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3-Aminoazetidin-2-one Derivatives as *N*-Acylethanolamine Acid Amidase (NAAA) Inhibitors Suitable for Systemic Administration

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N-Acylethanolamine acid amidase (NAAA) is a cysteine hydrolase that catalyzes the hydrolysis of endogenous lipid mediators such as palmitoylethanolamide (PEA). PEA has been shown to exert anti-inflammatory and antinociceptive effects in animals by engaging peroxisome proliferator-activated receptor α (PPAR-α). Thus, preventing PEA degradation by inhibiting NAAA may provide a novel approach for the treatment of pain and inflammatory states. Recently, 3-aminooxetan-2-one compounds were identified as a class of highly potent NAAA inhibitors. The utility of these compounds is limited, however, by their low chemical and plasma stabilities. In the present study, we synthesized and tested a series of *N*-(2-oxoazetidin-3-yl)amides as a novel class of NAAA inhibitors with good potency and improved physicochemical properties, suitable for systemic administration. Moreover, we elucidated the main structural features of 3-aminoazetidin-2-one derivatives that are critical for NAAA inhibition.

Introduction

Fatty acid ethanolamides (FAEs) are a family of lipid-derived messengers that play important roles in the control of pain, inflammation, and energy balance.[1] Two structurally and functionally distinct classes of FAEs have been described: polyunsaturated FAEs, such as arachidonoylethanolamide (anandamide), which are endogenous ligands for cannabinoid receptors and, among other functions, participate in the control of stress coping responses and pain initiation;[2] and monounsaturated and saturated FAEs, such as oleoylethanolamide (OEA) and palmitoylethanolamide (PEA), which are endogenous ligands for peroxisome proliferator-activated receptor α (PPAR-α) and are involved in energy balance, pain, and inflammation.[1a]

Within the latter class, PEA, the endogenous amide of palmitic acid and ethanolamine, has been shown to inhibit peripheral inflammation and mast cell degranulation[3] and to exhibit antinociceptive properties in rat and mouse models of acute and chronic pain.[3b,4] Those properties depend on PPAR-α activation because they are absent in PPAR-α-null mice, are mimicked by synthetic PPAR-α agonists, and are blocked by PPAR-α antagonists.[5] Furthermore, PEA has been reported to suppress pain behaviors induced by tissue injury, nerve damage, or inflammation in mice[5c] and to attenuate skin inflammation and neuropathic pain in humans.[6]

Tissue FAE levels are regulated at the level of biosynthesis and degradation.[7] A molecularly unique phospholipase D releases FAEs from precursor *N*-acylphosphatidylethanolamines (NAPEs) present in cell membranes.[8] Degradation of FAEs to the corresponding free fatty acids and ethanolamine is carried out by either fatty acid amide hydrolase (FAAH) or *N*-acylethanolamine acid amidase (NAAA).[9] These two intracellular lipid amidases show different cellular localization and substrate selectivity, with NAAA preferentially catalyzing the hydrolysis of PEA and OEA in innate immune cells.[4b]

NAAA[10] is a cysteine amidase that belongs to the N-terminal nucleophile (Ntn) family of enzymes,[11] and is characterized by a conserved amino acid catalytic triad Cys, Arg, and Asp. The primary structure of NAAA has no homology with that of FAAH,[11] while it shares 33–34% identity and 70% similarity with acid ceramidase (AC), a cysteine amidase involved in the hydrolysis of the sphingolipid messenger ceramide. NAAA is thought to be localized to lysosomes[12] and shares common features with other lysosomal hydrolases, that is, N-glycosylation, autoproteolytic cleavage,[13] and activation at acidic pH (around 4.5–5.0).[9a] After its maturation, the N-terminal cys-

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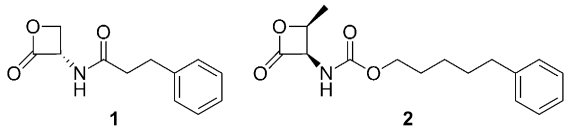
teine residue (Cys 131 in rodents; Cys 126 in humans) is responsible for the catalytic cleavage of nonpeptide C–N bonds in linear amides;[14] for this reason, NAAA is also linked to the choloylglycine hydrolase superfamily.[11] Site-directed mutagenesis[15] and mass spectrometry studies[16] have unambiguously identified human Cys 126 as the nucleophile responsible for both autoproteolysis and activity.

Recently, inhibition of NAAA activity has attracted increasing interest as a strategy to sustain endogenous PEA and OEA levels, and this enzyme has been envisaged as a new potential therapeutic target for inflammation.[5a] So far, only a limited number of natural[17] or synthetic[18] NAAA inhibitors have been reported in the literature. Among these, α-amino-β-lactone (3-aminooxetan-2-one) derivatives have shown considerable promise because of their high inhibitory potency and target selectivity.[19] In particular, in vitro and in vivo studies have demonstrated that (*S*)-*N*-(2-oxo-3-oxetanyl)-3-phenylpropionamide ((*S*)-OOPP, **1**), which inhibits rat-NAAA (*r*-NAAA) with



1 (*S*)-OOPP

2 ARN077

a median inhibitory concentration (IC_{50}) of 0.42 μM,[19a,f] blocks FAE hydrolysis in activated inflammatory cells and dampens tissue reactions to various pro-inflammatory stimuli.[19b] More recently, 5-phenylpentyl *N*-[(2*S*,3*R*)-2-methyl-4-oxo-oxetan-3-yl]-carbamate (ARN077, **2**; *r*-NAAA IC_{50} = 0.050 μM),[19c,d,f] a new potent and selective β-lactone NAAA inhibitor, was shown to elevate PEA and OEA levels in mouse skin and sciatic nerve tissues and to attenuate nociception in mice and rats following topical administration.[20]

Despite their high potency and efficacy, β-lactone-based NAAA inhibitors suffer from limited chemical and plasma stabilities,[19c,e] due to facile ring-opening to the corresponding β-hydroxy acid. Their low chemical stability makes these derivatives suitable for topical applications[19c,e] but prevents their use as systemic agents.

To further explore the therapeutic utility of NAAA inhibitors, we started a program aimed at identifying new chemical series that might be suitable for systemic administration. Within this program, we investigated functionalized α-amino-β-lactam (3-amidoazetidin-2-one) derivatives, a chemotype structurally similar to 3-aminooxetan-2-one, to test whether replacement of the β-lactone core with a β-lactam retained inhibitory activity toward NAAA, while increasing chemical and plasma stabilities.

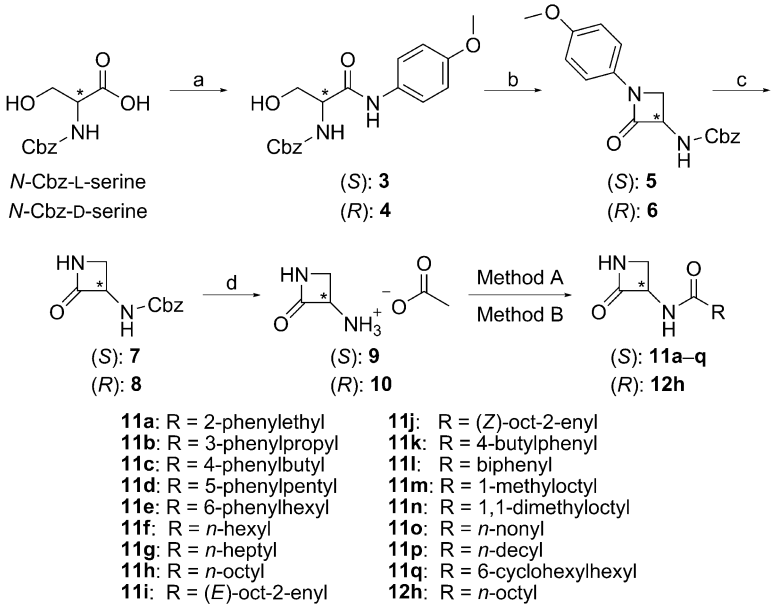
In the present work, we report the discovery of 3-aminoazetidin-2-ones as a new class of NAAA inhibitors endowed with good potency and promising chemical and plasma stabilities, which may make these derivatives suitable for systemic administration. In particular, we prepared a small series of β-lactam analogues and evaluated their inhibitory activities toward human NAAA (*h*-NAAA). This study allowed us to highlight key structural features for NAAA inhibition and derive an initial structure–activity relationship (SAR).

Results and Discussion

Chemistry

Differently substituted 3-aminoazetidin-2-one derivatives were efficiently accessed in an enantioselective fashion, starting from the corresponding D- or L-N-protected-serine derivatives. As reported in Scheme 1, the designed synthetic route envisaged both the (*S*)- and (*R*)-2-oxoazetidin-3-ylammonium acetate (**9** and **10**)[21] as key intermediates for the synthesis of 3-aminoazetidin-2-one amide derivatives bearing different side chains. Notably, by modifications and optimization of published procedures,[22] compounds **9** and **10** were prepared on a multigram scale, with 40–44% overall yield over four steps.[23]

First, benzyloxycarbonyl (Cbz)-protected azetidinones **7** and **8** were prepared following a three-step sequence.[22a] Amide coupling between *p*-anisidine and *N*-[(benzyloxycarbonyl)]-L- and D-serine afforded intermediates **3** and **4**, which were con-



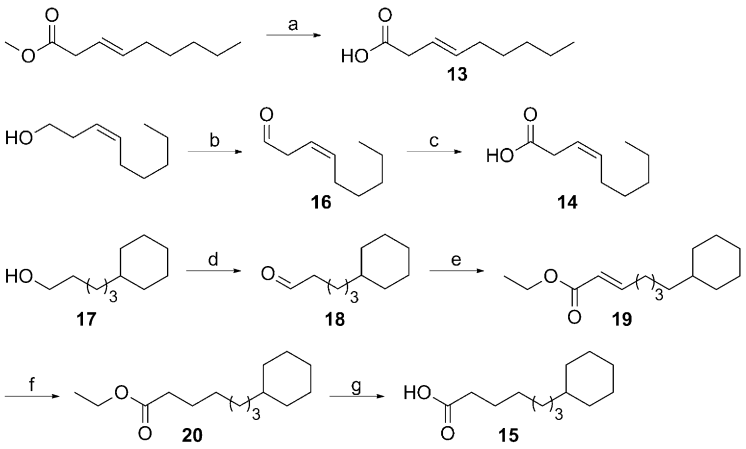
Scheme 1. Synthetic pathway for the preparation of amide derivatives **11a–q** and **12h**. *Reagents and conditions*: a) *p*-anisidine, EDC, THF/CH_2Cl_2 (3:1), RT, 16 h, 85–92%; b) $ImSO_2Im$, 0 °C, 1 h, then NaH, DMF, −20 °C, 1 h, 75%; c) CAN, MeCN/H_2O (1:1), 0 °C, 1 h, 80–85%; d) 1,4-cyclohexadiene, 10% Pd on charcoal, EtOH, RT, 12 h, then AcOH, EtOAc, 79%; Method A [for **11a**, **11f–h**, **11l**, **11o**, and **12h**]: RCOCl, Et_3N, CH_2Cl_2 or CH_2Cl_2/DMF (3:1), RT, 16 h,15–65%; Method B [for **11b–e**, **11i–k**, **11m–n**, and **11p–q**]: RCOOH, TBTU, Et_3N, CH_2Cl_2/DMF (3:1), RT, 16 h, 30–62%.

verted into the corresponding N-protected α-amino-β-lactams **5** and **6** by a one-pot cyclization reaction, which represents the key step in this synthesis. Subsequent *p*-methoxyphenyl deprotection with ceric ammonium nitrate in an acetonitrile/water (1:1) mixture led to β-lactams **7** and **8**. Cbz deprotection[22b] was then carried out by catalytic transfer hydrogenation, using 1,4-cyclohexadiene as a hydrogen source in the presence of 10% palladium on charcoal.[24] The resulting unprotected 3-aminoazetidin-2-ones were immediately trapped as acetate salts **9** and **10**, which could be easily stored for several months.

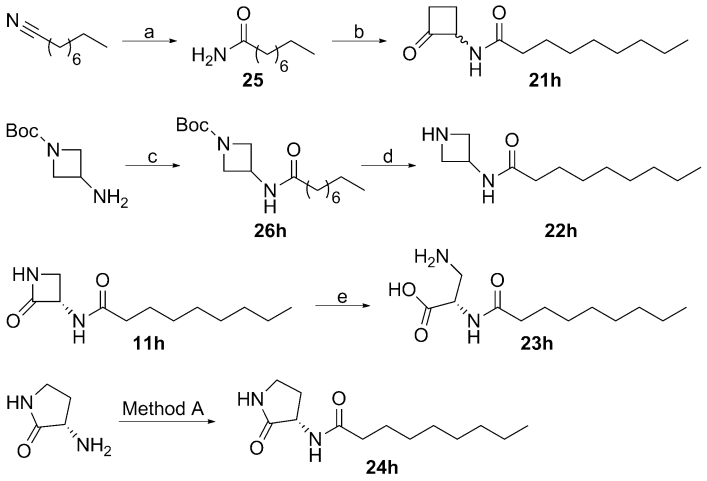
Coupling reactions of salt **9** with the suitable acyl chloride in the presence of triethylamine afforded amides **11a**, **11f**–**h**, **11l**, and **11o**, while reaction of enantiomer **10** with nonanoyl chloride led to compound **12h** (Method A, Scheme 1). Alternatively, the reaction of compound **9** with the appropriate carboxylic acid, activated with TBTU and triethylamine, gave amides **11b**–**e**, **11i**–**k**, **11m**–**n**, and **11p**–**q** (Method B, Scheme 1). In all coupling reactions, no racemization was detected at the stereogenic α-center.

All of the employed acyl chlorides and most of the carboxylic acids were commercially available; carboxylic acids **13**, **14**, and **15** were prepared as reported in Scheme 2. Compound **13** was synthesized by saponification of commercially available methyl (*E*)-3-nonenoate (Scheme 2). The preparation of (*Z*)-non-3-enoic acid (**14**) was accomplished in two steps starting from commercially available (*Z*)-non-3-en-1-ol. Oxidation with Dess–Martin periodinane[25] led to the exclusive formation of (*Z*)-non-3-enal (**16**) (Scheme 2). Further oxidation under Pinnick reaction conditions, in the presence of 2-methyl-2-butene as a scavenger, selectively yielded acid **14** as a single diastereomer.

Carboxylic acid **15** was prepared starting from 5-cyclohexylpentan-1-ol (**17**),[26] which was first oxidized to aldehyde **18**. Subsequent Horner–Emmons olefination afforded the α,β-unsaturated ester **19**, which was subjected to hydrogenation, followed by hydrolysis, to furnish compound **15** (Scheme 2).



Scheme 2. Syntheses of carboxylic acids **13**, **14**, and **15**. *Reagents and conditions*: a) LiOH, THF/MeOH/H_2O (1:1:1), RT, 1 h, quant.; b) Dess–Martin periodinane, CH_2Cl_2, 0 °C, 1 h, then RT, 1 h, 94%; c) $NaClO_2$, NaH_2PO_4, 2-methyl-2-butene, *t*BuOH/H_2O 4:1, RT, 1.5 h, quant.; d) DMSO, oxalyl chloride, CH_2Cl_2, −78 °C, 15 min then **17**, −78 °C, 1 h, then Et_3N, RT, quant.; e) $(EtO)_3POCH_2CO_2Et$, NaH (95%), THF, 0 °C→RT, 16 h, 77%; f) H_2, 10% Pd/C cartridge (H-Cube, EtOH, 45 °C, 20 bar, flow: 1.0 mL min^{-1}), 92%; g) LiOH, THF/MeOH/H_2O (1:1:1), RT, 1 h, quant.

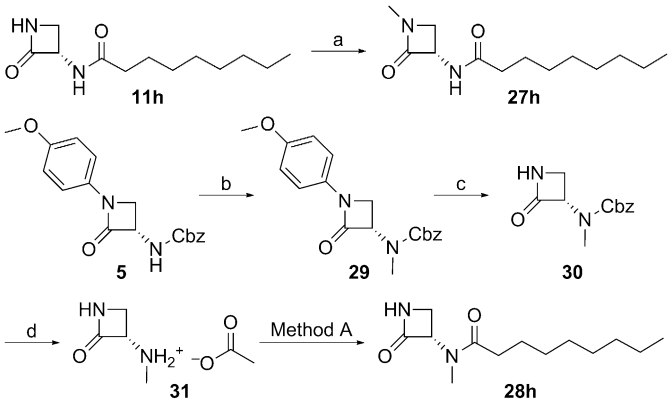


Scheme 3. Syntheses of compounds **21h**–**24h**. *Reagents and conditions*: a) 1 N NaOH, 35%, H_2O_2, 60% *t*BuOH·H_2O, RT, 16 h, 82%; b) 2 N HCl·Et_2O, 1,2-bis(trimethylsiloxy)cyclobutene, THF, reflux, 3 h, 37%; c) nonanoyl chloride, Et_3N, CH_2Cl_2, RT, 2 h, 94%; d) TFA/CH_2Cl_2 (1:3), 0 °C, 30 min, then RT, 45 min, quant.; e) 2 N HCl, RT, 30 min, then THF, RT, 16 h, 79%; Method A: nonanoyl chloride, Et_3N, CH_2Cl_2, RT, 16 h, 83%.

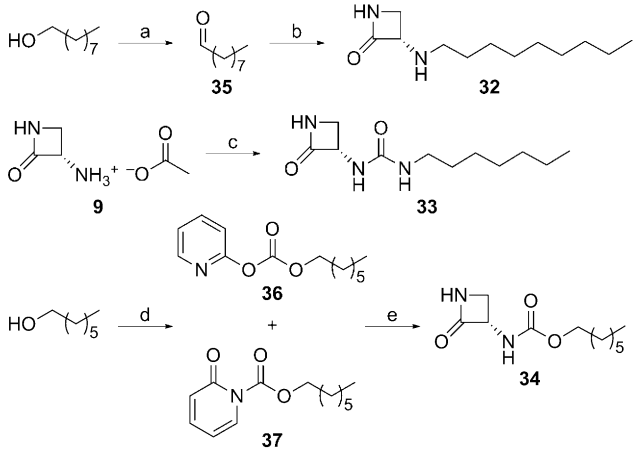
Compounds **21h**–**24h** were synthesized as reported in Scheme 3. Cyclobutanone derivative **21h** was prepared by the acid-catalyzed reaction of 1,2-bis(trimethylsilanoxy)cyclobutene with amide **25**, obtained by alkaline hydrolysis of nonanenitrile (Scheme 3).[19c] The synthesis of compound **22h** was accomplished starting from commercially available *N*-Boc-3-amino-azetidine by standard amide coupling with nonanoyl chloride, followed by Boc deprotection with TFA/CH_2Cl_2 (Scheme 3). Acid-mediated ring-opening of **11h** smoothly delivered compound **23h** as a hydrochloride salt in good yield (Scheme 3). Finally, standard amide coupling of nonanoyl chloride with commercially available (3*S*)-3-aminopyrrolidin-2-one led to derivative **24h** (Scheme 3).

N-methylated amides **27h** and **28h** were prepared as reported in Scheme 4. Alkylation of azetidinone **11h** with methyl iodide in the presence of sodium hydride in DMF selectively occurred on the N1 endocyclic nitrogen, providing compound **27h** (Scheme 4). Alkylation of protected β-lactam **5** with methyl iodide under standard conditions afforded compound **29** in good yield. Cleavage of the *p*-anisyl group, followed by catalytic hydrogenation, led to *N*-methyl acetate salt **31**, which gave *N*-methyl derivative **28h** after amide coupling with nonanoyl chloride using Method A (Scheme 4).

Acetate salt **9** was used to access amine, urea, and carbamate derivatives **32**, **33**, and **34**, respectively, as reported in Scheme 5. Amine derivative **32** was synthesized by reductive amination of aldehyde **35**, obtained by oxidation of *n*-nonanol



Scheme 4. Syntheses of N-methylated compounds **27h**–**28h**. *Reagents and conditions*: a) NaH (60% in mineral oil), MeI, THF, 0 °C, 1 h then RT, 4 h, 25%; b) NaH (95%), MeI, THF, 0 °C, 1 h then RT, 1 h, 90%; c) CAN, MeCN/H_2O (1:1), 0 °C, 1 h, 92%; d) H_2, 10% Pd/C cartridge (H-Cube, EtOAc, 30 °C, 1.0 bar, flow: 1.0 mL min^{-1}), then AcOH, EtOAc, 78%; Method A: nonanoyl chloride, Et_3N, CH_2Cl_2, RT, 16 h, 33%.



Scheme 5. Syntheses of amine, urea, and carbamate derivatives **32**–**34**. *Reagents and conditions*: a) DMSO, oxalyl chloride, CH_2Cl_2, −78 °C, 15 min, then *n*-nonanol −78 °C, 1 h, then Et_3N, RT, quant.; b) **9**, Et_3N, dichloroethane, 10 min, then $Na(OAc)_3BH$, RT, 1.5 h, 21%; c) nonyl isocyanate, DMAP, pyridine, RT, 16 h, 59%; d) DMAP, 2-DPC, CH_2Cl_2, RT, 16 h, quant.; e) **9**, DIPEA, CH_2Cl_2, RT, 16 h, 36%.

with salt **9**, while urea **33** was obtained by treating **9** with nonyl isocyanate in the presence of catalytic DMAP (Scheme 5). Carbamate **34** was synthesized in a two-step sequence. *n*-Heptanol was reacted with 2-pyridyl carbonate (2-DPC)[19d,27] and catalytic DMAP to give an isomeric mixture of 2-pyridyl carbonate **36** and 2-oxopyridine-1-carboxylate **37** (65:35 ratio), which was then coupled with **9** to afford desired compound **34** in good yield (Scheme 5).

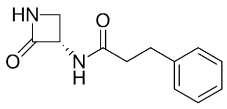
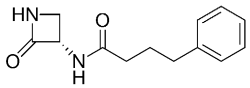
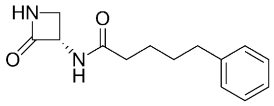
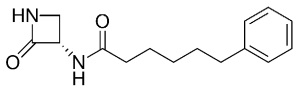
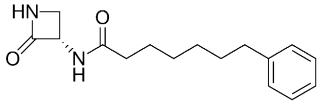
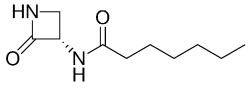
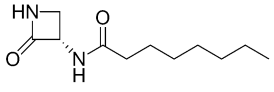
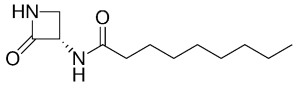
Structure–activity relationship (SAR) and stability studies

The aim of the present study was to explore substituted 3-aminoazetidin-2-one derivatives as a novel class of NAAA inhibitors potentially suitable for systemic administration. The new compounds were tested for their ability to inhibit the hydrolysis of *N*-(4-*m*ethyl-2-oxo-chromen-7-yl)hexadecanamide (PAMCA) by recombinant *h*-NAAA heterologously expressed in HEK293 human embryonic kidney cells (see Experimental Section).[16] Median inhibitory concentration (IC_{50}) values are reported in Tables 1, 3, and 4. A few representative compounds were also evaluated for their chemical stability in buffer at pH 7.4 and 5.0[28] and for their stability in mouse plasma (Table 2).

We used β-lactam derivative **11a** (Table 1), an analogue of the known β-lactone NAAA inhibitor (*S*)-OOPP (**1**, *h*-NAAA IC_{50}: 1.29 μM), as the starting point for our initial SAR work. Disappointingly, **11a** turned out to be inactive. However, the compound showed substantially higher hydrolytic stability ($t_{1/2}$ > 1440 min at both pH 7.4 and pH 5.0) and promising mouse plasma stability ($t_{1/2}$ > 120 min) and rat plasma stability ($t_{1/2}$ > 120 min) relative to (*S*)-OOPP ($t_{1/2}$ < 1 min) (Table 2).

Based on previous SAR results for the β-lactone class,[19d] we hypothesized that activity might be recovered by increasing the length of the amide side chain. Therefore, a small set of analogues was prepared in which the length of the phenylalkyl chain of **11a** was progressively increased from two to six methylene units (**11b**–**e**, Table 1). Confirming our hypothesis, NAAA inhibitory activity, albeit in the high micromolar range,

Table 1. Inhibitory potencies of compounds **11a**–**h** against human *N*-acylethanolamine acid amidase (NAAA).

Compd	Structure	IC_{50} [μM][a]
11a		n.a.[b]
11b		73.36 ± 10.68
11c		27.16 ± 1.89
11d		12.06 ± 2.18
11e		0.60 ± 0.17
11f		27.02 ± 6.15
11g		1.91 ± 0.28
11h		0.34 ± 0.03

[a] Values are the mean ± SEM of three or more determinations. [b] Not active (n.a.): < 10% inhibition at 50 μM.

Table 2. Stability data of (*S*)-OOPP (**1**) and compounds **11 a** and **11 h** at various pH values and in mouse (*m*) and rat (*r*) plasma.[a]

Compd	$t_{1/2}$ [min][b]		$t_{1/2}$ [min]	
	pH 7.4	pH 5.0	*m* plasma[c]	*r* plasma[d]
1	30	45	< 1	n.t.[e]
11 a	> 1440 (*92*)	> 1440 (*97*)	> 120	> 120
11 h	> 1440 (*100*)	> 1440 (*98*)	103	> 120

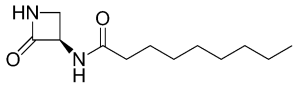
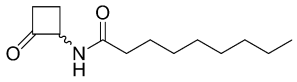
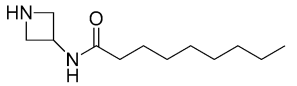
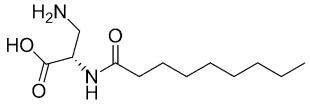
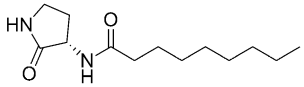
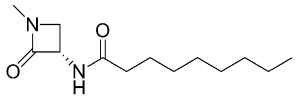
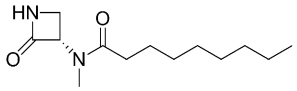
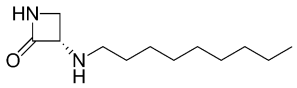
[a] All stability values are the mean ± SEM of three or more determinations. [b] Chemical stability measured after 24 h incubation at 37 °C in PBS + 10% MeCN, at pH 7.4 and 5.0; values in parentheses represent the compound remaining (%) after 24 h (1440 min). [c] Measured in 100% mouse plasma. [d] Compounds tested at 2.5 μM in rat plasma with 2.5% DMSO. [e] Not tested (n.t.); compound **1** is reported to have $t_{1/2} < 10$ s in 80% rat plasma.[19c]

was observed for compounds **11 b** ($IC_{50} = 73.36$ μM) and **11 c** ($IC_{50} = 27.16$ μM), while 6-phenylhexyl analogue **11 e** showed sub-micromolar potency ($IC_{50} = 0.60$ μM). Removal of the phenyl group from **11 e** led to compound **11 f** ($IC_{50} = 27.02$ μM), which showed a greater than 40-fold drop in potency relative to **11 e**. Good inhibitory activity against NAAA was again recovered by increasing the length of the alkyl chain from six to eight carbon atoms, as in analogue **11 h** ($IC_{50} = 0.34$ μM), which turned out to be the most potent derivative within this small series of compounds. Notably, β-lactam **11 h** retained a favorable chemical and plasma stability profile (Table 2), proving stable to chemical hydrolysis over 24 h ($t_{1/2} >$ 1440 min) and showing an acceptable half-life in mouse and rat plasma ($t_{1/2} = 103$ min and > 120 min, respectively). These initial results suggested that *N*-(2-oxoazetidin-3-yl)amide derivatives may have potential as a new potent and stable class of NAAA inhibitors.

Encouraged by these results, we considered compound **11 h** as the prototype for this novel class of inhibitors and synthesized an initial set of analogues to identify the key structural features for NAAA inhibition. We first modified the 3-aminoazetidin-2-one scaffold while maintaining fixed the amide chain; the stereochemistry at the C3 position was inverted, the nitrogen and the carbonyl group of the azetidinone ring were replaced by a methylene, and the β-lactam ring was hydrolyzed and expanded to the five-atom γ-lactam homologue. Finally, the endocyclic nitrogen was methylated to assess the stereoelectronic requirements at this position.

The importance of the *S* configuration for the substituent at the C3 position was clearly demonstrated by the greater than 200-fold loss in potency observed for compound **12 h** ($IC_{50} = 74.52$ μM, Table 3), the *R* enantiomer of **11 h**. This stereochemical preference is in agreement with previous observations of serine-derived β-lactone inhibitors.[19a,b] Replacement of the endocyclic nitrogen with a methylene (**21 h**), reduction of the azetidinone to an azetidine ring (**22 h**), ring-opening of the azetidinone (**23 h**), and ring-expansion to a γ-lactam (**24 h**) all led to loss of activity, as previously observed in the β-lactone series.[19a,b] Methylation of the N1 endocyclic nitrogen (**27 h**) abolished NAAA inhibitory activity. These findings unambiguously indicated that an intact β-lactam moiety was mandatory for NAAA inhibition.

Table 3. Inhibitory potencies of analogues of compound **11 h** against human *N*-acylethanolamine acid amidase (NAAA).

Compd	Structure	IC_{50} [μM][a]
12 h		74.52 ± 9.08
21 h		n.a.[b]
22 h		n.a.[b]
23 h		n.a.[b]
24 h		n.a.[b]
27 h		n.a.[b]
28 h		n.a.[b]
32		n.a.[b]

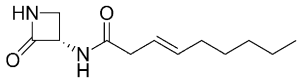
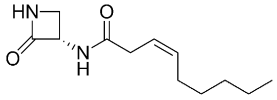
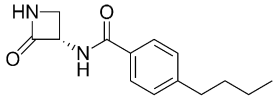
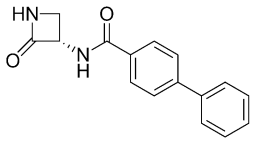
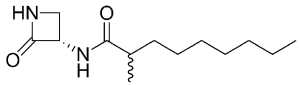
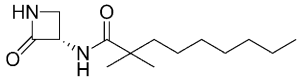
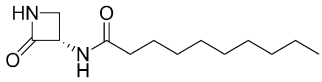
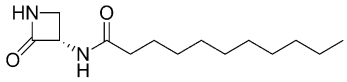
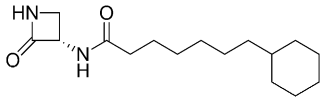
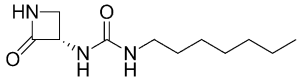
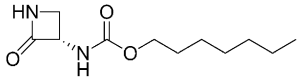
[a] Values are the mean ± SEM of three or more determinations. [b] Not active (n.a.): < 10% inhibition at 50 μM.

We then focused our attention on the amide functionality at the C3 position of the azetidinone ring. The exocyclic nitrogen was methylated, leading to compound **28 h**, and the amide was replaced by an *n*-nonylamino group, as in compound **32**. Both derivatives turned out to be inactive. A secondary amide at the C3 position of the azetidinone ring appears, therefore, to be required for NAAA inhibition.

As the next step in our SAR study, we directed our attention to the amide side chain and explored the importance of the conformational flexibility, substitution, and length of the alkyl chain. First, we introduced a steric constraint in compounds **11 i–j** (Table 4), two analogues of **11 h** that contain a double bond in the (*E*) and (*Z*) configuration, respectively. Both changes resulted in a 10-fold drop in potency, with no preference for the alkene configuration (**11 i**, $IC_{50} = 3.09$ μM; **11 j**, $IC_{50} = 3.90$ μM). Further reduction of the side chain flexibility by introduction of a *para*-substituted phenyl ring, as in compounds **11 k–l**, led to a decrease (**11 k**, $IC_{50} = 13.85$ μM) or total loss (**11 l**) of inhibitory activity. These findings indicated that the insertion of sterically constrained amide chains is detrimental for activity, contrary to what was observed for β-lactone amides.[19c]

We also synthesized compounds bearing a branched aliphatic side chain (**11 m** and **11 n**). A single methyl group close to

Table 4. Inhibitory potencies of compounds **11 i–q**, **33**, and **34** against human *N*-acylethanolamine acid amidase (NAAA).

Compd	Structure	IC_{50} [μM][a]
11 i		3.09 ± 0.50
11 j		3.90 ± 0.86
11 k		13.85 ± 3.41
11 l		n.a.[b]
11 m		0.22 ± 0.03
11 n		0.76 ± 0.26
11 o		0.24 ± 0.03
11 p		0.10 ± 0.02
11 q		0.28 ± 0.07
33		5.76 ± 0.90
34		0.24 ± 0.02

[a] Values are the mean ± SEM of three or more determinations. [b] Not active (n.a.): < 10% inhibition at 50 μM.

the amide functionality appeared to be well accommodated as compound **11 m** (IC_{50} = 0.22 μM), even as a mixture of diastereomers, showed a slight increase in potency relative to compound **11 h**. However, the introduction of a *gem*-dimethyl group in the same position, as in derivative **11 n** (IC_{50} = 0.76 μM), was detrimental for potency.

We then turned our attention to analogues with longer alkyl chain and prepared compounds containing a decanamide (**11 o**) and an undecanamide residue (**11 p**). These compounds showed improved NAAA inhibitory activity (**11 o**, IC_{50} = 0.24 μM; **11 p**, IC_{50} = 0.10 μM). This result supports the idea that linear lipophilic moieties, which resemble the natural enzyme substrate, are well accommodated in the active site.

The insertion of a terminal cyclohexyl moiety, as in amide **11 q** (IC_{50} = 0.28 μM), resulted in a slight decrease in potency compared with **11 p** (Table 4) but a 2-fold potency increase with respect to phenyl analogue **11 e** (Table 1). Finally, we investigated the impact of the functionality at the N3 exocyclic amino group of the β-lactam on NAAA inhibition by synthetizing the corresponding urea (**33**) and carbamate (**34**) analogues of amide **11 h** (Table 4). Replacement of the amide moiety with a urea, as in derivative **33** (IC_{50} = 5.76 μM), resulted in a marked decrease in potency. By contrast, carbamate **34** (IC_{50} = 0.24 μM) was notably more potent than amide **11 h**.

NAAA shows 33–34% identity and 70% similarity to AC, a cysteine amidase that catalyzes the deactivating hydrolysis of the pro-inflammatory lipid messenger, ceramide. We therefore tested the most representative compounds identified in our study (**11 h**, its enantiomer **12 h**, and **11 p**) for their selectivity against human AC and FAAH. The selectivity of compounds **11 h**, **12 h**, and **11 p** versus AC was evaluated using a UPLC–MS-based assay[19a,29] to measure IC_{50} values under similar experimental conditions. The results are reported in Table 5.

Table 5. Inhibition of human *N*-acylethanolamine acid amidase (NAAA), human acid ceramidase (AC), and human fatty acid amide hydrolase (FAAH) by compounds **11 h**, **11 p**, and **12 h**.

Compd	IC_{50} [μM][a]		Inhib. [%][b]
	h-NAAA	*h*-AC	*h*-FAAH
11 h	0.13 ± 0.03	2.53 ± 0.06	n.a.
12 h	45.54 ± 19.94	27.81 ± 5.38	n.a.
11 p	0.056 ± 0.010	0.33 ± 0.10	n.a.

[a] Values were determined by LC–MS; data are the mean ± SD of three or more determinations. [b] Percent inhibitory activity; not active (n.a.): < 10% inhibition at 100 μM.

Compound **11 h** showed ~19-fold selectivity for NAAA vs. AC (*h*-NAAA IC_{50} = 0.13 μM; *h*-AC IC_{50} = 2.53 μM) while its enantiomer, **12 h**, displayed low potency toward the two enzymes (*h*-NAAA IC_{50} = 45.5 μM; *h*-AC IC_{50} = 27.8 μM). Interestingly, lengthening the amide alkyl chain, as in compound **11 p**, led to an approximate 7-fold increase in potency toward *h*-AC (IC_{50} = 0.33 μM) and an approximate 2-fold increase in potency toward *h*-NAAA (IC_{50} = 0.056 μM). Lengthening of the alkyl chain appeared, therefore, to have a more pronounced effect on AC than on NAAA, as indicated by the drop in selectivity toward AC for analogue **11 p** versus **11 h**. None of the three selected compounds showed inhibitory activity toward *h*-FAAH.

The higher selectivity toward AC of **11 h** with respect to **11 p**, coupled with its greater chemical and plasma stability, make this compound a potential probe for further exploration of the functional role of NAAA. To test whether **11 h** could be administered systemically, we dosed the compound intravenously (3.0 mg kg^{-1}) and orally (10 mg kg^{-1}) in rats and determined its pharmacokinetic (PK) profile. Relevant PK parameters are reported in Table 6. Although **11 h** showed a relatively high clearance (Cl = 702 mL min^{-1} kg^{-1}) by the intravenous route, the oral PK profile was characterized by a maximal plasma con-

Table 6. Plasma pharmacokinetic parameters of compound **11 h** after single intravenous (i.v.) or oral (p.o.) administration in rats.[a]

Parameter	3.0 mg kg^{-1} (i.v.)	10 mg kg^{-1} (p.o.)[a]
C_{max} [ng mL^{-1}]	397	570
T_{max} [h]	0.08	0.25
AUC [ng h mL^{-1}]	111	247
Cl [mL min^{-1} kg^{-1}]	702	–
F [%]	–	67

[a] Maximum observed concentration (C_{max}); cumulative area under curve (AUC) for experimental time points (0–24 h); systemic clearance (Cl) based on observed data points (0–24 h); bioavailability (F). Compound was dosed in 10% PEG400/10% Tween 80/80% saline solution; three animals per dosage were treated.

centration (C_{max}) of 570 ng mL^{-1} at 15 min post-dosing and a bioavailability of 67% (Table 6). These results support the use of **11 h** as an NAAA inhibitor for systemic administration.

Conclusions

In the present work, we report the discovery of 3-aminoazetidin-2-one derivatives as a novel class of NAAA inhibitors. A series of *N*-(2-oxoazetidin-3-yl)amides were synthesized and tested to identify key structural features for NAAA inhibition. Our results showed that the β-lactam moiety is mandatory for activity, and that alkylation of the endocyclic nitrogen is not tolerated. The *S* configuration of the acylamino substituent at C3 position is strongly preferred over the *R* configuration. The potency of *N*-(2-oxoazetidin-3-yl)amides as NAAA inhibitors is modulated by the amide alkyl chain, with long and flexible alkyl residues being preferred over sterically constrained chains. Interestingly, we found out that the amino group on the 3-aminoazetidin-2-one scaffold could be further derivatized as a carbamate (**34**) with retention of NAAA inhibitory activity (Figure 1). Further studies on this new series of derivatives are

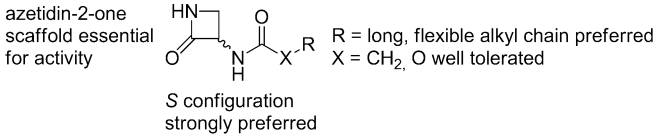


Figure 1. Summary of key structural features for β-lactam-based NAAA inhibitors.

currently ongoing. Among the synthesized compounds, *N*-[(*S*)-2-oxoazetidin-3-yl]nonanamide (**11 h**) showed good inhibitory potency (IC_{50} = 0.34 μM) against NAAA, ~19-fold selectivity versus AC (a closely related cysteine amidase), excellent chemical stability in buffer, and acceptable plasma stability. Importantly, this compound showed good oral bioavailability in rats.

Compound **11 h** and other potent *N*-(2-oxoazetidin-3-yl)-amide analogues represent promising probes that may help further characterize the functional roles of NAAA and assess the therapeutic potential of systemically available NAAA inhibitors.

Experimental Section

Chemistry

Chemicals, materials and methods: All commercially available reagents and solvents were used as purchased from vendors without further purification. Dry solvents (CH_2Cl_2, THF, DMF, pyridine, dichloroethane) were purchased from Sigma–Aldrich. Optical rotations were measured on a Rudolf Research Analytical Autopol II automatic polarimeter using a sodium lamp (589 nm) as the light source; concentrations are expressed in g/100 mL using MeOH as solvent and a 1 dm cell. Automated column chromatography purifications were done using a Teledyne ISCO apparatus (CombiFlash R_f) with pre-packed silica gel columns (4 g). Mixtures of increasing polarity of cyclohexane (Cy) and ethyl acetate (EtOAc) were used as eluents. Column chromatography was performed manually on pre-packed silica cartridges (2 g or 5 g) from Biotage or on glass columns using Merck silica gel 60 (230–400 mesh) as the stationary phase. Purifications by preparative HPLC–MS were run on a Waters Autopurification system consisting of a 3100 single quadrupole mass spectrometer equipped with an electrospray ionization interface and a 2998 photodiode array detector. The HPLC system included a 2747 sample manager, a 2545 binary gradient module, a system fluidic organizer, and a 515 HPLC pump. The PDA range was 210–400 nm. Purifications were performed on an XBridge™ Prep C_{18} OBD (100×19 mm i.d., particle size 5 μm) with a XBridge™ Prep C_{18} (10×19 mm i.d., particle size 5 μm) Guard Cartridge. The mobile phase was 10 mM NH_4OAc in MeCN/H_2O (95:5) at pH 5. Electrospray ionization (ESI) was used in positive and negative mode. Hydrogenation reactions were performed using H-Cube continuous hydrogenation equipment (SS-reaction line version), employing disposable catalyst cartridges (CatCart) preloaded with the required heterogeneous catalyst. NMR experiments were run on a Bruker Avance III 400 system (400.13 MHz for ^{1}H, and 100.62 MHz for ^{13}C) equipped with a BBI probe and Z-gradients. Spectra were acquired at 300 K using deuterated dimethyl sulfoxide ([D_6]DMSO) or deuterated chloroform ($CDCl_3$) as solvents. UPLC–MS analyses were run on a Waters Acquity UPLC–MS system consisting of a SQD (single quadrupole detector) mass spectrometer equipped with an electrospray ionization interface and a photodiode array detector. The PDA range was 210–400 nm. Analyses were performed on an Acquity UPLC HSS T3 C_{18} column (50×2.1 mm i.d., particle size 1.8 μm) with a VanGuard HSS T3 C_{18} pre-column (5×2.1 mm i.d., particle size 1.8 μm). The mobile phase was either 10 mM NH_4OAc in H_2O at pH 5, adjusted with AcOH (A) and 10 mM NH_4OAc in MeCN/H_2O (95:5) at pH 5 (B). ESI was applied in positive and negative mode. Accurate mass measurement (HMRS) was performed on a Synapt G2 quadrupole-Tof instrument (Waters, USA), equipped with an ESI ion source.

All final compounds (**11 a**–**q**, **12 h**, **21 h**–**24 h**, **27 h**, **28 h**, and **32**–**34**) showed ≥95% purity by NMR and UPLC–MS analysis. The syntheses of reaction intermediates **9**, **10**, **13**–**15**, **25**, **26 h**, **31**, and **35**–**37** are described in the Supporting Information.

General procedures for the synthesis of amide derivatives 11 a, 11 f–h, 11 l, 11 o and 12 h via Method A (Scheme 1): Under nitrogen atmosphere, dry Et_3N (2.1 equiv) and the suitable acid chloride (1.1 equiv) were added to a cooled (0 °C), stirred suspension of (2-oxoazetidin-3-yl)ammonium acetate (**9** or **10**, 1.0 equiv) in dry CH_2Cl_2 (0.07 M solution) or to a mixture of dry CH_2Cl_2/DMF (3:1; 0.07 M solution). The resulting mixture was stirred at room temperature for 16 h, then diluted with CH_2Cl_2 and washed with saturated NH_4Cl solution, saturated $NaHCO_3$ solution, and brine. The organic phase was dried over Na_2SO_4, filtered, concentrated to dryness,

and purified according to the specific conditions described in each example.

3-Phenyl-*N*-[(*S*)-2-oxoazetidin-3-yl]propanamide (11 a): The reaction was carried out following Method A, using salt **9** (0.050 g, 0.34 mmol), commercially available hydrocinnamoyl chloride (0.056 mL, 0.38 mmol), and dry Et_3N (0.10 mL, 0.71 mmol) in dry CH_2Cl_2 (5.0 mL). After workup, trituration with EtOAc afforded compound **11 a** (0.080 g, 15%) as a white solid: $R_t = 1.40$ min; $[\alpha]_D^{25} = -10.8\ cm^3\,g^{-1}\,dm^{-1}$ ($c = 0.09$ in MeOH); ^1H NMR (400 MHz, [D$_6$]DMSO): $\delta = 8.51$ (d, 1H, $J = 8.2$ Hz), 7.96 (bs, 1H), 7.29–7.24 (m, 2H), 7.22–7.14 (m, 3H), 4.87–4.80 (m, 1H), 3.38 (t, 1H, $J = 5.4$ Hz), 2.99 (dd, 1H, $J = 5.4$, 2.6 Hz), 2.81 (t, 2H, $J = 7.9$ Hz), 2.41 ppm (t, 2H, $J = 7.9$ Hz); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta = 171.4$, 168.0, 141.1, 128.3, 128.2, 125.4, 56.9, 42.9, 36.8, 30.9 ppm; MS (ESI, +) *m/z*: 219 $[M+H]^+$, 241 $[M+Na]^+$, 257 $[M+K]^+$; MS (ESI, −) *m/z*: 217 $[M-H]^-$; HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{12}H_{15}N_2O_2$: 219.1134, found: 219.1136.

***N*-[(*S*)-2-Oxoazetidin-3-yl]heptanamide (11 f)**: The reaction was carried out following Method A, using salt **9** (0.050 g, 0.34 mmol), commercially available heptanoyl chloride (0.058 mL, 0.38 mmol), and dry Et_3N (0.1 mL, 0.71 mmol) in dry CH_2Cl_2 (5.0 mL). After workup, purification by typical silica gel flash chromatography (CH_2Cl_2/MeOH, from 100:0 to 96:4) afforded compound **11 f** (0.023 g, 34%) as a white solid: $R_t = 1.63$ min; $[\alpha]_D^{25} = -20.3\ cm^3\,g^{-1}\,dm^{-1}$ ($c = 0.07$ in MeOH); ^1H NMR (400 MHz, [D$_6$]DMSO): $\delta = 8.43$ (d, 1H, $J = 8.3$ Hz), 7.94 (bs, 1H), 4.82 (ddd, 1H, $J = 8.3$, 5.4, 2.7 Hz), 3.38 (t, 1H, $J = 5.4$ Hz), 3.02 (dd, 1H, $J = 5.4$, 2.7 Hz), 2.08 (t, 2H, $J = 7.4$ Hz), 1.53–1.42 (m, 2H), 1.32–1.17 (m, 6H), 0.85 ppm (t, 3H, $J = 7.0$ Hz); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta = 172.7$, 168.7, 57.3, 43.3, 35.6, 31.5, 28.7, 25.5, 22.4, 14.4 ppm; MS (ESI, +) *m/z*: 199 $[M+H]^+$, 221 $[M+Na]^+$, 237 $[M+K]^+$; MS (ESI, −) *m/z*: 197 $[M-H]^-$; HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{10}H_{19}N_2O_2$: 199.1447, found: 199.1449.

***N*-[(*S*)-2-Oxoazetidin-3-yl]octanamide (11 g)**: The reaction was carried out following Method A, using salt **9** (0.060 g, 0.41 mmol), commercially available octanoyl chloride (0.076 mL, 0.45 mmol), and dry Et_3N (0.11 mL, 0.86 mmol) in dry CH_2Cl_2 (6.0 mL). After workup, trituration with EtOAc afforded compound **11 g** (0.019 g, 22%) as a white solid: $R_t = 1.88$ min; $[\alpha]_D^{25} = -10.5\ cm^3\,g^{-1}\,dm^{-1}$ ($c = 0.07$ in MeOH); ^1H NMR (400 MHz, [D$_6$]DMSO): $\delta = 8.43$ (d, 1H, $J = 8.2$ Hz), 7.94 (bs, 1H), 4.82 (ddd, 1H, $J = 8.2$, 5.4, 2.4 Hz), 3.38 (t, 1H, $J = 5.4$ Hz), 3.02 (dd, 1H, $J = 5.4$, 2.4 Hz), 2.08 (t, 2H, $J = 7.4$ Hz), 1.53–1.42 (m, 2H), 1.32–1.17 (m, 8H), 0.85 ppm (t, 3H, $J = 7.0$ Hz); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta = 172.2$, 168.2, 56.8, 42.8, 35.1, 31.1, 28.5, 28.4, 25.1, 22.0, 13.9 ppm; MS (ESI, +) *m/z*: 213 $[M+H]^+$, 251 $[M+K]^+$; HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{11}H_{21}N_2O_2$: 213.1603, found: 213.1611.

***N*-[(*S*)-2-Oxoazetidin-3-yl]nonanamide (11 h)**: The reaction was carried out following Method A, using salt **9** (0.60 g, 4.1 mmol), commercially available nonanoyl chloride (0.85 mL, 4.51 mmol), and dry Et_3N (1.2 mL, 8.6 mmol) in dry CH_2Cl_2 (60 mL). After workup, trituration with EtOAc afforded compound **11 h** (0.60 g, 65%) as a white solid: $R_t = 2.13$ min; $[\alpha]_D^{25} = -18.6\ cm^3\,g^{-1}\,dm^{-1}$ ($c = 0.07$ in MeOH); ^1H NMR (400 MHz, [D$_6$]DMSO): $\delta = 8.42$ (d, 1H, $J = 8.3$ Hz), 7.94 (bs, 1H), 4.83 (ddd, 1H, $J = 8.3$, 5.3, 2.7 Hz), 3.38 (t, 1H, $J = 5.3$ Hz), 3.02 (dd, 1H, $J = 5.3$, 2.7 Hz), 2.08 (t, 2H, $J = 7.3$ Hz), 1.53–1.42 (m, 2H), 1.31–1.18 (m, 10H), 0.86 ppm (t, 3H, $J = 6.8$ Hz); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta = 172.2$, 168.2, 56.8, 42.8, 35.1, 31.2, 28.7, 28.6, 28.5, 25.1, 22.1, 13.9 ppm; MS (ESI, +) *m/z*: 227 $[M+H]^+$, 249 $[M+Na]^+$, 265 $[M+K]^+$; MS (ESI, −) *m/z*: 225 $[M-H]^-$; HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{12}H_{23}N_2O_2$: 227.1760, found: 227.1771.

***N*-[(*S*)-2-Oxoazetidin-3-yl]-4-phenylbenzamide (11 l)**: The reaction was carried out following Method A, using salt **9** (0.13 g, 0.89 mmol), commercially available 4-phenylbenzoyl chloride (0.21 g, 0.98 mmol), and dry Et_3N (0.26 mL, 1.87 mmol) in dry CH_2Cl_2/DMF (4.0 mL). After workup, trituration with EtOAc afforded compound **11 l** (0.10 g, 42%) as a white solid: $R_t = 1.89$ min; ^1H NMR (400 MHz, [D$_6$]DMSO): $\delta = 9.14$ (d, 1H, $J = 8.5$ Hz), 8.05 (bs, 1H), 7.97 (d, 2H, $J = 8.4$ Hz), 7.79 (d, 2H, $J = 8.4$ Hz), 7.74 (d, 2H, $J = 7.4$ Hz), 7.50 (t, 2H, $J = 7.6$ Hz), 7.45–7.38 (m, 1H), 5.09 (ddd, 1H, $J = 8.5$, 5.2, 2.5 Hz), 3.49 (t, 1H, $J = 5.2$ Hz), 3.27 ppm (dd, 1H, $J = 5.2$, 2.5 Hz); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta = 168.6$, 166.1, 143.5, 139.5, 132.8, 129.4, 128.5, 127.3, 126.9, 58.5, 43.3; MS (ESI, +) *m/z*: 267 $[M+H]^+$, 289 $[M+Na]^+$; MS (ESI, −) *m/z*: 265 $[M-H]^-$; HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{16}H_{15}N_2O_2$: 267.1134, found: 267.1133.

***N*-[(*S*)-2-Oxoazetidin-3-yl]decanamide (11 o)**: The reaction was carried out following Method A, using salt **9** (0.050 g, 0.34 mmol), commercially available decanoyl chloride (0.077 mL, 0.38 mmol), and dry Et_3N (0.11 mL, 0.71 mmol) in dry CH_2Cl_2 (6.0 mL). After workup, purification by typical silica gel flash chromatography (CH_2Cl_2/MeOH, from 100:0 to 96:4) afforded compound **11 o** (0.051 g, 63%) as a white solid: $R_t = 2.31$ min; $[\alpha]_D^{25} = -16.2\ cm^3\,g^{-1}\,dm^{-1}$ ($c = 0.07$ in MeOH); ^1H NMR (400 MHz, [D$_6$]DMSO): $\delta = 8.43$ (d, 1H, $J = 8.4$ Hz), 7.94 (s, 1H), 4.82 (ddd, 1H, $J = 8.4$, 5.4, 2.7 Hz), 3.38 (t, 1H, $J = 5.4$ Hz), 3.02 (dd, 1H, $J = 5.4$, 2.7 Hz), 2.08 (t, 2H, $J = 7.5$ Hz), 1.53–1.42 (m, 2H), 1.33–1.16 (m, 12H), 0.86 ppm (t, 3H, $J = 7.1$ Hz); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta = 172.7$, 168.7, 57.3, 43.3, 35.6, 31.7, 29.3, 29.2, 29.1, 29.0, 25.5, 22.6, 14.4 ppm; MS (ESI, +) *m/z*: 241 $[M+H]^+$, 263 $[M+Na]^+$, 279 $[M+K]^+$; MS (ESI, −) *m/z*: 239 $[M-H]^-$; HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{13}H_{25}N_2O_2$: 241.1916, found: 241.1920.

***N*-[(*R*)-2-Oxoazetidin-3-yl]nonanamide (12 h)**: The reaction was carried out following Method A, using salt **10** (0.090 g, 0.62 mmol), commercially available nonanoyl chloride (0.13 mL, 0.68 mmol), and dry Et_3N (0.18 mL, 1.03 mmol) in dry CH_2Cl_2 (9.0 mL). After workup, trituration with EtOAc afforded compound **12 h** (0.074 g, 53%) as a white solid: $R_t = 2.13$ min; $[\alpha]_D^{25} = +14.3\ cm^3\,g^{-1}\,dm^{-1}$ ($c = 0.07$ in MeOH); ^1H NMR (400 MHz, [D$_6$]DMSO): $\delta = 8.42$ (d, 1H, $J = 8.3$ Hz), 7.94 (bs, 1H), 4.83 (ddd, 1H, $J = 8.3$, 5.3, 2.7 Hz), 3.38 (t, 1H, $J = 5.3$ Hz), 3.02 (dd, 1H, $J = 5.3$, 2.7 Hz), 2.08 (t, 2H, $J = 7.3$ Hz), 1.53–1.42 (m, 2H), 1.31–1.18 (m, 10H), 0.86 ppm (t, 3H, $J = 6.8$ Hz); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta = 172.2$, 168.2, 56.8, 42.8, 35.1, 31.2, 28.7, 28.6, 28.5, 25.1, 22.1, 13.9 ppm; MS (ESI, +) *m/z*: 227 $[M+H]^+$, 249 $[M+Na]^+$, 265 $[M+K]^+$; MS (ESI, −) *m/z*: 225 $[M-H]^-$; HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{12}H_{23}N_2O_2$: 227.1760, found: 227.1766.

General procedures for the synthesis of amide derivatives 11 b–e, 11 i–k, 11 m–n, and 11 p–q via Method B (Scheme 1): Under nitrogen atmosphere, dry Et_3N (2.2 equiv) was added to a cooled (0 °C) solution of the suitable carboxylic acid (1.1 equiv) in dry CH_2Cl_2 (0.07 M solution) or in a 3:1 mixture of dry CH_2Cl_2/DMF (0.07 M solution), followed by addition of *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(benzotriazol-1-yl)uronium tetrafluoroborate (TBTU, 1.1 equiv). The resulting reaction mixture was stirred for 10 min, then (*S*)-2-oxoazetidin-3-yl)ammonium acetate (**9**, 1.0 equiv) was added. The reaction mixture was stirred at room temperature for 16 h, then diluted with CH_2Cl_2 and washed with saturated NH_4Cl solution, saturated $NaHCO_3$ solution, and brine. The organic phase was dried over Na_2SO_4, filtered, concentrated to dryness, and purified according to the specific conditions described in each example.

***N*-[(*S*)-2-Oxoazetidin-3-yl]-4-phenylbutanamide (11 b)**: The reaction was carried out following Method B, using salt **9** (0.060 g, 0.41 mmol), commercially available 4-phenylbutanoic acid (0.074 g, 0.45 mmol), TBTU (0.144 g, 0.45 mmol), and dry Et_3N (0.12 mL, 0.90 mmol) in dry CH_2Cl_2/DMF (6.0 mL). After workup, purification by silica gel column chromatography (Cy/EtOAc, from 100:0 to 10:90) afforded compound **11 b** (0.032 g, 34%) as a white solid: $R_t = 1.62$ min; $[\alpha]_D^{25} = -10.8\ cm^3\,g^{-1}\,dm^{-1}$ ($c = 0.09$ in MeOH); ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 8.46$ (d, 1H, $J = 8.4$ Hz), 7.94 (bs, 1H), 7.33–7.24 (m, 2H), 7.20–7.16 (m, 3H), 4.82 (ddd, 1H, $J = 8.4$, 5.4, 2.5 Hz), 3.39 (t, 1H, $J = 5.4$ Hz), 3.03 (dd, 1H, $J = 5.4$, 2.5 Hz), 2.55 (t, 2H, $J = 7.5$ Hz), 2.12 (t, 2H, $J = 7.5$ Hz), 1.79 ppm (p, 2H, $J = 7.5$ Hz); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta = 171.9$, 168.4, 141.7, 128.9, 126.0, 125.7, 56.9, 43.1, 34.5, 34.6, 26.9 ppm; MS (ESI, +) *m/z*: 233 $[M+H]^+$, 250 $[M+Na]^+$, 271 $[M+K]^+$; HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{13}H_{17}N_2O_2$: 233.129, found: 233.1299.

5-Phenyl-*N*-[(*S*)-2-oxoazetidin-3-yl]pentanamide (11 c): The reaction was carried out following Method B, using salt **9** (0.050 g, 0.34 mmol), commercially available 5-phenylpentanoic acid (0.067 g, 0.38 mmol), TBTU (0.12 g, 0.38 mmol), and dry Et_3N (0.10 mL, 0.71 mmol) in dry CH_2Cl_2 (6.0 mL). After workup, trituration with Et_2O afforded compound **11 c** (0.028 g, 33%) as a white solid: $R_t = 1.79$ min; $[\alpha]_D^{25} = -40.2\ cm^3\,g^{-1}\,dm^{-1}$ ($c = 0.07$ in MeOH); ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 8.46$ (d, 1H, $J = 8.3$ Hz), 7.94 (bs, 1H), 7.30–7.23 (m, 2H), 7.21–7.13 (m, 3H), 4.82 (ddd, 1H, $J = 8.3$, 5.4, 2.6 Hz), 3.38 (t, 1H, $J = 5.4$ Hz), 3.02 (dd, 1H, $J = 5.4$, 2.6 Hz), 2.56 (t, 2H, $J = 7.2$ Hz), 2.12 (t, 2H, $J = 6.8$ Hz), 1.60–1.45 ppm (m, 4H); ^{13}C NMR (100 MHz, [D^6]DMSO): $\delta = 172.6$, 168.6, 142.5, 128.7, 128.6, 126.1, 57.3, 43.31, 35.4, 35.3, 30.9, 25.2 ppm; MS (ESI, +) *m/z*: 247 $[M+H]^+$, 269 $[M+Na]^+$, 285 $[M+K]^+$; MS (ESI, −) *m/z*: 245 $[M-H]^-$; HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{14}H_{19}N_2O_2$: 247.1447, found: 247.1458.

***N*-[(*S*)-2-Oxoazetidin-3-yl]-6-phenylhexanamide (11 d)**: The reaction was carried out following Method B, using salt **9** (0.060 g, 0.41 mmol), commercially available 6-phenylhexanoic acid (0.084 mL, 0.45 mmol), TBTU (0.144 g, 0.45 mmol), and dry Et_3N (0.12 mL, 0.90 mmol) in dry CH_2Cl_2/DMF (6.0 mL). After workup, trituration with Et_2O afforded compound **11 d** (0.032 g, 30%) as a white solid: $R_t = 1.98$ min; $[\alpha]_D^{25} = -12.9\ cm^3\,g^{-1}\,dm^{-1}$ ($c = 0.07$ in MeOH); ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 8.44$ (d, 1H, $J = 8.2$ Hz), 7.94 (bs, 1H), 7.29–7.23 (m, 2H), 7.20–7.13 (m, 3H), 4.82 (ddd, 1H, $J = 8.2$, 5.4, 2.5 Hz), 3.38 (t, 1H, $J = 5.4$ Hz), 3.01 (dd, 1H, $J = 5.4$, 2.5 Hz), 2.58–2.52 (m, 2H), 2.08 (t, 2H, $J = 7.4$ Hz), 1.60–1.42 (m, 4H), 1.32–1.20 ppm (m, 2H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta =$ 172.5, 168.5, 142.2, 128.3, 128.2, 125.6, 56.8, 42.8, 35.5, 35.4, 30.7, 28.2, 24.9 ppm; MS (ESI, +) *m/z*: 261 $[M+H]^+$, 283 $[M+Na]^+$, 299 $[M+K]^+$; HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{15}H_{21}N_2O_2$: 261.1603, found: 261.1603.

7-Phenyl-*N*-[(*S*)-2-oxoazetidin-3-yl]heptanamide (11 e): The reaction was carried out following Method B, using salt **9** (0.030 g, 0.21 mmol), commercially available 7-phenylheptanoic acid (0.065 mL, 0.23 mmol), TBTU (0.073 g, 0.23 mmol), and dry Et_3N (0.06 mL, 0.46 mmol) in dry CH_2Cl_2 (3.0 mL). After workup, trituration with EtOAc afforded compound **11 e** (0.022 g, 38%) as a white solid: $R_t = 2.19$ min; $[\alpha]_D^{25} = -17.6\ cm^3\,g^{-1}\,dm^{-1}$ ($c = 0.07$ in MeOH); ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 8.43$ (d, 1H, $J = 8.4$ Hz), 7.94 (bs, 1H), 7.29–7.23 (m, 2H), 7.20–7.13 (m, 3H), 4.82 (ddd, 1H, $J = 8.4$, 5.3, 2.6 Hz), 3.38 (t, 1H, $J = 5.3$ Hz), 3.02 (dd, 1H, $J = 5.3$, 2.6 Hz), 2.59–2.53 (m, 2H), 2.08 (t, 2H, $J = 7.4$ Hz), 1.60–1.42 (m, 4H), 1.32–1.21 ppm (m, 4H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta = 172.7$, 168.6, 142.8, 128.7, 128.6, 126.0, 57.4, 43.3, 35.6, 35.5, 31.4, 28.9, 28.8, 25.5 ppm; MS (ESI, +) *m/z*: 275 $[M+H]^+$, 297 $[M+Na]^+$, 313 $[M+K]^+$; HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{16}H_{23}N_2O_2$: 275.1760, found: 275.1766.

(*E*)-*N*-[(*S*)-2-Oxoazetidin-3-yl]non-3-enamide (11 i): The reaction was carried out following Method B, using salt **9** (0.050 g, 0.34 mmol), (*E*)-3-nonenoic acid (**13**) (0.058 g, 0.37 mmol), TBTU (0.12 g, 0.37 mmol), and dry Et_3N (0.10 mL, 0.75 mmol) in dry CH_2Cl_2 (5.0 mL). After workup, trituration with EtOAc afforded compound **11 i** (0.035 g, 46%) as a white solid: $R_t = 2.04$ min; $[\alpha]_D^{25} = -16.5\ cm^3\,g^{-1}\,dm^{-1}$ ($c = 0.07$ in MeOH); ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 8.45$ (d, 1H, $J = 8.4$ Hz), 7.96 (bs, 1H), 5.54–5.41 (m, 2H), 4.81 (ddd, 1H, $J = 8.4$, 5.5, 2.6 Hz), 3.38 (t, 1H, $J = 5.5$ Hz), 3.03 (dd, 1H, $J = 5.5$, 2.6 Hz), 2.83 (d, 2H, $J = 5.6$ Hz), 2.01–1.93 (m, 2H), 1.37–1.19 (m, 6H), 0.86 ppm (t, 3H, $J = 7.1$ Hz); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta = 171.0$, 168.5, 133.5, 124.0, 57.3, 43.2, 39.6, 32.3, 31.3, 28.9, 22.4, 14.4 ppm; MS (ESI, +) *m/z*: 225 $[M+H]^+$, 247 $[M+Na]^+$, 263 $[M+K]^+$; HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{12}H_{21}N_2O_2$: 225.1603, found: 225.1614.

(*Z*)-*N*-[(*S*)-2-Oxoazetidin-3-yl]non-3-enamide (11 j): The reaction was carried out following Method B, using salt **9** (0.050 g, 0.34 mmol), (*Z*)-non-3-enoic acid (**14**; 0.059 g, 0.38 mmol), TBTU (0.12 g, 0.38 mmol), and dry Et_3N (0.10 mL, 0.71 mmol) in dry CH_2Cl_2 (3.0 mL). After workup, trituration with EtOAc afforded compound **11 j** (0.034 g, 45%) as a white solid: $R_t = 1.98$ min; $[\alpha]_D^{25} = -7.6\ cm^3\,g^{-1}\,dm^{-1}$ ($c = 0.07$ in MeOH); ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 8.50$ (d, 1H, $J = 8.3$ Hz), 7.97 (bs, 1H), 5.52–5.42 (m, 2H), 4.82 (ddd, 1H, $J = 8.3$, 5.4, 2.5 Hz), 3.39 (t, 1H, $J = 5.4$ Hz), 3.03 (dd, 1H, $J = 5.4$, 2.5 Hz), 2.94–2.86 (m, 2H), 2.04–1.96 (m, 2H), 1.37–1.18 (m, 6H), 0.86 ppm (t, 3H, $J = 7.1$ Hz); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta = 170.4$, 168.0, 131.8, 122.9, 56.9, 42.7, 34.0, 30.8, 28.5, 26.8, 21.9, 13.9 ppm; MS (ESI, +) *m/z*: 225 $[M+H]^+$, 247 $[M+Na]^+$, 263 $[M+K]^+$; MS (ESI, −) *m/z*: 223 $[M-H]^-$; HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{12}H_{21}N_2O_2$: 225.1603, found: 225.1612.

4-Butyl-*N*-[(*S*)-2-oxoazetidin-3-yl]benzamide (11 k): The reaction was carried out following Method B, using salt **9** (0.060 g, 0.41 mmol), commercially available 4-butylbenzoic acid (0.080 g, 0.45 mmol), TBTU (0.144 g, 0.45 mmol), and dry Et_3N (0.12 mL, 0.90 mmol) in dry CH_2Cl_2 (6.0 mL). After workup, trituration with Et_2O afforded compound **11 k** (0.029 g, 29%) as a white solid: $R_t =$ 2.07 min; $[\alpha]_D^{25} = -7.5\ cm^3\,g^{-1}\,dm^{-1}$ ($c = 0.07$ in MeOH); ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 8.99$ (d, 1H, $J = 8.4$ Hz), 8.02 (bs, 1H), 7.78 (d, 2H, $J = 8.2$ Hz), 7.29 (d, 2H, $J = 8.2$ Hz), 5.05 (ddd, 1H, $J = 8.4$, 5.4, 2.6 Hz), 3.46 (t, 1H, $J = 5.4$ Hz), 3.24 (dd, 1H, $J = 5.4$, 2.6 Hz), 2.63 (t, 2H, $J = 7.5$ Hz), 1.63–1.50 (m, 2H), 1.37–1.20 (m, 2H), 0.89 ppm (t, 3H, $J = 7.4$ Hz); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta =$ 168.3, 166.0, 146.3, 131.1, 128.3, 127.4, 57.3, 42.5, 34.6, 32.8, 217, 13.7 ppm; MS (ESI, +) *m/z*: 247 $[M+H]^+$, 269 $[M+Na]^+$, 285 $[M+K]^+$; HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{14}H_{19}N_2O_2$: 247.1447, found: 247.1457.

(2*R*)- and (2*S*)-2-Methyl-*N*-[(3*S*)-2-oxoazetidin-3-yl]nonanamide (11 m): The reaction was carried out following Method B, using salt **9** (0.045 mg, 0.31 mmol), 2-methylnonanoic acid (0.059 mg, 0.34 mmol), TBTU (0.109 mg, 0.34 mmol), and dry Et_3N (0.090 mL, 0.68 mmol) in dry CH_2Cl_2 (4.5 mL). After workup, purification by silica gel flash chromatography using a Teledyne ISCO apparatus (Cy/EtOAc from 90:10 to 0:100) afforded compound **11 m** (0.024 g, 32%) as a clear liquid 1:1 mixture of isomers: $R_t = 2.28$ min; ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 8.41$ (t, 2H, $J = 8.0$ Hz), 7.94 (bs, 2H), 4.85–4.78 (m, 2H), 3.44–3.36 (m, 2H), 3.01 (ddd, 2H, $J = 8.0$, 5.2, 2.7 Hz), 2.30–2.15 (m, 2H), 1.50–1.44 (m, 2H), 1.23 (s, 22H), 0.99–0.97 (m, 6H), 0.85 ppm (t, 6H, $J = 6.8$ Hz); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta = 175.8$, 168.2, 56.9, 43.0, 42.9, 33.8, 31.2, 29.0, 28.9,

28.7, 26.9, 26.8, 22.1, 17.9, 17.8, 14.0 ppm; MS (ESI, +) *m/z*: 241 $[M+H]^+$, 258 $[M+NH_4]^+$, 279 $[M+K]^+$; HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{13}H_{25}N_2O_2$: 241.1916, found: 241.1923.

2,2-Dimethyl-*N*-[(*S*)-2-oxoazetidin-3-yl]nonanamide (11 n): The reaction was carried out following Method B, using salt **9** (0.045 mg, 0.31 mmol), commercially available 2,2-dimethylnonanoic acid (0.064 mg, 0.34 mmol), TBTU (0.144 g, 0.45 mmol), and dry Et_3N (0.090 mL, 0.68 mmol) in dry CH_2Cl_2/DMF (3:1, 4.4 mL). After workup, purification by preparative HPLC afforded compound **11 n** (0.026 g, 33%) as a clear liquid: $R_t = 2.47$ min; $[\alpha]_D^{25} = -3.50\ cm^3\,g^{-1}\,dm^{-1}$ ($c = 0.10$ in MeOH); ^1H NMR (400 MHz, [D$_6$]DMSO) $\delta = 8.01$ (d, 1H, $J = 8.4$ Hz), 7.88 (bs, 1H), 4.79 (ddd, 1H, $J = 8.4$, 5.4, 2.8 Hz), 3.34 (t, 1H, $J = 5.4$ Hz), 3.09 (dd, 1H, $J = 5.4$, 2.8 Hz), 1.45–1.36 (m, 2H), 1.29–1.07 (m, 10H), 1.04 (s, 6H), 0.84 (t, 3H, $J = 6.9$ Hz); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta = 177.0$, 168.6, 57.1, 42.4, 41.5, 40.6, 31.3, 29.6, 28.7, 25.3, 25.2, 24.2, 22.1, 14.0 ppm; MS (ESI, +) *m/z*: 255 $[M+H]^+$, 272 $[M+NH_4]^+$, 277 $[M+Na]^+$; HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{14}H_{27}N_2O_2$: 255.2073, found: 255.2084.

***N*-[(*S*)-2-Oxoazetidin-3-yl]undecanamide (11 p)**: The reaction was carried out following Method B, using salt **9** (0.060 g, 0.41 mmol), commercially available undecanoic acid (0.084 g, 0.45 mmol), TBTU (0.144 g, 0.45 mmol), and dry Et_3N (0.12 mL, 0.90 mmol) in dry CH_2Cl_2 (6.0 mL). After workup, trituration with EtOAc afforded compound **11 p** (0.065 g, 62%) as a white solid: $R_t = 2.54$ min; $[\alpha]_D^{25} = -15.6\ cm^3\,g^{-1}\,dm^{-1}$ ($c = 0.08$ in MeOH); ^1H NMR (400 MHz, [D$_6$]DMSO): $\delta = 8.43$ (d, 1H, $J = 8.3$ Hz), 7.94 (bs, 1H), 4.82 (ddd, 1H, $J = 8.3$, 5.4, 2.5 Hz), 3.38 (t, 1H, $J = 5.4$ Hz), 3.02 (dd, 1H, $J = 5.4$, 2.5 Hz), 2.08 (t, 2H, $J = 7.4$ Hz), 1.54–1.42 (m, 2H), 1.33–1.18 (m, 14H), 0.84 ppm (t, 3H, $J = 6.4$ Hz); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta = 172.3$, 168.2, 55.9, 42.9, 35.2, 31.3, 29.0, 28.9, 28.8, 28.7, 28.6, 25.1, 22.1, 14.0 ppm; MS (ESI, +) *m/z*: 255 $[M+H]^+$, 293 $[M+K]^+$; HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{14}H_{27}N_2O_2$: 255.2073, found: 255.2083.

6-Cyclohexyl-*N*-[(*S*)-2-oxoazetidin-3-yl]hexanamide (11 q): The reaction was carried out following Method B, using salt **9** (0.050 g, 0.34 mmol), 7-cyclohexylheptanoic acid (**15**) (0.08 g, 0.38 mmol), TBTU (0.12 g, 0.38 mmol), and dry Et_3N (0.100 mL, 0.75 mmol) in dry CH_2Cl_2 (6.0 mL). After workup, trituration with Et_2O afforded compound **11 q** (0.033 g, 35%) as a white solid: $R_t = 2.73$ min; $[\alpha]_D^{25} = -14.5\ cm^3\,g^{-1}\,dm^{-1}$ ($c = 0.07$ in MeOH); ^1H NMR (400 MHz, [D$_6$]DMSO): $\delta = 8.42$ (d, 1H, $J = 8.3$ Hz), 7.94 (bs, 1H), 4.85–4.79 (m, 1H), 3.38 (t, 1H, $J = 5.3$ Hz), 3.02 (dd, 1H, $J = 5.3$, 2.7 Hz), 2.08 (t, 2H, $J = 7.4$ Hz), 1.70–1.55 (m, 5H), 1.53–1.42 (m, 2H), 1.28–1.07 (m, 12H), 0.89–0.77 ppm (m, 2H); ^{13}C NMR (400 MHz, [D$_6$]DMSO) $\delta = 172.7$, 168.6, 57.3, 43.3, 37.5, 37.4, 35.6, 33.4, 29.6, 29.1, 26.7, 26.6, 26.3, 25.5 ppm; MS (ESI, +) *m/z*: 281 $[M+H]^+$, 303 $[M+Na]^+$, 319 $[M+K]^+$; MS (ESI, −) *m/z*: 279 $[M-H]^-$; HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{16}H_{29}N_2O_2$: 281.2229, found: 281.2237.

***N*-(2-Oxocyclobutyl)nonanamide (21 h)**: Under nitrogen atmosphere, amide **25** (0.210 g, 1.34 mmol) was dissolved in dry THF (15.0 mL), and commercially available 2 N HCl·Et_2O solution (15.0 mL) was added at 0 °C. Commercially available bis(trimethylsilyloxy)cyclobutene (0.33 mL, 1.27 mmol) was then added in one portion. The solution was refluxed for 3 h and then concentrated to dryness. Purification by typical silica flash chromatography (Cy/EtOAc, 60:40), and preparative HPLC afforded compound **21 h** (0.110 g, 37%) as a white solid: $R_t = 2.48$ min; ^1H NMR (400 MHz, [D$_6$]DMSO): $\delta = 8.27$ (d, 1H, $J = 7.9$ Hz), 4.77 (dt, 1H, $J = 10.3$, 7.9 Hz), 2.93–2.80 (m, 1H), 2.79–2.68 (m, 1H), 2.19 (qd, 1H, $J = 10.3$, 4.5 Hz), 2.06 (t, 2H, $J = 7.3$ Hz), 2.02–1.91 (m, 1H), 1.52–1.40 (m, 2H), 1.32–1.17 (m, 10H), 0.86 ppm (t, 3H, $J = 6.8$ Hz); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta = 207.5$, 172.3, 64.0, 41.6, 35.2, 31.7, 29.2, 29.1, 29.0, 25.5, 22.5, 18.8, 14.4 ppm. MS (ESI, +) *m/z*: 226 $[M+H]^+$, 248 $[M+Na]^+$, 264 $[M+K]^+$; HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{13}H_{24}NO_2$: 226.1807, found: 226.1814.

***N*-(Azetidin-3-yl)nonanamide (22 h)**: A mixture of TFA/CH_2Cl_2 (1:3; 4.0 mL) was added dropwise to a cooled (0 °C), stirred solution of Boc-derivative **26** (0.060 g, 0.19 mmol) in CH_2Cl_2 (3.0 mL). The reaction mixture was stirred at 0 °C for 30 min, then at room temperature for an additional 30 min. The solution was diluted with sat. Na_2CO_3 solution until neutralization. The organic phase was separated, and the aqueous phase was extracted from CH_2Cl_2 (15.0 mL) and EtOAc (15.0 mL). The combined organic phases were dried over Na_2SO_4, filtered, and concentrated to dryness, affording compound **22 h** (0.095 g, quant.), as a colorless oil: $R_t = 1.92$ min; ^1H NMR (400 MHz, [D$_6$]DMSO): $\delta = 8.62$ (bs, 1H), 8.50 (d, 1H, $J = 6.5$ Hz), 4.55 (sex, 1H, $J = 15.1$, 7.6 Hz), 4.11–4.04 (m, 1H), 3.92–3.85 (m, 1H), 2.08 (t, 2H, $J = 7.4$ Hz), 1.55–1.40 (m, 2H), 1.32–1.17 (m, 10H), 0.86 ppm (t, 3H, $J = 6.8$ Hz); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta = 172.9$, 52.6, 41.4, 35.6, 31.7, 29.2, 29.1, 29.0, 25.4, 22.5, 14.4 ppm; MS (ESI, +) *m/z*: 213 $[M+H]^+$, 235 $[M+Na]^+$; HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{12}H_{25}N_2O$: 213.1967, found: 213.1977.

[(*S*)-3-Hydroxy-2-(nonanoylamino)-3-oxo-propyl]ammonium chloride (23 h): β-lactam amide **11 h** (0.040 g, 0.18 mmol) was suspended in 2 N HCl (3.0 mL), the mixture was vigorously stirred at room temperature for 30 min. THF (2.0 mL) was then added to the suspension to dissolve any insoluble residue. The resulting solution was stirred at room temperature for 16 h, the solvents were evaporated, and trituration with EtOAc afforded compound **23 h** (0.040 g, 79%) as a white solid: $R_t = 1.68$ min; $[\alpha]_D^{25} = -10.0\ cm^3\,g^{-1}\,dm^{-1}$ ($c = 0.11$ in MeOH); ^1H NMR (400 MHz, [D$_6$]DMSO): $\delta = 13.00$ (bs, 1H), 8.31 (d, 1H, $J = 8.0$ Hz), 8.17 (bs, 3H), 4.47 (dt, 1H, $J = 8.0$, 5.2 Hz), 3.19 (dd, 1H, $J = 13.0$, 5.2 Hz), 3.00 (dd, 1H, $J = 13.0$, 8.9 Hz), 2.15 (t, 2H, $J = 7.6$ Hz), 1.56–1.46 (m, 2H), 1.33–1.19 (m, 10H), 0.90–0.82 ppm (m, 3H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta = 173.4$, 171.3, 50.4, 35.7, 31.7, 29.3, 29.1, 25.4, 22.6, 14.4 ppm; MS (ESI, +) *m/z*: 245 $[M+H]^+$, 267 $[M+Na]^+$, 283 $[M+K]^+$; HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{12}H_{25}N_2O_3$: 245.1865, found: 245.1873.

***N*-[(3*S*)-2-Oxopyrrolidin-3-yl]nonanamide (24 h)**: The reaction was carried out following Method A, using commercially available (3*S*)-3-aminopyrrolidin-2-one (0.100 g, 1.0 mmol), commercially available nonanoyl chloride (0.24 mL, 1.10 mmol), and dry Et_3N (0.15 mL, 1.10 mmol) in dry CH_2Cl_2 (8 mL). After workup, trituration with Et_2O afforded compound **24 h** (0.20 g, 83%) as a white solid: $R_t = 2.09$ min; ^1H NMR (400 MHz, [D$_6$]DMSO): $\delta = 7.99$ (d, 1H, $J = 8.3$ Hz), 7.76 (bs, 1H), 4.27 (dt, 1H, $J = 10.3$, 8.3 Hz), 3.20–3.11 (m, 2H), 2.32–2.23 (m, 1H), 2.07 (t, 2H, $J = 7.4$ Hz), 1.81–1.69 (m, 1H), 1.53–1.43 (m, 2H), 1.31–1.20 (m, 10H), 0.85 ppm (t, 3H, $J = 6.6$ Hz); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta = 174.5$, 172.2, 49.3, 38.0, 35.2, 31.2, 28.7, 28.6, 28.5, 25.2, 22.1, 13.9 ppm; MS (ESI, +) *m/z*: 241 $[M+H]^+$; MS (ESI, −) *m/z*: 239 $[M-H]^-$; HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{13}H_{25}N_2O_2$: 241.1916, found: 241.192.

***N*-[(*S*)-1-methyl-2-oxo-azetidin-3-yl]nonanamide (27 h)**: Under nitrogen atmosphere, a suspension of NaH (60% mineral oil, 0.015 g, 0.362 mmol) in dry THF (4.0 mL) was added dropwise to a cooled (0 °C) solution of amide **11 h** in dry THF (2.5 mL). The mixture was warmed to room temperature, stirred for an additional 20 min, and cooled again to 0 °C. MeI (0.022 mL, 0.362 mmol) was added dropwise, and the resulting reaction mixture was maintained at 0 °C for 3 h, warmed to room temperature, and stirred for additional 2 h and 30 min. The reaction mixture was diluted with CH_2Cl_2 (25 mL)

and water (2.0 mL). The aqueous layer was extracted with CH_2Cl_2 (3 x10 mL), and the combined organic layers were dried over Na_2SO_4, filtered, and concentrated to dryness. Purification by preparative HPLC afforded pure compound **27h** (0.020 g, 25% yield) as a white solid: $R_t = 2.23$ min; $[\alpha]_D^{25} = -6.7$ cm^3 g^{-1} dm^{-1} ($c = 0.07$ in MeOH); ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 8.42$ (d, 1H, $J = 8.1$ Hz), 4.81 (ddd, 1H, $J = 8.1$, 5.2, 2.4 Hz), 3.46 (t, 1H, $J = 5.2$ Hz), 3.08 (dd, 1H, $J = 5.2$, 2.4 Hz), 2.73 (s, 3H), 2.07 (t, 2H, $J = 7.4$ Hz), 1.55–1.42 (m, 2H), 1.33–1.17 (m, 10H), 0.86 ppm (t, 3H, $J = 6.8$ Hz); ^{13}C NMR (100 MHz, [D$_6$]DMSO: $\delta = 172.2$, 167.1, 56.0, 49.0, 35.1, 31.2, 28.7, 28.6, 28.5, 28.1, 25.1, 22.1, 13.9 ppm; MS (ESI, +) *m/z*: 241 $[M+H]^+$, 263 $[M+Na]^+$, 279 $[M+K]^+$; HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{13}H_{25}N_2O_2$: 241.1916, found: 241.1918.

***N*-Methyl-*N*-[(*S*)-2-oxoazetidin-3-yl]nonanamide (28h)**: The reaction was carried out following Method A, using salt **31** (0.050 g, 0.31 mmol), commercially available nonanoyl chloride (0.076 mL, 0.34 mmol), and dry Et_3N (0.091 mL, 0.66 mmol) in dry CH_2Cl_2 (5.0 mL). After workup, purification by preparative HPLC afforded compound **28h** (0.027 g, 33%) as an oil in a 1:1 mixture of two rotamers: $R_t = 2.31$ min; $[\alpha]_D^{25} = -16.3$ cm^3 g^{-1} dm^{-1} ($c = 0.12$ in MeOH); ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 8.14$ (bs, 1H), 8.07 (bs, 1H), 5.50–5.45 (m, 1H), 5.33–5.27 (m, 1H), 3.43 (t, 1H, $J = 5.8$ Hz), 3.35 (t, 1H, $J = 5.8$ Hz), 3.22 (dd, 1H, $J = 5.8$, 2.5 Hz), 3.17 (dd, 1H, $J = 5.8$, 2.5 Hz), 2.90 (s, 3H), 2.74 (s, 3H), 2.42–2.23 (m, 4H), 1.53–1.40 (m, 4H), 1.33–1.16 (m, 20H), 0.86 ppm (t, 6H, $J = 7.0$ Hz); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta = 172.6$, 172.2, 167.4, 166.8, 64.6, 62.0, 32.6, 32.4, 31.4, 31.2, 28.8, 28.7, 28.6, 28.0, 24.9, 24.4, 22.1, 14.0 ppm; MS (ESI, +) *m/z*: 241 $[M+H]^+$, 263 $[M+Na]^+$, 279 $[M+K]^+$; HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{13}H_{25}N_2O_2$: 241.1916, found: 241.1918.

(*S*)-3-(Nonylamino)azetidin-2-one (32): Under nitrogen atmosphere, dry Et_3N (0.050 mL, 0.38 mmol) was added to a stirred suspension of salt **9** (0.050 g, 0.34 mmol) in dry dichloroethane (3.5 mL). The resulting suspension was stirred for 10 min, then a solution of aldehyde **35** (0.049 g, 0.34 mmol) in dry dichloroethane (0.5 mL) and $Na(OAc)_3BH$ (0.100 g, 0.48 mmol) were added to the reaction mixture. The solution was stirred for 90 min, then diluted with EtOAc (20.0 mL) and washed with saturated $NaHCO_3$ solution (10.0 mL). The organic phase was dried over Na_2SO_4, filtered, and concentrated to dryness. Purification by typical silica flash chromatography (CH_2Cl_2/MeOH, from 97:3 to 95:5) afforded compound **32** (0.015 g, 21%) as a white solid: $R_t = 2.37$ min; ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 7.72$ (bs, 1H), 4.00 (bs, 1H), 3.24 (t, 1H, $J = 5.2$ Hz), 2.92 (dd, 1H, $J = 5.6$, 2.4 Hz), 2.63–2.52 (m, 2H), 1.42–1.16 (s, 14H), 0.86 ppm (d, 3H, $J = 7.0$ Hz); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta =$ 168.5, 67.5, 46.5, 43.0, 31.8, 30.3, 29.5, 29.4, 29.1, 27.2, 22.6, 14.4 ppm; MS (ESI, +) *m/z*: 213 $[M+H]^+$, 235 $[M+Na]^+$; HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{12}H_{25}N_2O$: 213.1967, found: 213.1977.

1-Heptyl-3-[(*S*)-2-oxoazetidin-3-yl]urea (33): DMAP (0.048 g, 0.39 mmol) was added to a solution of salt **9** (0.047 g, 0.32 mmol) in dry pyridine (4.0 mL), followed by the addition of heptyl isocyanate (0.057 mL, 0.36 mmol). The reaction mixture was stirred at room temperature for 16 h and concentrated to dryness. Trituration with CH_2Cl_2 afforded compound **33** (0.040 g, 59%) as a white solid: $R_t = 1.90$ min; $[\alpha]_D^{25} = +2.07$ cm^3 g^{-1} dm^{-1} ($c = 0.08$ in MeOH); ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 7.83$ (bs, 1H), 6.50 (d, 1H, $J =$ 8.4 Hz), 5.94 (t, 1H, $J = 5.4$ Hz), 4.80–4.63 (m, 1H), 3.34 (t, 1H, $J =$ 5.4 Hz), 3.03–2.99 (m, 1H), 2.99–2.92 (m, 2H), 1.31–1.14 (m, 10H), 0.94–0.81 ppm (m, 3H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta = 169.4$, 157.0, 57.9, 43.8, 31.3, 29.9, 28.4, 26.3, 22.0, 13.9 ppm; MS (ESI, +) *m/z*: 228 $[M+H]^+$, 250 $[M+Na]^+$; HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{11}H_{22}N_3O_2$: 228.1712, found: 228.1718.

Heptyl *N*-[(*S*)-2-oxoazetidin-3-yl]carbamate (34): Under nitrogen atmosphere, DIPEA (0.053 mL, 0.32 mmol) was added dropwise to a suspension of salt **9** (0.040 g, 0.27 mmol) in dry CH_2Cl_2 (4.0 mL). Subsequently, the crude mixture (0.179 g) containing **37** (0.064 g, 0.27 mmol) in dry CH_2Cl_2 (2.0 mL) was added. The reaction mixture was stirred at room temperature for 15 h, then concentrated to dryness. Purification by typical silica gel flash chromatography (Cy/EtOAc, from 100:0 to 40:60) afforded compound **34** (0.022 g, 36%) as a white solid: $R_t = 2.18$ min; $[\alpha]_D^{25} = -15.1$ cm^3 g^{-1} dm^{-1} ($c = 0.05$ in MeOH); ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 7.90$ (bs, 1H), 7.78 (d, 1H, $J = 8.6$ Hz), 4.58–4.62 (m, 1H), 3.95 (t, 2H, $J = 6.7$ Hz), 3.37 (t, 1H, $J = 5.4$ Hz), 3.07 (dd, 1H, $J = 5.4$, 2.7 Hz), 1.59–1.48 (m, 2H), 1.35–1.21 (m, 8H), 0.86 ppm (t, 3H, $J = 6.9$ Hz); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta = 168.2$, 155.6, 64.1, 58.3, 42.6, 31.2, 28.6, 28.3, 25.3, 22.0, 13.9 ppm; MS (ESI, +) *m/z*: 229 $[M+H]^+$, 251 $[M+Na]^+$, 267 $[M+K]^+$; HRMS-ESI: *m/z* $[M+Na]^+$ calcd for $C_{11}H_{20}N_2O_3Na$: 251.1372, found: 251.1374.

Pharmacology

Fluorogenic h-NAAA assay: HEK293 human embryonic kidney cells stably transfected with the human NAAA coding sequence cloned from a human spleen cDNA library were used as the enzyme source. Recombinant HEK-*h*-NAAA pellets were resuspended in homogenizing buffer and sonicated. Samples were centrifuged at 800 *g* for 15 min at 4 °C, and the resulting supernatants were then ultracentrifuged at 12000 *g* for 30 min at 4 °C. The pellets were resuspended in PBS at pH 7.4 on ice and subjected to two freeze/thaw cycles at −80 °C. The suspension was finally centrifuged at 105000 *g* for 1 h at 4 °C. Protein concentration was measured, and samples were aliquoted and stored at −80 °C until use. The assay was run in Optiplate 96-well black plates (PerkinElmer Inc., Boston, MA, USA) in a total reaction volume of 200 μL. NAAA protein preparation (20 μg) was pre-incubated for 10 min with various concentrations of test compounds or vehicle control (5% DMSO) in 100 mM citrate/phosphate buffer (pH 4.5) containing 3 mM DTT, 0,1% Triton X-100, 0.05% BSA, and 150 mM NaCl. *N*-(4-*m*ethyl-2-oxo-chromen-7-yl)hexadecanamide (PAMCA)[16] was used as a substrate (5 μM), and the reaction was carried out over 30 min at 37 °C. The samples were then read in a PerkinElmer Envision plate reader (PerkinElmer Inc., Boston, MA, USA) using an excitation wavelength of 360 nm and emission of 460 nm. IC_{50} values were calculated by non-linear regression analysis of log[concentration]/inhibition curves using GraphPad Prism 5 (GraphPad Software Inc., CA, USA) applying a standard slope curve fitting.

In vitro chemical stability: Chemical stability of selected compounds was evaluated under physiological pH conditions (0.01 M phosphate-buffered saline, pH 7.4) and acidic pH conditions (0.01 M phosphate buffer, pH 5.0) for up to 24 h. Both buffers were added with 10% of CH_3CN. Stock solutions of each compound (10 mM) were prepared freshly in DMSO. Each compound was incubated at a final concentration of 1 μM (1% DMSO) in both preheated buffers (37 °C). The sample solutions were divided into aliquots in glass vials (preheated at 37 °C) for each time point. The samples were maintained at 37 °C in the UPLC–MS autosampler during the study (without shaking). A reference solution of each compound (final concentration: 1 μM at 1% DMSO) in preheated CH_3CN (37 °C) was prepared from the stock solutions (10 mM in DMSO) and maintained at 37 °C in the UPLC–MS autosampler during the study (without shaking). The analyses were performed on a Waters Acquity UPLC–MS triple quadrupole detector (TQD) system consisting of a TQD mass spectrometer equipped with an ESI interface and a photodiode array detector. The 24 h time course analyses for

1, **11 a**, and **11 h** were carried out by UPLC-UV on the same instrument described above. A calibration curve in the 0.2–50 μM concentration range was prepared for each parent compound by serial dilution in CH_3CN (R^2 values were >0.999 for all compounds). The concentrations of the rearranged and hydrolyzed products of each parent were then calculated on the corresponding calibration curve, assuming no changes in the molar absorptivity values (ε). To test this assumption, each parent was fully hydrolyzed with 1 M NaOH solution, and the concentration of the corresponding product was calculated on the parent compound calibration curve. The measured concentration matched the expected 30 μM value. The analyses were run on an ACQUITY UPLC BEH C_{18} 1.7 μm 2.1× 50 mm column with a VanGuard BEH C_{18} 1.7 μm pre-column at 40 °C. The mobile phase was 0.1% HCOOH in H_2O (A) and 0.1% HCOOH in CH_3CN (B) using the following gradient: 0–0.5 min: 5% B, 0.5–2.5 min: 5–100% B, 2.5–2.7 min: 100% B, 2.7–2.8 min: 100–5% B, 2.8–3.5 min: 5% B at a flow rate of 0.5 mL min^{-1}.

In vitro mouse plasma stability: Compounds were diluted in mouse plasma with 5% DMSO added to help solubilization. Plasma was pre-heated at 37 °C (10 min). The final compound concentration was 2 μM. At selected time points (immediately after dilution, 5, 15, 30, 60, and 120 min), a 40 μL aliquot of the incubation solution was diluted in 120 μL of cold CH_3CN, spiked with warfarin (200 nM) as an internal standard. After vortexing for 30 sec, the solution was centrifuged at 3500 *g* for 15 min at 4 °C, and the supernatant was transferred for LC–MS analysis on a Waters Acquity UPLC–MS TQD system consisting of a TQD mass spectrometer equipped with an ESI interface. Briefly, 3 μL of the supernatant were injected into a reversed phase column (BEH C_{18} 2.1X50 mm) and separated with a linear CH_3CN gradient. Compounds were quantified on the basis of their multiple reaction monitoring (MRM) peak areas. The response factors, calculated on the basis of the internal standard peak areas, were then plotted over time. When possible, response vs. time profiles were fitted with Prism (GraphPad Software, Inc., USA) to estimate compound half-lives in plasma.

In vitro rat plasma stability: Compounds were added to rat plasma pre-incubated at 37 °C at a final molecule concentration of 2 μM and a final DMSO concentration of 2.5%. The mixture was kept at 37 °C while shaking. Aliquots (50 μL) were taken at various time points (0, 5, 15, 30, 60, and 120 min), and 150 μL of CH_3CN spiked with 200 nM warfarin (internal standard) were added. After vortexing and centrifugation, 3 μL of supernatant were analyzed by LC–MS/MS by MRM. The corresponding time vs. response factor profiles were fitted with PRISM (GraphPad Software) to derive the experimental half-lives of the compounds.

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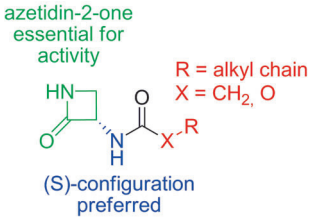
3-Aminoazetidin-2-one Derivatives as *N*-Acylethanolamine Acid Amidase (NAAA) Inhibitors Suitable for Systemic Administration



Stability is the key: α-Amino-β-lactams were synthesized as amide derivatives, and the effect of the azetidin-2-one ring, the stereochemistry at the α-position, and the functionalization of the α-amino group were studied with regard to *N*-acylethanolamine acid amidase inhibitory potency and hydrolytic and plasma stability.