCA	NM_001122838.1
RPS18	TAGCCTTTGCCATCACTGCC	TCACACGTTCCACCTCATCC	NM_022551.3

CB_2 receptor radioligand displacement assay

The CB_2 receptor radioligand displacement assay was performed as described in Chapter 2.

hNAPE-PLD surrogate substrate activity assay

The hNAPE-PLD activity assay was performed as described in Chapter 2.

Natural substrate-based fluorescence assay hMAGL

The hMAGL activity assay was performed as described in Chapter 2.

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

Gel-based activity-based protein profiling (ABPP) was performed as described in Chapter 2.

B. Targeted lipidomics in U2OS cells

The targeted lipidomics experiments are based on previously reported methods with small alterations.[21]

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 plasmid DNA (2.7 μg/dish) and polyethyleneimine (PEI, 1 μg/μL) were incubated in serum-free culture medium (15 min, rt), and then added dropwise to the cells. 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

Lipids extraction was performed on ice. In brief, cell pellets with $2 \cdot 10^6$ cells were transferred to 1.5 mL Eppendorf tubes, 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 speed-vac 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. 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.

C. Computational Chemistry

Ligand preparation

Molecular structures of **LEI-301** and **1** were drawn with specified chirality and prepared for docking using Ligprep from Schrödinger.[22] Default Ligprep settings were applied: states of heteroatoms were generated using Epik at a pH 7 ± 2.[23] 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).[20] The apo protein structure was prepared for docking with the Protein Preparation tool from the Schrödinger 2017-4 suite. Waters were removed and default protein preparation settings were applied: explicit hydrogens were added and states of heteroatoms were generated using Epik at a pH 7 ± 2, resulting in a protonated state of binding pocket His23. Missing side chains and loops were added using Prime[24]: loop 39-53 based on sequence and loop 105-111 based on the structure of PLAAT3 (PDB ID: 4DOT).[20]

Docking

The PLAAT2 binding pocket was induced using the binding pose from **1** in PLAAT3 as previously reported and molecular dynamic simulations (10 ns).[17,25] Both **LEI-301** and **1** were docked with induced-fit, followed by covalent docking to Cys113. Compounds were docked using the Schrödinger 2017-4 suite[25] with SP precision. 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.

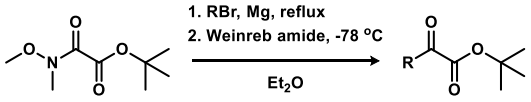
D. Synthetic procedures

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 ^oC. ^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)

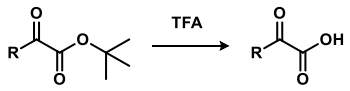
mass spectrometer or a Synapt G2-Si high definition mass spectrometer (Waters) equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10 mL min-1, capillary temperature 250 °C) with resolution R = 60000 at m/z 400 (mass range m/z = 150-2000) and dioctylphtalate (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



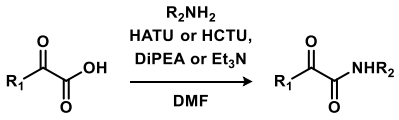
Magnesium turnings were activated by stirring in a 3 M solution of HCl for 5 min. The magnesium was then washed with water and acetone and dried under reduced pressure. A round-bottom flask connected to a reflux condenser was flame dried before addition of activated magnesium turnings (2 eq) under an 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



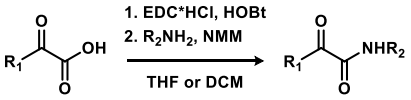
A round bottom flask was charged with α-ketoester (1 eq), DCM (0.3 M) and TFA (5-10 eq) and stirred for 1-24 h at rt. The reaction mixture was concentrated under reduced pressure after TLC analysis showed complete consumption of the starting material, followed by coevaporation with toluene (3x). The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

General procedure C



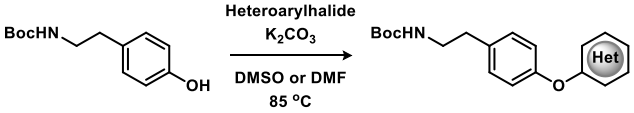
A round bottom flask was charged with α-ketoacid (1 eq) and DMF (0.2 M). HATU or HCTU (1-1.2 eq), DiPEA (1-2 eq) or Et_3N (1-2 eq), and amine (1-1.1 eq) were added and the mixture was stirred for 2-24 h at rt. Water was added and the mixture was extracted with DCM (2x). The combined organic layers were 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



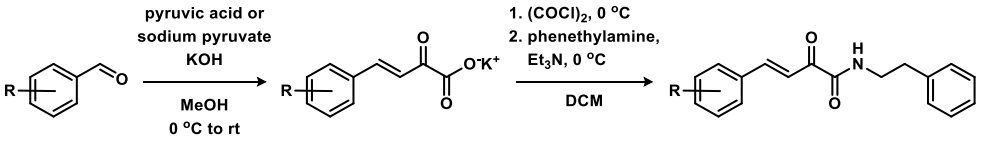
A round bottom flask was charged with α-ketoacid (1 eq) and THF or DCM (0.2 M) at 0 °C. EDC·HCl (1-1.5 eq) and HOBt (1-1.5 eq) were added and the mixture was stirred for 30 min, followed by addition of NMM (optional, 4 eq) and the amine (1.2 eq). The mixture was stirred for 1-4 days warming to rt. Work-up 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

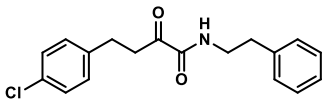


A microwave vial was charged with *N*-Boc-tyramine **64** (1 eq), heteroaryl halide (1 eq), K_2CO_3 (2 eq) in DMSO or DMF (0.2 – 1 M) and capped. The mixture was stirred for 24 - 42 h at 85°C in an oil bath until TLC showed complete conversion. The mixture was diluted with H_2O and extracted with EtOAc (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.

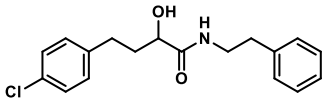
General procedure F



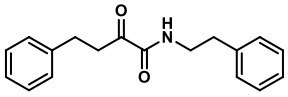
A round bottom flask was charged with pyruvic acid or sodium pyruvate (1 eq), aldehyde (1 eq) and MeOH (1 M) and cooled to 0 °C. A solution of KOH (2 M, 1.5 eq) in MeOH was added dropwise while keeping the temperature below 25 °C. The reaction was stirred at rt overnight, forming a yellow precipitate. 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.



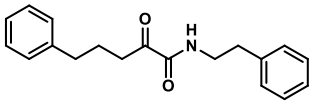
4-(4-Chlorophenyl)-2-oxo-*N*-phenethylbutanamide (1). *t-Butyl deprotection **59a:*** the α-ketoacid was prepared according to general procedure B using α-ketoester **59a** (0.85 g, 3.2 mmol, 1 eq) and TFA (2.5 mL, 32 mmol, 10 eq) affording the α-ketoacid **60a** (0.68 g, 3.2 mmol, quant.). *Amide coupling:* 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 found.



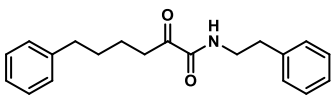
4-(4-Chlorophenyl)-2-hydroxy-*N*-phenethylbutanamide (23). A round bottom flask was charged with α-ketoamide **1** (70 mg, 0.22 mmol, 1 eq) and THF (1 mL). $NaBH_4$ (12 mg, 0.33 mmol, 1.5 eq) was added and the mixture was stirred for 15 min. The reaction was quenched with water (10 mL) and extracted 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.

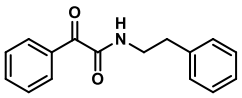


2-Oxo-*N*-phenethyl-5-phenylpentanamide (25). The title compound was prepared according to general procedure C using the α-ketoacid **60c** (0.12 g, 0.63 mmol, 1 eq), phenethylamine (86 μL, 0.69 mmol, 1.1 eq), HCTU (0.26 g, 0.63 mmol, 1 eq) and DiPEA (0.12 mL, 0.70 mmol, 1.1 eq) in DCM, affording the 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.

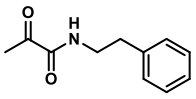


2-Oxo-*N*-phenethyl-6-phenylhexanamide (26). *t-Butyl deprotection **59d:*** the α-ketoacid was prepared according to general procedure B using α-ketoester **59d** (0.33 g, 1.3 mmol, 1 eq) and TFA (1.9 mL, 25 mmol, 19 eq) affording the α-ketoacid **60d** (0.26 g, 1.3 mmol, quant.). *Amide coupling:* 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

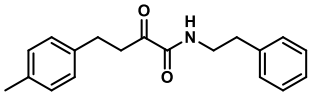
(0.30 mL, 1.72 mmol, 1.3 eq), affording the product (0.34 g, 1.11 mmol, 71%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.43 – 7.08 (m, 10H), 7.09 – 6.89 (m, 1H), 3.52 (q, *J* = 6.9 Hz, 2H), 3.01 – 2.86 (m, 2H), 2.81 (t, *J* = 7.1 Hz, 2H), 2.61 (t, *J* = 7.0 Hz, 2H), 1.62 (p, *J* = 3.5 Hz, 4H). ^{13}C NMR (75 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.



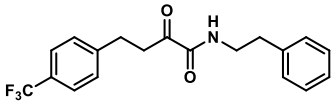
2-Oxo-*N*-phenethyl-2-phenylacetamide (27). *t-Butyl deprotection **59e:*** the α-ketoacid was prepared according to general procedure B using α-ketoester **59e** (0.68 g, 3.3 mmol, 1 eq) and TFA (2.4 mL, 32 mmol, 10 eq) affording the α-ketoacid **60e** (0.58 g, 3.2 mmol, quant.). *Amide coupling:* the 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.

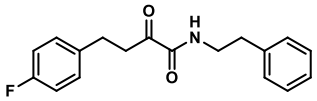


2-Oxo-*N*-phenethyl-4-(*p*-tolyl)butanamide (29). *t-Butyl deprotection **59f:*** the α-ketoacid was prepared according to general procedure B using α-ketoester **59f** (0.54 g, 2.2 mmol, 1 eq) and TFA (1.6 mL, 22 mmol, 10 eq) affording the α-ketoacid **60f** (0.42 g, 2.2 mmol, quant.). *Amide coupling:* 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.

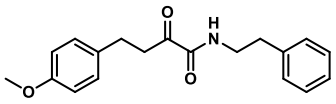


2-Oxo-*N*-phenethyl-4-(4-(trifluoromethyl)phenyl)butanamide (30). *t-Butyl deprotection **59g:*** the α-ketoacid was prepared according to general procedure B using α-ketoester **59g** (0.25 g, 0.83 mmol, 1 eq) and TFA (0.62 mL, 8.3 mmol, 10 eq) affording the α-ketoacid **60g** (0.20 g, 0.83 mmol, quant.). *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),

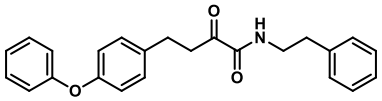
7.39 – 7.31 (m, 4H), 7.31 – 7.25 (m, 1H), 7.25 – 7.19 (m, 2H), 7.12 – 7.00 (m, 1H), 3.60 (q, *J* = 7.0 Hz, 2H), 3.32 (t, *J* = 7.4 Hz, 2H), 3.01 (t, *J* = 7.4 Hz, 2H), 2.88 (t, *J* = 7.1 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 197.70, 159.88, 144.56 (q, *J* = 1.3 Hz), 138.19, 128.78, 128.70, 128.48, 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.



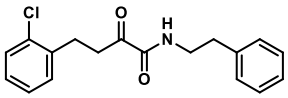
4-(4-Fluorophenyl)-2-oxo-*N*-phenethylbutanamide (31). *t-Butyl deprotection **59h:*** the α-ketoacid was prepared according to general procedure B using α-ketoester **59h** (0.15 g, 0.59 mmol, 1 eq) and TFA (0.44 mL, 5.9 mmol, 10 eq) affording the α-ketoacid **60h** (0.12 g, 0.59 mmol, quant.). *Amide 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.



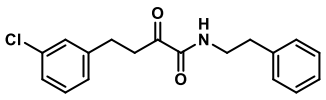
4-(4-Methoxyphenyl)-2-oxo-*N*-phenethylbutanamide (32). A round bottom flask was charged with unsaturated α-ketoamide **39** (0.10 g, 0.32 mmol, 1 eq) and MeOH (1 mL) and flushed with N_2. Pd/C (10 wt. %, 10 mg, 9.4 µmol, 3 mol%) was added and the flask was purged again with N_2, followed by H_2 and 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.



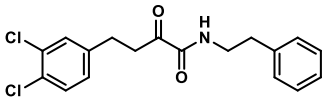
2-Oxo-*N*-phenethyl-4-(4-phenoxyphenyl)butanamide (33). A round bottom flask was charged with unsaturated α-ketoamide **67** (0.12 g, 0.33 mmol, 1 eq) and MeOH (1 mL) and flushed with N_2. Pd/C (10 wt. %, 10 mg, 9.4 µmol, 3 mol%) was added and the flask was purged again with N_2, followed by H_2 and 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.



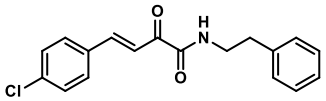
4-(2-Chlorophenyl)-2-oxo-*N*-phenethylbutanamide (34). *t-Butyl deprotection **59i:*** the α-ketoacid was prepared according to general procedure B using α-ketoester **59i** (0.23 g, 0.87 mmol, 1 eq) and TFA (0.94 mL, 13 mmol, 15 eq) affording the α-ketoacid **60i** (0.18 g, 0.87 mmol, quant.). *Amide 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.



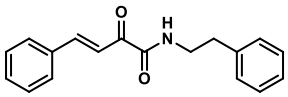
4-(3-Chlorophenyl)-2-oxo-*N*-phenethylbutanamide (35). *t-Butyl deprotection **59j:*** the α-ketoacid was prepared according to general procedure B using α-ketoester **59j** (0.55 g, 2.0 mmol, 1 eq) and TFA (2 mL, 26 mmol, 13 eq) affording the α-ketoacid **60j** (0.47 g, 2.0 mmol, quant.). *Amide coupling:* 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.



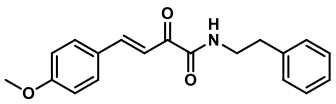
4-(3,4-Dichlorophenyl)-2-oxo-*N*-phenethylbutanamide (36). *t-Butyl deprotection **59k:*** the α-ketoacid was prepared according to general procedure B using α-ketoester **59k** (90 mg, 0.30 mmol, 1 eq) and TFA (0.25 mL, 32 mmol, 10 eq) affording the α-ketoacid **60k** (74 mg, 0.30 mmol, quant.). *Amide 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.



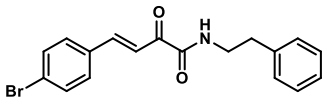
(*E*)-4-(4-Chlorophenyl)-2-oxo-*N*-phenethylbut-3-enamide (37). *α-Ketoacid formation:* the α-ketoacid salt was prepared according to general procedure F using pyruvic acid (1.7 mL, 18 mmol, 1 eq), 4-chlorobenzaldehyde (2.2 mL, 19 mmol, 1 eq), KOH (2.1 g, 38 mmol, 2 eq) in MeOH affording potassium 4-(4-chlorophenyl)-2-oxobut-3-enoate **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.



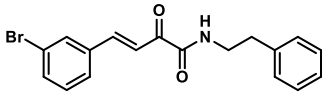
(*E*)-2-Oxo-*N*-phenethyl-4-phenylbut-3-enamide (38). *α-Ketoacid formation:* the α-ketoacid salt was prepared according to general procedure F using pyruvic acid (0.79 mL, 11 mmol, 1 eq), benzaldehyde (1.2 g, 11 mmol, 1 eq), KOH (0.98 g, 17 mmol, 1.5 eq) in MeOH affording potassium 2-oxo-4-phenylbut-3-enoate **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.



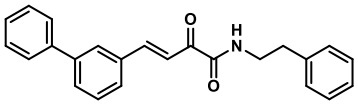
(*E*)-4-(4-Methoxyphenyl)-2-oxo-*N*-phenethylbut-3-enamide (39). *α-Ketoacid formation:* the α-ketoacid salt was prepared according to general procedure F using sodium pyruvate (3.0 g, 27 mmol, 1 eq), 4-methoxybenzaldehyde (3.3 mL, 27 mmol, 1 eq), KOH (2.3 g, 41 mmol, 1.5 eq) in MeOH affording potassium 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.



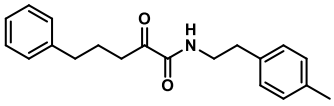
(*E*)-4-(4-Bromophenyl)-2-oxo-*N*-phenethylbut-3-enamide (40). *α-Ketoacid formation:* the α-ketoacid salt was prepared according to general procedure F using pyruvic acid (1.4 mL, 16 mmol, 1 eq), 4-bromobenzaldehyde (1.6 mL, 16 mmol, 1 eq), KOH (1.8 g, 32 mmol, 2 eq) in MeOH affording potassium 4-(4-bromophenyl)-2-oxobut-3-enoate **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.



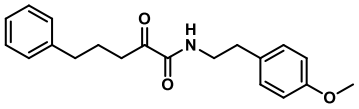
(*E*)-4-(3-Bromophenyl)-2-oxo-*N*-phenethylbut-3-enamide (41). *α-Ketoacid formation:* the α-ketoacid salt was prepared according to general procedure F using sodium pyruvate (1.0 g, 11 mmol, 1 eq), 3-bromobenzaldehyde (1.3 mL, 11 mmol, 1 eq), KOH (0.96 g, 17 mmol, 1.5 eq) in MeOH affording potassium 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.



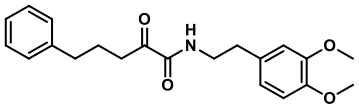
(*E*)-4-([1,1'-Biphenyl]-3-yl)-2-oxo-*N*-phenethylbut-3-enamide (42). A round bottom flask was charged with aryl bromide **41** (0.20 g, 0.56 mmol, 1 eq) and toluene/EtOH (4:1, 3 mL) and degassed for 20 min with sonication. $Pd(PPh_3)_4$ (13 mg, 0.01 mmol, 2 mol%), phenylboronic acid (0.10 g, 0.84 mmol, 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.



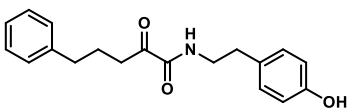
***N*-(4-Methylphenethyl)-2-oxo-5-phenylpentanamide (43).** The title compound was prepared according to general procedure D using α-ketoacid **60c** (57 mg, 0.29 mmol, 1 eq), 2-(*p*-tolyl)ethan-1-amine (47 μL, 0.32 mmol, 1.1 eq), EDC·HCl (85 mg, 0.44 mmol, 1.5 eq), HOBt (60 mg, 0.44 mmol, 1.5 eq) in 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.

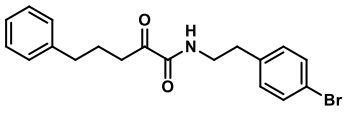


***N*-(3,4-Dimethoxyphenethyl)-2-oxo-5-phenylpentanamide (45).** The title compound was prepared according to general procedure D using α-ketoacid **60c** (57 mg, 0.30 mmol, 1 eq), 2-(3,4-dimethoxyphenyl)ethan-1-amine (56 μL, 0.33 mmol, 1.1 eq), EDC·HCl (86 mg, 0.45 mmol, 1.5 eq), HOBt (61 mg, 0.45 mmol, 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.

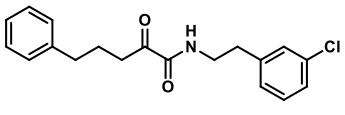


***N*-(4-Hydroxyphenethyl)-2-oxo-5-phenylpentanamide (46).** The title compound was prepared according to general procedure D using α-ketoacid **60c** (98 mg, 0.51 mmol, 1 eq), 4-(2-aminoethyl)phenol (77 mg, 0.56 mmol, 1.1 eq), EDC·HCl (0.15 g, 0.77 mmol, 1.5 eq), HOBt (0.10 g, 0.77 mmol, 1.5 eq) in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

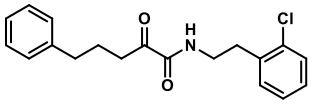
(18 mg, 58 μmol, 20%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.32 – 7.23 (m, 2H), 7.23 – 7.12 (m, 3H), 7.04 (d, *J* = 8.5 Hz, 2H), 7.01 – 6.90 (m, 1H), 6.83 – 6.72 (m, 2H), 5.26 (br s, 1H), 3.50 (q, *J* = 7.0 Hz, 2H), 2.93 (t, *J* = 7.3 Hz, 2H), 2.76 (t, *J* = 7.1 Hz, 2H), 2.64 (t, *J* = 7.6 Hz, 2H), 1.93 (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.



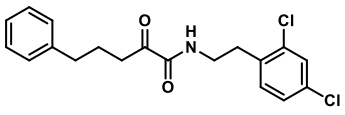
***N*-(4-Bromophenethyl)-2-oxo-5-phenylpentanamide (47).** The title compound was prepared according to general procedure D using α-ketoacid **60c** (0.96 g, 5.0 mmol, 1 eq), 2-(4-bromophenyl)ethan-1-amine (0.85 mL, 5.5 mmol, 1.1 eq), EDC·HCl (1.5 g, 7.5 mmol, 1.5 eq), HOBt (1.2 g, 7.5 mmol, 1.5 eq) 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.



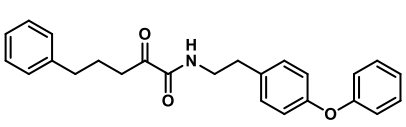
***N*-(3-Chlorophenethyl)-2-oxo-5-phenylpentanamide (48).** The title compound was prepared according to general procedure D using α-ketoacid **60c** (72 mg, 0.37 mmol, 1 eq), 2-(3-chlorophenyl)ethan-1-amine (57 μl, 0.41 mmol, 1.1 eq), EDC·HCl (0.11 g, 0.56 mmol, 1.5 eq), HOBt (86 mg, 0.56 mmol, 1.5 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.



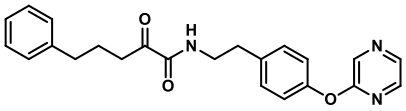
***N*-(2-Chlorophenethyl)-2-oxo-5-phenylpentanamide (49).** The title compound was prepared according to general procedure D using α-ketoacid **60c** (81 mg, 0.42 mmol, 1 eq), 2-(2-chlorophenyl)ethan-1-amine (66 μl, 0.47 mmol, 1.1 eq), EDC·HCl (0.12 g, 0.64 mmol, 1.5 eq) and HOBt (86 mg, 0.64 mmol, 1.5 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.



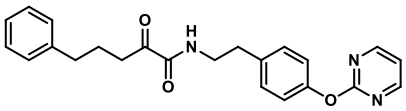
***N*-(2,4-Dichlorophenethyl)-2-oxo-5-phenylpentanamide (50).** The title compound was prepared according to general procedure D using α-ketoacid **60c** (95 mg, 0.49 mmol, 1 eq), 2-(2,4-dichlorophenyl)ethan-1-amine (89 μl, 0.54 mmol, 1.1 eq), EDC·HCl (0.14 g, 0.74 mmol, 1.5 eq) and HOBt (0.10 g, 0.74 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.



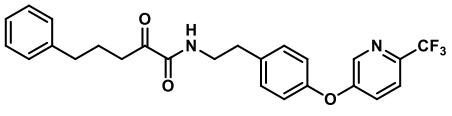
2-Oxo-*N*-(4-phenoxyphenethyl)-5-phenylpentanamide (51, LEI-301). The title compound was prepared according to general procedure D using α-ketoacid **60c** (0.19 g, 1.0 mmol, 1 eq), 2-(4-phenoxyphenyl)ethan-1-amine TFA salt (0.36 g, 1.1 mmol, 1.1 eq), EDC·HCl (0.29 g, 1.5 mmol, 1.5 eq), HOBt (0.23 g, 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.

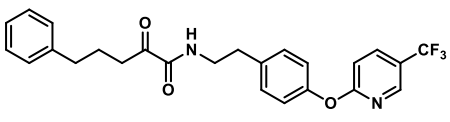


2-Oxo-5-phenyl-*N*-(4-(pyrimidin-2-yloxy)phenethyl)pentanamide (53). *Boc-deprotection **65b**:* a round bottom flask was charged with Boc-protected amine **65b** (0.30 g, 0.94 mmol, 1 eq) and HCl (4 M in dioxane, 4.5 mL, 18 mmol, 19 eq). After stirring for 1 h the mixture was concentrated under reduced 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.

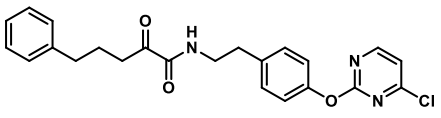


2-Oxo-5-phenyl-*N*-(4-((6-(trifluoromethyl)pyridin-3-yl)oxy) phenethyl)pentanamide (54). *Boc-deprotection **65c**:* a round bottom flask was charged with Boc-protected amine **65c** (0.18 g, 0.48 mmol, 1 eq) and HCl (4 M in dioxane, 3 mL, 12 mmol, 25 eq). After stirring for 1 h the mixture was concentrated 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,

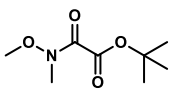
0.20 mmol, 1 eq), EDC·HCl (46 mg, 0.24 mmol, 1.2 eq), HOBt (32 mg, 0.24 mmol, 1.2 eq), NMM (87 μL, 0.80 mmol, 4 eq) and the amine HCl salt **66c** (68 mg, 0.24 mmol, 1.2 eq). Purification by preparative HPLC (C18 reverse phase, 65% to 75% ACN/H_2O + 0.2% TFA, RT 8.55 min) afforded the product (21 mg, 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.

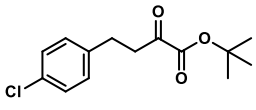


***N*-(4-((4-Chloropyrimidin-2-yl)oxy)phenethyl)-2-oxo-5-phenyl pentanamide (56).** *Boc-deprotection **65e**:* a round bottom flask was charged with Boc-protected amine **65e** (0.18 g, 0.50 mmol, 1 eq) and HCl (4 M in dioxane, 3 mL, 12 mmol, 24 eq). After stirring for 1 h the mixture was concentrated 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.

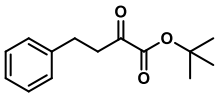


***tert*-Butyl 2-(methoxyamino)-2-oxoacetate (58).** A round bottom flask was charged with oxalyl chloride (13.5 ml, 158 mmol, 1 eq) in dry THF (200 mL) under an inert atmosphere and was cooled to 0 °C. *tert*-Butanol (14.7 ml, 154 mmol, 0.975 eq.) was added in one batch and the mixture was stirred for 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

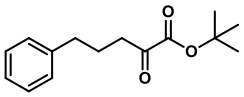
mL), brine (1 x 200 mL), dried ($MgSO_4$), filtered and concentrated under reduced pressure. The crude residue was purified using silica gel column chromatography (0% -> 20% EtOAc in pentane) affording the product (22.3 g, 117 mmol, 75%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.76 (s, 3H), 3.20 (s, 3H), 1.56 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 161.98, 161.63, 83.89, 61.82, 30.88, 27.62.



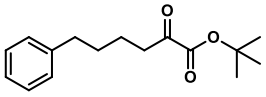
***tert*-Butyl 4-(4-chlorophenyl)-2-oxobutanoate (59a).** The title compound was prepared according to general procedure A using magnesium (0.42 gram, 18.3 mmol, 2.0 eq), 1-(2-bromoethyl)-4-chlorobenzene (1.3 mL, 9.1 mmol, 1 eq) and Weinreb amide **58** (1.7 gram, 9.1 mmol, 1.0 eq), affording the product (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.



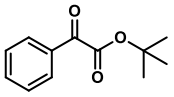
***tert*-Butyl 2-oxo-4-phenylbutanoate (59b).** The title compound was prepared according to general procedure A using magnesium (72 mg, 3.0 mmol, 0.7 eq), (2-bromoethyl)benzene (0.58 mL, 4.2 mmol, 1 eq) and Weinreb amide **58** (0.80 g, 4.2 mmol, 1 eq), affording the product (0.22 g, 0.95 mmol, 22%). ^{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.



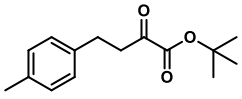
***tert*-Butyl 2-oxo-5-phenylpentanoate (59c).** The title compound was prepared according to general procedure A using magnesium (2.3 g, 94 mmol, 2.0 eq), (3-bromopropyl)benzene (11 mL, 71 mmol, 1.5 eq) and Weinreb amide **58** (8.9 g, 47 mmol, 1.0 eq). Column chromatography (0% -> 10% EtOAc in pentane)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



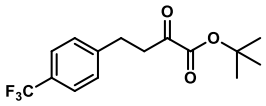
***tert*-Butyl 4-(2-chlorophenyl)-2-oxobutanoate (59d).** The title compound was prepared according to general procedure A using magnesium (0.17 g, 7.0 mmol, 2 eq), (4-bromobutyl)benzene (0.62 mL, 3.5 mmol, 1 eq) and Weinreb amide **58** (0.67 g, 3.5 mmol, 1 eq), affording the product (0.33 g, 1.3 mmol, 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.



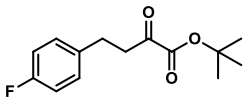
***tert*-Butyl 2-oxo-2-phenylacetate (59e).** The title compound was prepared according to general procedure A using magnesium (0.21 g, 8.5 mmol, 2 eq), bromobenzene (0.43 mL, 4.2 mmol, 1 eq) and Weinreb amide **58** (0.80 g, 4.2 mmol, 1 eq), affording the product (0.73 g, 3.5 mmol, 83%).^{1}H NMR (400 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.



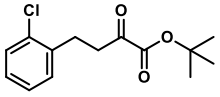
***tert*-Butyl 2-oxo-4-(*p*-tolyl)butanoate (59f).** The title compound was prepared according to general procedure A using magnesium (0.24 g, 10 mmol, 2 eq), 1-(2-bromoethyl)-4-methylbenzene (0.77 mL, 5.0 mmol, 1 eq) and Weinreb amide **58** (0.95 g, 5.0 mmol, 1 eq), affording the product (0.54 g, 2.2 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.



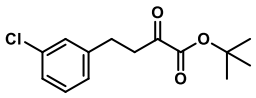
***tert*-Butyl 2-oxo-4-(4-(trifluoromethyl)phenyl)butanoate (59g).** The title compound was prepared according to general procedure A using magnesium (0.18 g, 7.9 mmol, 2 eq), 1-(2-bromoethyl)-4-(trifluoromethyl)benzene (0.67 mL, 4.0 mmol, 1 eq) and Weinreb amide **58** (0.75 g, 4.0 mmol, 1 eq), affording 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.



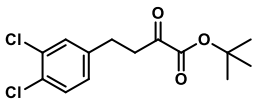
***tert*-Butyl 4-(4-fluorophenyl)-2-oxobutanoate (59h).** The title compound was prepared according to general procedure A using magnesium (0.24 g, 10.6 mmol, 2 eq), 1-(2-bromoethyl)-4-fluorobenzene (0.72 mL, 5.3 mmol, 1 eq) and Weinreb amide **59** (1.1 g, 5.3 mmol, 1 eq), affording the product (0.28 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.



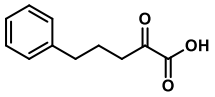
***tert*-Butyl 4-(2-chlorophenyl)-2-oxobutanoate (59i).** The title compound was prepared according to general procedure A using magnesium (0.22 g, 9.1 mmol, 2 eq), 1-(2-bromoethyl)-2-chlorobenzene (0.69 mL, 4.6 mmol, 1 eq) and Weinreb amide **58** (0.86 g, 4.6 mmol, 1 eq), affording the product (0.34 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.



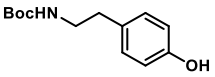
***tert*-Butyl 4-(3-chlorophenyl)-2-oxobutanoate (59j).** The title compound was prepared according to general procedure A using magnesium (0.22 g, 9.1 mmol, 2 eq), 1-(2-bromoethyl)-3-chlorobenzene (0.67 mL, 4.6 mmol, 1 eq) and Weinreb amide **58** (0.86 g, 4.6 mmol, 1 eq), affording the product (0.51 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.



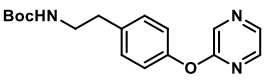
***tert*-Butyl 4-(3,4-dichlorophenyl)-2-oxobutanoate (59k).** The title compound was prepared according to general procedure A using magnesium (34 mg, 1.4 mmol, 1.2 eq), 4-(2-bromoethyl)-1,2-dichlorobenzene (0.30 g, 1.2 mmol, 1 eq) and Weinreb amide **58** (0.27 g, 1.4 mmol, 1.2 eq), affording the product (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.



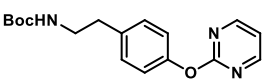
2-Oxo-5-phenylpentanoic acid (60c). The title compound was prepared according to general procedure B using α-ketoester **59c** (7.4 g, 30 mmol, 1 eq) and TFA (23 mL, 300 mmol, 10 eq) affording the product (5.8 g, 30 mmol, quant.). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.33 – 7.12 (m, 5H), 2.91 (t, *J* = 7.2 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.



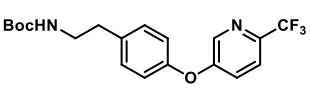
***N*-Boc-tyramine (64).** A round bottom flask was charged with tyramine (5.0 gram, 36 mmol, 1.0 eq), and dissolved in THF (160 mL). Boc_2O (8.1 gram, 37 mmol, 1.0 eq) and a solution of $NaHCO_3$ (3.4 gram, 40 mmol, 1.1 eq) in water (80 mL) was added and the reaction was stirred vigorously overnight. 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.



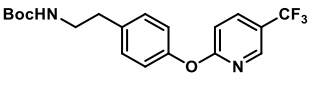
***tert*-Butyl (4-(pyrazin-2-yloxy)phenethyl)carbamate (65a).** The title compound was prepared according to general procedure E using *N*-Boc-tyramine **64** (0.48 g, 2.0 mmol, 1 eq), 2-chloropyrazine (0.18 mL, 2.0 mmol, 1 eq) and K_2CO_3 (0.55 g, 4.0 mmol, 2 eq) in DMSO (2 mL). Column chromatography (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.



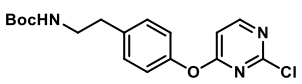
***tert*-Butyl (4-(pyrimidin-2-yloxy)phenethyl)carbamate (65b).** The title compound was prepared according to general procedure E using *N*-Boc-tyramine **64** (0.48 g, 2 mmol, 1 eq), 2-chloro-pyrimidine (0.23 g, 2 mmol, 1 eq) and K_2CO_3 (0.55 g, 4 mmol, 2 eq) in DMSO (2 mL). Column chromatography (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.



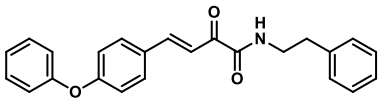
***tert*-Butyl (4-((6-(trifluoromethyl)pyridin-3-yl)oxy)phenethyl) carbamate (65c).** The title compound was prepared according to general procedure E using *N*-Boc-tyramine **64** (0.25 g, 1.05 mmol, 1.05 eq), 2-trifluoromethyl-5-fluoropyridine (0.12 mL, 1 mmol, 1 eq) and K_2CO_3 (0.21 g, 1.5 mmol, 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.

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Supplementary Information

Supplementary Table 1. PLAAT2 overexpression does not significantly increase fatty acid levels in U2OS cells, except for arachidonic acid. Data represent mean values ± SD for 4 biological replicates. *P*-values were determined by one-way ANOVA.

Fatty acid	**Fold change ± SD PLAAT2 vs. mock**	***P*-value**
palmitic acid (16:0)	1.28 ± 0.24	0.208
stearic acid (18:0)	1.41 ± 0.23	0.113
oleic acid (18:1-ω9)	1.05 ± 0.32	0.847
α-linoleic acid (18:2-ω6)	0.55 ± 0.18	0.100
α-linolenic acid (18:3-ω3)	0.71 ± 0.28	0.370
γ-linolenic acid (18:3-ω6)	0.36 ± 0.13	0.026
mead acid (20:3-ω9)	0.79 ± 0.26	0.399
arachidonic acid (20:4-ω6)	1.81 ± 0.45	0.040
FFA (20:3-ω6-&-ω3)	0.79 ± 0.22	0.345
eicosapentaenoic acid (20:5-ω3)	1.27 ± 0.42	0.366
docosapentaenoic acid (22:5-ω3)	0.95 ± 0.31	0.856