DOI: 10.1002/cmdc.201300416

Synthesis, Structure–Activity, and Structure–Stability Relationships of 2-Substituted-*N*-(4-oxo-3-oxetanyl) *N*-Acylethanolamine Acid Amidase (NAAA) Inhibitors

Romina Vitale,[a] Giuliana Ottonello,[a] Rita Petracca,[a] Sine Mandrup Bertozzi,[a] Stefano Ponzano,[a] Andrea Armirotti,[a] Anna Berteotti,[a] Mauro Dionisi,[a] Andrea Cavalli,[a, b] Daniele Piomelli,[a, c] Tiziano Bandiera,*[a] and Fabio Bertozzi*[a]

N-Acylethanolamine acid amidase (NAAA) is a cysteine amidase that preferentially hydrolyzes saturated or monounsaturated fatty acid ethanolamides (FAEs), such as palmitoylethanolamide (PEA) and oleoylethanolamide (OEA), which are endogenous agonists of nuclear peroxisome proliferator-activated receptor-α (PPAR-α). Compounds that feature an α-amino-β-lactone ring have been identified as potent and selective NAAA inhibitors and have been shown to exert marked anti-inflammatory effects that are mediated through FAE-dependent activation of PPAR-α. We synthesized and tested a series of racemic, diastereomerically pure β-substituted α-amino-β-lactones, as either carbamate or amide derivatives, investigating the structure–activity and structure–stability relationships (SAR and SSR) following changes in β-substituent size, relative stereochemistry at the α- and β-positions, and α-amino functionality. Substituted carbamate derivatives emerged as more active and stable than amide analogues, with the *cis* configuration being generally preferred for stability. Increased steric bulk at the β-position negatively affected NAAA inhibitory potency, while improving both chemical and plasma stability.

Introduction

Palmitoylethanolamide (PEA), the endogenous amide of palmitic acid and ethanolamine, belongs to the family of fatty acid ethanolamides (FAEs), a class of lipid-derived messengers that participate in the control of multiple physiological functions, including pain and inflammation.[1] PEA is produced by most mammalian cells,[2] and 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.[4] It has also been suggested that PEA might attenuate skin inflammation and neuropathic pain in humans.[5] These effects are mainly attributed to the ability of PEA to activate peroxisome proliferator-activated receptor-α (PPAR-α), a member of the steroid/nuclear receptor superfamily.[6]

FAEs such as PEA and its monounsaturated analogue oleoylethanolamide (OEA) are produced on demand and their endogenous levels are regulated by enzymes responsible for their formation and degradation.[7] They are enzymatically biosynthesized from membrane glycerophospholipids via their corresponding *N*-acylphosphatidylethanolamines (NAPEs).[8] The major pathway for degradation of FAEs is the hydrolysis to free fatty acids and ethanolamine, which is carried out by two intracellular lipid amidases: fatty acid amide hydrolase (FAAH) and *N*-acylethanolamine acid amidase (NAAA).[9] Although NAAA and FAAH share the ability to cleave lipid amide bonds, they show major differences in primary structure, substrate selectivity, and cellular localization. NAAA is a cysteine amidase that belongs to the *N*-terminal nucleophile (Ntn) family of enzymes,[9c,10] preferentially hydrolyzing saturated or monounsaturated FAEs that activate PPAR-α, such as PEA and OEA, over the polyunsaturated endocannabinoid anandamide.[9c,11] NAAA has no sequence homology to FAAH,[9c] but is linked to the choloylglycine hydrolase family of enzymes, members of which are characterized by the ability to cleave carbon–nitrogen bonds in linear amides, with the exception of peptide bonds.[12] Like other Ntn enzymes, NAAA is produced as an inactive pro-enzyme and is activated at acidic pH by autocatalytic cleavage at a specific site of the peptide chain.[13] Site-directed mutagenesis experiments have univocally identified Cys131 (in mice) and Cys126 (in humans) as the catalytic residue responsible for both auto-proteolysis and FAE hydrolysis,[12,14]

[a] *R. Vitale,[+] Dr. G. Ottonello,[+] R. Petracca, S. M. Bertozzi, Dr. S. Ponzano, Dr. A. Armirotti, Dr. A. Berteotti, Dr. M. Dionisi, Prof. A. Cavalli, Prof. D. Piomelli, Dr. T. Bandiera, Dr. F. Bertozzi*
Drug Discovery & Development
Italian Institute of Technology (IIT), Via Morego 30, 16163 Genova (Italy)
E-mail: tiziano.bandiera@iit.it
fabio.bertozzi@iit.it

[b] *Prof. A. Cavalli*
Department of Pharmacy & Biotechnology
University of Bologna, Via Belmeloro 6, 40126 Bologna (Italy)

[c] *Prof. D. Piomelli*
Departments of Anatomy & Neurobiology, Pharmacology & Biological Chemistry
University of California, Irvine, Neuroscience Research Facility
3216 Gillespie Building, Irvine, CA 92697-4621 (USA)

[+] *These authors contributed equally to this work.*

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cmdc.201300416.

and have indicated Cys126, Arg142, and Asp154 as the residues that constitute the catalytic triad of human NAAA.[14a]

Because NAAA has been shown to primarily deactivate PEA and OEA,[9c,11] selective NAAA inhibitors could be envisaged to increase local levels of these FAEs, which may lead to anti-inflammatory and analgesic effects via PPAR-α signaling. This hypothesis was confirmed experimentally.[15] As the discovery and initial characterization of NAAA are quite recent, only a limited number of NAAA inhibitors have been reported. Among them, palmitic acid derivatives,[16] lipophilic amides[17] and amines[18] were reported to inhibit NAAA activity with medium-to-low micromolar potencies. Recently, compounds featuring an α-amino-β-lactone ring as a reactive electrophilic warhead were identified as the first class of potent and selective NAAA inhibitors.[19]

The β-lactone ring is present in many biologically active natural products such as lipstatin **1**,[20] the *clasto*-lactacystin β-lactone (omuralide) **2**,[21] salinosporamide A **3**,[22] and obafluorin **4**[23] (Figure 1) and represents a promising privileged structure

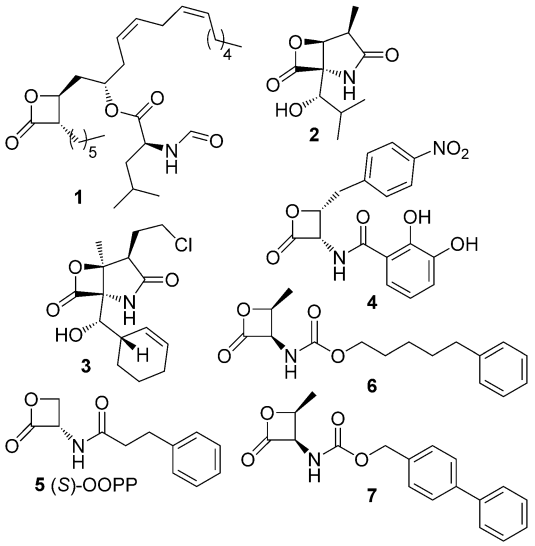


Figure 1. Naturally occurring β-lactones **1**–**4** and α-amino-β-lactone NAAA inhibitors **5**–**7**.

that can react covalently with the active sites of certain enzymes, such as lipases,[20b] cysteine[24] and serine[25] proteases, and the proteasome.[22b,23a,26] Over the last 20 years naturally occurring β-lactone (oxetan-2-one) derivatives have been extensively studied as potential drug candidates for several human disease conditions, including hypercholesterolemia and cancer.[27] Among them, α-amino-β-lactone derivatives have been studied as proteasome inhibitors and have progressed to clinical studies as novel anticancer agents.[22a,23a,27d]

Within this class of compounds, (*S*)-*N*-(2-oxo-3-oxetanyl)-3-phenylpropionamide [(*S*)-OOPP, **5**, Figure 1] was reported to be a relatively potent NAAA inhibitor [rat NAAA (rNAAA): IC_{50} = 0.42 μM], and was shown to increase PEA and OEA levels in activated leukocytes and decrease responses induced by inflammatory stimuli both in vitro and in vivo.[19a,b] Further investigations aiming at a structure–activity relationship (SAR) expansion of α-amino-β-lactone derivatives examined the effects of side chain modifications on NAAA inhibition and the stereochemical requirements for introduction of a methyl group in the β-position. These studies led to the identification of compounds that are highly potent at inhibiting both rNAAA and human NAAA (hNAAA), such as β-lactones **6** (rNAAA: IC_{50} = 0.050 μM, hNAAA: IC_{50} = 0.007 μM)[19c,28] and **7**[28b] (r and hNAAA: IC_{50} = 0.007 μM; Figure 1). The in vitro characterization of **6** proved that this compound inhibits rNAAA through a mechanism that is rapid, noncompetitive, and fully reversible after overnight dialysis.[29] Furthermore, topical administration of **6** was shown to elevate FAE levels in mouse skin and sciatic nerve tissues, and to attenuate nociception in mice and rats through a mechanism engaging PPAR-α activation.[29]

The β-lactone moiety, which is crucial for NAAA inhibitory activity, displays low chemical and plasma stability, thus limiting the therapeutic applications of these compounds.[19c,30] However, threonine-derived β-lactones, which bear a β-methyl substituent, are known to have enhanced stability in aqueous media relative to their serine-derived β-lactone counterparts, which are readily hydrolyzed to the corresponding β-hydroxy acids.[19c,24b,30]

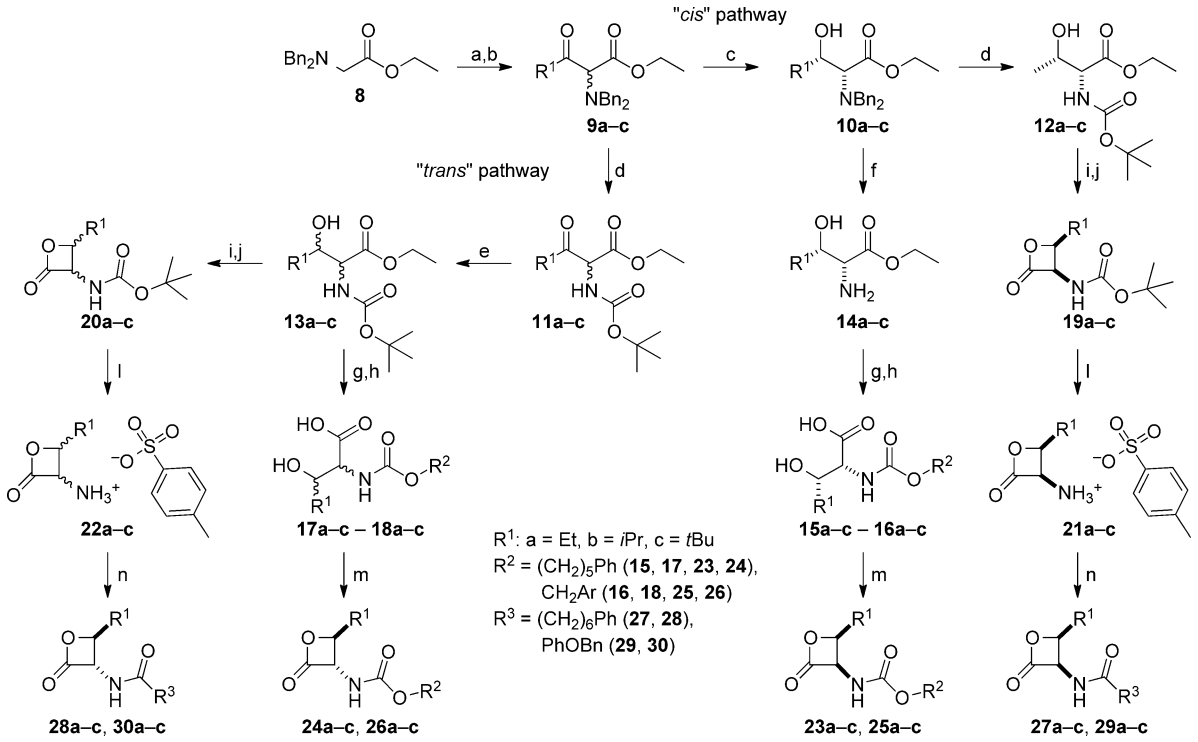
In the present work, we focused our attention on β-substituted α-amino-β-lactones, envisioning that the presence of a bulkier substituent at the β-position of the oxetan-2-one scaffold could improve the hydrolytic stability in aqueous media and provide new insight into substitution patterns that may lead to improved potency. To address the SARs as well as the structure–stability relationships (SSRs), we synthesized and tested a series of racemic, diastereomerically pure β-substituted α-amino-β-lactones as *cis*- and *trans*-carbamic acid esters and amide derivatives and examined both their potency and stability. In particular, we investigated the effect of the β-substituent size (Me, Et, *i*Pr, *t*Bu) and relative stereochemistry at positions 2 and 3 on NAAA inhibitory activity and chemical and plasma stability. Finally, quantum chemical calculations at the density functional theory (DFT) level were carried out to help explain the results of our experimental SSR studies.

Results and Discussion

Chemistry

The synthesis of β-substituted α-amino-β-lactones as carbamate **23a–c**–**26a–c** and amide **27a–c**–**30a–c** derivatives was undertaken following a racemic, diastereoselective synthetic pathway, which allowed the independent generation of *cis*- and *trans*-stereoisomers (Scheme 1).

Racemic *cis*-β-substituted α-amino-β-lactones **23a–c** and **25a–c** were obtained by [benzotriazol-1-yloxy(dimethylamino)-methylidene]dimethylazanium tetrafluoroborate (TBTU)-mediated cyclization from the corresponding *erythro*-α-substituted β-hydroxy acids **15a–c**–**16a–c**, prepared following a previously reported protocol by Guanti et al. (Scheme 1).[31] Microwave irradiation of a mixture of dibenzylamine and ethyl chloroacetate gave dibenzylamino ethyl acetate (**8**),[32] which was acylat-

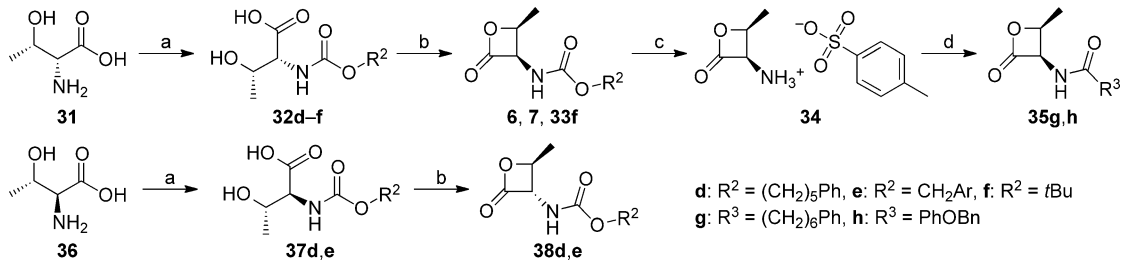


Scheme 1. General strategy for the synthesis of *cis*- and *trans*-β-substituted α-amino-β-lactones, as carbamates (**23**–**26**) and amides (**27**–**30**). *Reagents and conditions*: a) LDA, THF, −78 °C, 30 min; b) R^1COCl, −78 °C, 10 min, 70–80%; c) $NaBH_4$, NH_4Cl, EtOH/H_2O 4:1, RT, 3 h, 55–86%; d) H_2, 10% $Pd(OH)_2$/C, Boc_2O (H-Cube, EtOH, 70 °C, 1.0 bar), 55%–quant.; e) $NaBH_4$, THF/EtOH 1:1, RT, 1 h, 48–83%; f) H_2, 10% $Pd(OH)_2$/C (H-Cube, EtOH, 70 °C, 1.0 bar), 70%–quant.; g) 6.0 M HCl, microwave, 130 °C, 30 min, 81–93%; h) mixture of **41**–**42** [$R^2 = (CH_2)_5Ph$] or **43**–**44** [$R^2 = CH_2Ar$], $NaHCO_3$, H_2O/THF 1:1, RT, 16 h, 32–93%; i) 1.0 M NaOH, acetone/DMF 6:1, RT, 1 h; j) TBTU, Et_3N, CH_2Cl_2, RT, 16 h, 22–92%; l) CF_3COOH, *p*-TsOH, 0 °C, 30 min, 83%–quant.; m) TBTU, Et_3N, CH_2Cl_2, RT, 16 h, 10–65%; n) [$R^3 = (CH_2)_6Ph$ (**27a**–**c**, **28a**–**c**)] R^3COOH, TBTU, Et_3N, CH_2Cl_2, RT, 16 h, 30–46%; [R^3 = PhOBn (**29a**–**c**, **30a**–**c**)] R^3COCl, Et_3N, CH_2Cl_2, 0 °C, 2 h, 8.5–56% [Ar = 4-phenylphenyl].

ed with the desired acyl chloride (R^1 = Et, *i*Pr, *t*Bu) to give the corresponding β-keto esters **9a**–**c**. Acidic chemoselective reduction of the keto functionality with sodium borohydride in the presence of ammonium chloride[31] allowed complete stereoselective synthesis of racemic *erythro*-β-hydroxyamino esters **10a**–**c**. After deprotection of the amino functionality by hydrogenation, hydrolysis of the corresponding ester moiety of **14a**–**c** followed by carbamoylation of the free amino group with activated alcohols **41**–**44**[28b](see Supporting Information) led to the desired carbamic acid ester intermediates **15a**–**c**–**16a**–**c**.

Diastereomeric *threo*-β-substituted α-amino-β-hydroxy acids **17a**–**c**–**18a**–**c** were obtained by a modification of the procedure described above. One-pot deprotection of dibenzylamino derivatives **9a**–**c**, followed by in situ Boc protection of the free amino group in the H-Cube, furnished β-keto esters **11a**–**c** in good yields (55–75%). As previously reported,[33] sodium borohydride reduction of compounds **11a**–**c** led to *threo*/*erythro* mixtures of the corresponding β-hydroxy esters **13a**–**c** in ratios of 8:2 (R^1 = Et and *i*Pr) to 9:1 (R^1 = *t*Bu). After one-pot acidic *N*-Boc deprotection and hydrolysis of esters **13a**–**c**,[34] isomeric mixtures of compounds **17a**–**c**–**18a**–**c** were obtained by *N*-carbamoylation with activated alcohols **41**–**44**. Cyclization performed in the presence of TBTU led to the final β-substituted α-amino-β-lactones as a 8:2–9:1 mixtures of *trans*/*cis* isomers, which after chromatographic purification afforded the diastereomerically pure *trans*-β-lactone carbamates **24a**–**c** and **26a**–**c**. Because the cyclization of *N*-acylated-β-hydroxyamino acids to β-lactones has been reported to mainly produce the corresponding five-membered ring azalactones,[35] an alternative route to racemic *cis*- and *trans*-β-substituted α-amino-β-lactone amide derivatives **27a**–**c**–**30a**–**c** was envisaged, starting from intermediates **10a**–**c** and **13a**–**c**, respectively. Concerning *cis*-amide derivatives, in situ one-pot hydrogenation/*N*-Boc protection of intermediates **10a**–**c** provided diastereochemically pure *erythro*-β-hydroxy esters **12a**–**c**, which, after basic hydrolysis and TBTU cyclization, gave *N*-Boc-α-amino-β-lactones **19a**–**c** (Scheme 1). As already reported for similarly protected α-amino-β-lactones,[19b,24b] reaction of **19a**–**c** with *p*-toluenesulfonic acid in TFA gave the corresponding toluenesulfonate salts **21a**–**c**, which could be functionalized by coupling reaction with the desired carboxylic acid or acyl chloride in the presence of TBTU to give the final amides **27a**–**c** and **29a**–**c**. *trans*-Amide analogues **28a**–**c** and **30a**–**c** were prepared by following a similar procedure as for *cis* derivatives, starting from intermediates **13a**–**c**. Diastereomerically pure *trans*-β-lactone amides were isolated after chromatographic purification of the corresponding mixtures of *trans*/*cis* isomers.

The preparation of (2*S*,3*R*)-2-methyl-4-oxo-oxetan-3-yl-carbamates **6**,[19c,28b] **7**[28b] and amides **35g**,[28a] **h**, and (2*S*,3*S*)-2-methyl-4-oxo-oxetan-3-yl-carbamates **38d**,[28b] **e** was accomplished in a similar manner as the above-described β-substitut-



Scheme 2. Preparation of (2*S*,3*R*)-2-methyl-4-oxo-oxetan-3-yl-carbamates **6**, **7** and amides **35 g**,**h**, and (2*S*,3*S*)-2-methyl-4-oxo-oxetan-3-yl-carbamates **38 d**,**e**. *Reagents and conditions*: a) mixture of **41**–**42** [$R^2=(CH_2)_5Ph$] or **43**–**44** [$R^2=CH_2Ar$] or Boc_2O [$R^2=t$Bu], $NaHCO_3$, H_2O/THF 1:1, RT, 16 h, 88–92%; b) TBTU, Et_3N, CH_2Cl_2, RT, 16 h, 17–30%; c) [$R^2=t$Bu (**33 f**)] CF_3COOH, *p*-TsOH, 0 °C, 30 min, 95%; d) [$R^3=(CH_2)_6Ph$ (**35 g**)] R^3COOH, TBTU, Et_3N, CH_2Cl_2, RT, 16 h, 40%; [$R^3=$PhOBn (**35 h**)] R^3COCl, Et_3N, CH_2Cl_2, 0 °C, 2 h, 36% [Ar = 4-phenylphenyl].

ed analogues, starting from D- (**31**) and L-*allo*-threonine (**36**), respectively (Scheme 2). Functionalization of the amino group as for carbamates **15 a**–**c**–**18 a**–**c** was performed according to a previously reported protocol[28b,36] involving the activation of the desired alcohols **39** and **40** with di-2-pyridyl carbonate (DPC) followed by coupling with the β-hydroxy-α-amino acid (see Supporting Information).

Structure–activity relationship (SAR)

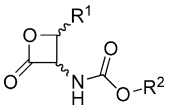
In our previous studies on serine- and threonine-derived α-amino-β-lactones, we found that the introduction of a methyl group at the β-position of the oxetan-2-one moiety proved to be beneficial for potency on both rat and human NAAA.[19c,28b] Furthermore, the type of functionalization of the amino group of the β-lactone, that is, amide versus carbamate, proved to be important in terms of activity and stability.[19b,c,28b]

Here, to investigate the possible influence of various alkyl groups at the β-position on potency, a series of β-substituted α-amino-β-lactone derivatives were designed and synthesized as carbamic acid esters (hereafter referred to as the "carbamate series") and amides (hereafter referred to as the "amide series"). The amino group was functionalized with different chains, selected among those previously disclosed in potent β-lactone NAAA inhibitors. In particular, we selected the 5-phenylpentyl[19c,28b] and the 4-phenylphenylmethyl[28b] chains for the carbamate series (Table 1), and the 4-benzyloxyphenyl[19b,c] and 6-phenylhexyl[28a] chain for the amide analogues (Table 2).

All the compounds were tested for their ability to inhibit the hydrolysis of *N*-(4-methyl-2-oxo-chromen-7-yl)hexadecanamide (PAMCA) by recombinant hNAAA heterologously expressed in HEK293 cells (see Experimental Section below).[14b,c] IC_{50} values are listed in Tables 1 and 2.

We first focused on the carbamate series, investigating the role of the alkyl groups—Et, *i*Pr, and *t*Bu—at position 2 of the β-lactone ring. Compound **23 a**, the racemic *cis*-2-ethyl stereoisomer carrying a 5-phenylpentyl side chain, turned out to be a nanomolar inhibitor of hNAAA ($IC_{50}=0.009$ μM), showing similar potency to **6** ($IC_{50}=0.014$ μM), the (2*S*,3*R*)-2-methyl analogue (Table 1). Increasing the steric bulk at the β-position while maintaining a *cis* configuration led to compounds **23 b** ($R^1=i$Pr, $IC_{50}=0.063$ μM) and **23 c** ($R^1=t$Bu, $IC_{50}=0.302$ μM), which respectively showed 6- and 30-fold lower potency than **23 a**. We then investigated the effect of stereochemistry on

Table 1. Inhibitory potency toward hNAAA activity, and in vitro chemical and rat plasma stabilities of carbamates **6**,[a] **7**,[a] **23 a**–**c**–**26 a**–**c**[b] and **38 d**,**e**.[a]



Compd	Config	R^1	R^2	IC_{50} [μM][c]	$t_{1/2}$ [min][d] pH 7.4[e]	pH 5.0[f]	RP[g]
6	*cis*-(2*S*,3*R*)	Me	$(CH_2)_5Ph$	0.014 ± 0.006	215 ± 26	522 ± 44	< 5
23 a	*cis*	Et	$(CH_2)_5Ph$	0.009 ± 0.003	223 ± 9	550 ± 64	< 5
23 b	*cis*	*i*Pr	$(CH_2)_5Ph$	0.063 ± 0.015	354 ± 19	> 1440 (*76*)[h]	< 5
23 c	*cis*	*t*Bu	$(CH_2)_5Ph$	0.302 ± 0.064	611 ± 42	> 1440 (*73*)[h]	< 5
38 d	*trans*-(2*S*,3*S*)	Me	$(CH_2)_5Ph$	0.008 ± 0.001	184 ± 15	340 ± 28	< 5
24 a	*trans*	Et	$(CH_2)_5Ph$	0.004 ± 0.001	145 ± 4	339 ± 46	< 5
24 b	*trans*	*i*Pr	$(CH_2)_5Ph$	0.019 ± 0.004	259 ± 13	1258 ± 83	< 5
24 c	*trans*	*t*Bu	$(CH_2)_5Ph$	0.234 ± 0.054	235 ± 40	> 1440 (*68*)[h]	5.3 ± 1.0
7	*cis*-(2*S*,3*R*)	Me	CH_2Ar[i]	0.015 ± 0.004	201 ± 41	491 ± 65	< 5
25 a	*cis*	Et	CH_2Ar[i]	0.015 ± 0.003	183 ± 28	415 ± 91	< 5
25 b	*cis*	*i*Pr	CH_2Ar[i]	0.039 ± 0.004	305 ± 36	> 1440 (*65*)[h]	< 5
25 c	*cis*	*t*Bu	CH_2Ar[i]	0.576 ± 0.128	605 ± 53	> 1440 (*65*)[h]	< 5
38 e	*trans*-(2*S*,3*S*)	Me	CH_2Ar[i]	0.008 ± 0.002	159 ± 20	395 ± 45	< 5
26 a	*trans*	Et	CH_2Ar[i]	0.006 ± 0.001	151 ± 22	457 ± 37	< 5
26 b	*trans*	*i*Pr	CH_2Ar[i]	0.023 ± 0.007	191 ± 35	1121 ± 201	< 5
26 c	*trans*	*t*Bu	CH_2Ar[i]	0.507 ± 0.196	181 ± 12	> 1440 (*56*)[h]	6.5 ± 1.8

[a] Compounds tested as pure enantiomers. [b] Compounds tested as racemic mixtures. [c] Values are the mean ± SD of three or more determinations. [d] Stability values are the mean ± SD of three or more determinations. [e] PBS (0.01 M) + 10% CH_3CN, pH 7.4, 37 °C. [f] Phosphate buffer (0.01 M) + 10% CH_3CN, pH 5.0, 37 °C. [g] Rat plasma (80% *v*/*v*) + 20% PBS, 5% DMSO, pH 7.4. [h] Compound remaining (%) after 24 h (1440 min). [i] Ar = 4-phenylphenyl.

NAAA inhibition, while keeping the same substituents on the α-amino group. Along with a generally slight increase in inhibitory activity, compounds with a *trans* configuration displayed an analogous pattern to that of the corresponding *cis* stereoisomers, showing a decrease in potency for more sterically hindered β-substituted compounds (IC_{50} values from 0.004 μM for **24a** to 0.234 μM for **24c**).

Moving to compounds **25a–c** and **26a–c**, which carry a 4-phenylphenylmethyl chain, we similarly observed a positive influence on NAAA inhibitory potency of a *trans* relationship between the substituents at positions α and β, with compounds **26a–c** (IC_{50} = 0.006–0.507 μM) slightly more active than stereoisomers **25a–c** (IC_{50} = 0.015–0.576 μM). The detrimental effect of bulky substituents at the β-position was also evident in this subset of compounds. Notably, increasing steric hindrance at the β-position (from methyl to *tert*-butyl) led to a progressive decrease in NAAA inhibition of this series of compounds, independently from the carbamate functionalization (5-phenylpentyl or 4-phenylphenylmethyl).

Replacement of the carbamate function with an amide, as in derivatives **27a–c–30a–c** and **35g,h**, resulted in an evident decrease in potency (Table 2). A >40-fold drop in NAAA inhibitory activity was observed for the β-lactone amide **27a** (IC_{50} = 0.408 μM) relative to the corresponding carbamate analogue **23a** (IC_{50} = 0.009 μM). A more pronounced effect was observed for the enantiomerically pure threonine-derived compounds **6** (IC_{50} = 0.014 μM) and **35g** (IC_{50} = 1.56 μM), which showed a ~100-fold difference in potency.

While the activity of β-ethyl (**27a**) and β-isopropyl (**27b**) analogues turned out to be quite similar (IC_{50} values of 0.408 and 0.379 μM, respectively), a significant drop in potency was observed in the case of the *tert*-butyl group in β-position (**27c**, IC_{50} = 2.87 μM), confirming the trend observed in the carbamate series. Although the previously described L-serine-derived amide carrying a 4-benzyloxyphenyl chain had shown nanomolar potency against NAAA,[19b] all substituted cis-amides **29a–c** and **35h** disappointingly turned out to be poorly active (IC_{50} > 100 μM). The data obtained for D-threonine analogue **35h** suggests that the low activity observed for this set of compounds relative to the L-serine-derived amide, could be ascribed to the opposite configuration at α-position (*R* vs. *S* configuration) and/or the substitution at the β-position (Me vs. H).

Moving to amides with the *trans* configuration, whereas analogues **28a,b** and **30a** could not be tested due to their low intrinsic chemical stability (see the following section), compounds **28c** and **30b,c** showed moderate NAAA inhibition (IC_{50} values from 0.277 μM for **28c** to 1.98 μM for **30c**).

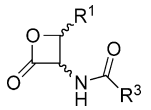
Chemical stability

β-Lactones are known to exhibit limited stability in water due to ring-opening to the corresponding β-hydroxy acids, which relieves the strain of the four-membered ring. In α-amino-β-lactone derivatives, it has been reported that a methyl group at the β-position of the oxetanone ring, as in threonine-derived β-lactones, improves the hydrolytic stability of such compounds with respect to the corresponding unsubstituted serine-derived analogues.[19c,24b]

On the other hand, replacement of the substituted benzyl group in the naturally occurring obafluorin (**4**, Figure 1) with a less bulky methyl led to a decrease in the hydrolytic stability of the β-lactone ring.[30] These findings prompted us to expand previous studies on the role of the β-substituent on the hydrolytic stability of α-amino-β-lactones.[19c] We focused our attention on the effect of the size of the β-substituent and on the role of the relative stereochemistry at positions 2 and 3. The role of the amino group functionalization, as amides and carbamic acid esters, was also investigated with the aim to understand the contribution of this modification to the susceptibility of disubstituted α-amino-β-lactones to chemical hydrolysis.

Chemical stability data for 2-substituted *N*-(4-oxo-3-oxetanyl)-carbamic acid esters **6**, **7**, **23a–c–26a–c**, **38d,e** and amides **27a–c–30a–c**, **35g,h** are summarized in Tables 1 and 2, respectively. Compound stability

Table 2. Inhibitory potency toward hNAAA activity, and in vitro chemical and rat plasma stabilities of amides **27a–c–30a–c**[a] and **35g,h**.[b]



Compd	Config	R^1	R^3	IC_{50} [μM][c]	$t_{1/2}$ [min][d] pH 7.4[e]	pH 5.0[f]	RP[g]
35g	*cis*-(2*S*,3*R*)	Me	$(CH_2)_6Ph$	1.56 ± 0.208	109 ± 9	229 ± 20	< 5
27a	*cis*	Et	$(CH_2)_6Ph$	0.408 ± 0.122	127 ± 10	387 ± 34	< 5
27b	*cis*	*i*Pr	$(CH_2)_6Ph$	0.379 ± 0.124	136 ± 21	> 1440 (*54*)[h]	< 5
27c	*cis*	*t*Bu	$(CH_2)_6Ph$	2.874 ± 0.176	356 ± 47	> 1440 (*73*)[h]	8.3 ± 2.3
28a	*trans*	Et	$(CH_2)_6Ph$	Unst.[i]	n.a.[j]	n.a.[j]	
28b	*trans*	*i*Pr	$(CH_2)_6Ph$	Unst.[i]	n.a.[j]	n.a.[j]	
28c	*trans*	*t*Bu	$(CH_2)_6Ph$	0.277 ± 0.022	152 ± 15	573 ± 51	< 5
35h	*cis*-(2*S*,3*R*)	Me	PhOBn	> 100[k]	50 ± 4	175 ± 5	10.3 ± 1.2
29a	*cis*	Et	PhOBn	> 100[k]	54 ± 8	298 ± 7	6.3 ± 1.8
29b	*cis*	*i*Pr	PhOBn	> 100[k]	62 ± 3	1345 ± 85	< 5
29c	*cis*	*t*Bu	PhOBn	> 100[k]	98 ± 8	> 1440 (*69*)[h]	10.2 ± 1.0
30a	*trans*	Et	PhOBn	Unst.[i]	n.a.[j]	n.a.[j]	
30b	*trans*	*i*Pr	PhOBn	0.581 ± 0.027	20 ± 2	24 ± 2	< 5
30c	*trans*	*t*Bu	PhOBn	1.98 ± 0.259	156 ± 17	494 ± 53	< 5

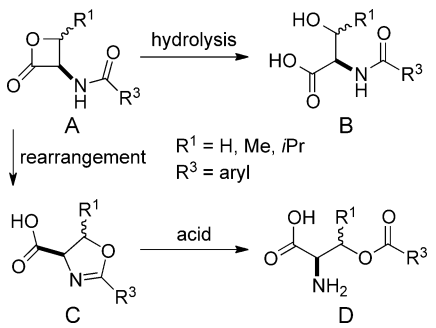
[a] Compounds tested as pure enantiomers. [b] Compounds tested as racemic mixtures. [c] Values are the mean ± SD of three or more determinations. [d] Stability values are the mean ± SD of three or more determinations. [e] PBS (0.01 M) + 10% CH_3CN, pH 7.4, 37 °C. [f] Phosphate buffer (0.01 M) + 10% CH_3CN, pH 5.0, 37 °C. [g] Rat plasma (80% *v/v*) + 20% PBS, 5% DMSO, pH 7.4. [h] Compound remaining (%) after 24 h (1440 min). [i] Unstable. [j] Not available ($t_{1/2}$ < 10 min). [k] IC_{50} > 100 μM refers to compounds showing < 30% inhibition at 100 μM.

was evaluated as the half-life ($t_{1/2}$), in minutes, in buffered solutions at pH 7.4 (physiological pH) and 5.0 (pH of the hNAAA assay), at 37 °C, by measuring the disappearance of each tested compound at various time points.

Previous stability studies had shown (2*S*,3*R*)-2-methyl-4-oxo-3-oxetanylcarbamic acid ester derivative **6** to have an improved chemical stability profile relative to serine-derived β-lactone analogues.[19c] These results led us to consider **6** as the starting point for our SSR investigations.

Replacement of the 5-phenylpentyl side chain with a 4-phenyphenylmethyl residue, as in compound **7**, or inversion of the stereochemistry at the α-position on both **6** and **7**, leading to compounds **38 d** and **38 e**, did not have major impacts on hydrolytic stability (Table 1). Conversely, amides **35 g**,**h** turned out to be considerably less stable (pH 7.4: $t_{1/2}$ = 50–109 min, pH 5.0: $t_{1/2}$ = 175–229 min) than carbamate **6** (pH 7.4: $t_{1/2}$ = 215 min, pH 5.0: $t_{1/2}$ = 522 min) (Table 2).

The effect of the relative stereochemistry at positions 2 and 3 of the β-lactone ring, carrying respectively the alkyl substituent and the functionalized amino group, was initially investigated. Whereas for the carbamate series, derivatives with a *cis* configuration (**23 a**–**c**: $t_{1/2}$ = 223–611 min, **25 a**–**c**: $t_{1/2}$ = 183–605 min) showed slightly higher hydrolytic stability at pH 7.4 than the corresponding *trans* counterparts (**24 a**–**c**: $t_{1/2}$ = 145–235 min, **26 a**–**c**: $t_{1/2}$ = 151–181 min; Table 1), for the amide analogues the relative stereochemistry had a more pronounced impact on the stability depending on the β-substitution. In fact, although *cis*-amides (**27 a**–**c**: $t_{1/2}$ = 127–356 min, **29 a**–**c**: $t_{1/2}$ = 54–98 min; Table 2) displayed an overall higher tendency toward hydrolysis than the carbamate analogues, a significant effect of the size of the β-substituent was observed with *trans*-substituted compounds. Replacement of the less sterically hindered ethyl group with the bulkier *tert*-butyl moiety resulted in a considerable increase in chemical stability (**28 a**, **30 a**: $t_{1/2}$ < 10 min, **28 c**, **30 c**: $t_{1/2}$ = 152–156 min; Table 2). A rationalization of the lower stability of α-amino-β-lactone amides with respect to the corresponding carbamic acid ester derivatives has been previously provided for serine- and threonine-derived β-lactone amides (A, R^1 = H, Me; Scheme 3). This type of compound has been described to undergo two possible degradation pathways, leading to either *N*-acylhydroxy acids (B), resulting from the hydrolytic opening of the β-lactone ring, or the corresponding *O*-acyl isomers (D), as a product of an intramolecular rearrangement followed by acid-mediated hydrolysis of the oxazoline-carboxylic acid intermediate (C) (Scheme 3).[19c,30,37]



Scheme 3. Substituted β-lactone amides (A) degradation products: *N*-acyl-β-hydroxy acids (B), and *O*-acyl-α-amino acids (D).

To determine whether a similar degradation mechanism also occurred to β-substituted higher homologues, we selected the two diastereomeric amides **29 b** and **30 b**, and we carried out a time course UPLC–UV analysis, at pH 7.4 and 5.0, of their chemical degradation. Similar to what we observed with serine- and threonine-derived β-lactones, both β-isopropylamide stereoisomers **29 b** and **30 b** showed the formation of a consistent amount of degradation products deriving from an intramolecular nucleophilic attack of the amide carbonyl group to the β-position of the lactone ring (see Supporting Information, figures S1 and S2).[19c,24b,30] Whereas at pH 7.4 *trans*-isomer **30 b** ($t_{1/2}$ = 20 min; Table 2) converted in a time dependent manner over 24 h, mainly (~70% mol) into the corresponding 4,5-dihydro-2-(4-benzyloxyphenyl)-5-isopropyl-4-oxazolecarboxylic acid (C, R^1 = *i*Pr; Scheme 3), the *O*-4-benzyloxybenzoyl-β-hydroxyamino acid D (R^1 = *i*Pr, Scheme 3) was the only species detected at pH 5.0 (Supporting Information, figure S1).[19c]

Interestingly, unlike the *trans* stereoisomer, *cis*-β-lactone amide **29 b** ($t_{1/2}$ = 62 min; Table 2) produced, both at pH 7.4 and 5.0, a consistent amount of diastereomeric *N*-acyl-β-hydroxy acid B (R^1 = *i*Pr, Scheme 3; 40 and 25% mol at pH 7.4 and 5.0, respectively), along with the products deriving from intramolecular rearrangement (Supporting Information, figure S2). These results clearly indicated for *cis*-amide **29 b** the presence of two competitive degradation pathways relative to *trans* analogue **30 b**.

Considering that the functionalization of the amino group of α-amino-β-lactones as carbamate has been reported to positively affect the chemical stability of such derivatives,[19c] a similar study was also conducted on *cis*- and *trans*-carbamate stereoisomers **25 b** and **26 b**. At pH 7.4, both β-lactones (**25 b**: $t_{1/2}$ = 305 min, **26 b**: $t_{1/2}$ = 191 min; Table 1) afforded exclusively the corresponding diastereomeric *N*-(4-phenylphenyl)methoxycarbonyl-β-hydroxyamino acids **16 b** and **18 b**. This indicates the hydrolytic opening of the β-lactone ring, leading to adduct B (Scheme 3), as the main degradation pathway for this kind of compound (Supporting Information, figures S3 and S4).

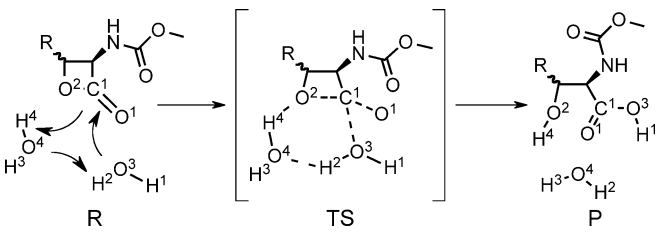
The peculiar behavior observed with α-amino-β-lactone amide derivatives could be reasonably explained by two possible competitive nucleophilic attacks occurring at different reaction rates, that is, intramolecular attack of the amide carbonyl moiety to the β-position of the β-lactone, versus attack of an external nucleophile (water) to the electrophilic endocyclic carbonyl moiety. The intramolecular pathway is expected to proceed more rapidly in *trans* isomers as a result of an antiperiplanar attack of the amide carbonyl group at the β-position relative to *cis* derivatives.[30,38] This degradation pathway, although possible also for the carbamate analogues, is expected to be more favored in amides due to the higher nucleophilicity of their carbonyl oxygen atom, as previously described.[19c] Therefore, the overall higher stability of β-lactone carbamate derivatives than amide analogues might be explained by the weak nucleophilic character of the carbamate carbonyl moiety, resulting in only one degradation pathway, that is, water attack with formation of the β-hydroxy acid.[39]

The influence of the various β-alkyl substitutions on stability was examined on both the carbamate and amide series of α-amino-β-lactones. A consistent trend was observed in the stability at pH 7.4 and 5.0 for carbamate and amide derivatives with the *cis* configuration. Increasing the steric demand of the alkyl group at the β-position led to an increase in the stability of such compounds. In fact, *cis*-amides **35g**,**h**, **27a**–**c** and **29a**–**c** bearing simple methyl to larger *tert*-butyl groups, displayed half-life ($t_{1/2}$) values ranging from 50 to 356 min at pH 7.4 and from 229 to more than 1440 min at pH 5.0 (Table 2). Interestingly, compounds **29a**–**c** and **35h**, featuring a benzamide side chain (R^2 = 4-benzyloxyphenyl) generally exhibited a lower half-life than the other substituted amides **27a**–**c** and **35g** having an aliphatic side chain (R^2 = 6-phenylhexyl).

The β-lactone carbamates with *cis* configuration—**6**, **7**, **23a**–**c** and **25a**–**c**—showed a similar trend concerning the effect of β-substitution (Table 1). Whereas methyl and ethyl substitution resulted in similar chemical stabilities (**6**, **23a**: $t_{1/2}$: ~220 min), increasing the size of the alkyl group at the β-position led to a significant improvement in hydrolytic stability (**23b**: $t_{1/2}$ = 354 min and **23c**: $t_{1/2}$ = 611 min), particularly at pH 7.4. Unlike amide derivatives, for *cis*-carbamates no relevant differences in hydrolytic stability were observed among compounds with different chains linked to the carbamate function.

Interestingly, the effect of the alkyl substituent at the β-position was much less evident for the series of *trans*-carbamates. In fact, contrary to what previously observed for *cis*-analogues, in compounds **24a**–**c**, **26a**–**c**, and **38d**,**e** the evident impact of steric hindrance at the β-position on degradation was somehow lost at pH 7.4. All *trans*-stereoisomers, regardless of the alkyl group at the β-position, showed a similar $t_{1/2}$ (**38d**, **24a**–**c**: $t_{1/2}$ = 184–235 min), with this result being more evident for the 4-phenylphenylmethyl side chain (**38e**, **26a**–**c**: $t_{1/2}$ = 159–181 min; Table 1).[40]

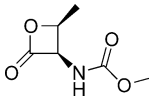
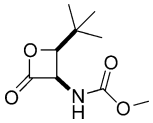
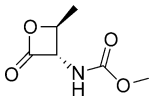
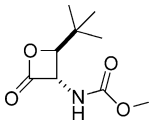
To provide a rational explanation of the variation in stability of the carbamate series at pH 7.4, we carried out DFT-based calculations simulating lactone hydrolysis in water of the truncated form of compounds **6**, **23c**, **24c** and **38d**, where the side chain of the carbamate function was replaced by a methyl group. In detail, we studied the nucleophilic attack of a water molecule at the carbonyl carbon of the β-lactone ring (Scheme 4). The hydrolysis was studied for both *cis* (**6** and **23c**) and *trans* (**24c** and **38d**) configurations, simulating the water nucleophilic attack from both sides of the ring (hereafter referred to as "*syn*" and "*anti*" attack with respect to the carbamate moiety; see Supporting Information). The reaction mechanism turned out to be quite similar for both the *syn* and *anti* attacks.



Scheme 4. Representation of the hydrolysis mechanism of β-lactones. The nucleophilic attack and protonation steps are concerted (R = reactants, TS = transition State, P = products).

Starting from the noncovalent complex, the reactants (R), the oxygen atom of one of the two water molecules (O^3, according to the numbering reported in Scheme 4) approached the carbonyl carbon of the lactone ring (C^1). The C^1–O^2 bond length increased, while the distance between O^2 and H^4 (i.e., a hydrogen atom of the second water molecule) decreased. O^4 was thus able to accept a proton (H^2) from the water carrying the nucleophilic attack. The main geometrical features characterizing the reaction mechanism are reported in the Supporting Information (table S2). All eight reactions occurred through a concerted mechanism, that is, at the transition state (TS) the nucleophilic attack and the proton transfers occurred simultaneously, leading to hydrolysis of the lactone ring (Scheme 4).

Table 3. In vitro chemical stabilities, representation of the truncated forms of compounds listed as determined by computational studies, and activation energy barriers for both the *syn* and *anti* water attacks.

Compd	$t_{1/2}$ [min][a]	Forms	E_a [kcal mol^{-1}] *syn*	*anti*
6	215		37.5	33.5
23c	611		42.2	33.9
38d	184		36.9	36.0
24c	235		37.0	37.7

[a] Determined at pH 7.4.

In Table 3, we report the in vitro chemical stability, the 2D representation of the truncated forms of compounds of column 1 studied by means of computational studies, and the activation energy (E_a) barriers for both the *syn* and *anti* attacks. For compounds bearing a *cis* configuration (**6** and **23c**), the difference between *syn* and *anti* attacks was higher than the same difference for compounds with a *trans* configuration (**24c** and **38d**). This outcome could be explained by considering that for **6** and **23c** the *cis* substituents (methyl or *tert*-butyl) hindered the water molecule from approaching the carbonyl carbon of the lactone ring. This is particularly evident for **23c**, where the presence of the *tert*-butyl group led to an E_a of 42.2 kcal mol^{-1}, almost 9 kcal mol^{-1} higher than the E_a for the same nucleophilic attack occurring from the *anti* side. This means that compound **23c** has a lower probability of being

hydrolyzed, as one of the two possible ways of attack is remarkably disfavored. The E_a difference between the *syn* and *anti* attack was 4 kcal mol^{-1} also in the case of the *cis* derivative **6**, carrying a methyl instead of a *tert*-butyl group, and was almost zero for the two *trans* derivatives (**24c** and **38d**). In addition, although comparison among different compounds could be questionable, *syn* attack on compound **23c** showed the highest E_a among all the reactions simulated here (Table 3). These DFT-based calculations help explain the half-life data obtained at pH 7.4 for these compounds (Table 1), pointing to **23c** as the compound with the highest stability in water among the whole series of lactones reported herein.

The influence of the β-alkyl substitution on hydrolytic decomposition for *trans*-carbamate analogues again became evident when studies were conducted at pH 5.0. In fact, along with an expected higher stability of these β-lactones at acidic pH relative to pH 7.4, high amounts of parent compound were observed for derivatives displaying either the *cis* or *trans* configuration (Table 1). This pH-dependency of hydrolytic stability on β-substitution was also recognizable to some extent for *cis*-amide derivatives **27a–c**, **29a–c** and **35g,h** (Table 2).

Rat plasma stability

β-Substituted α-amino-β-lactone derivatives were also tested for their stability in rat plasma (80% *v*/*v*; Tables 1 and 2). Most *cis*- and *trans*-carbamate analogues, regardless of β-substitution and side chain modification, showed low half-life values ($t_{1/2} < 5$ min) due to extensive degradation of the parent compounds. As observed for chemical stability, a bulky substituent at the β-position also contributed to improve plasma stability, with compounds **24c** and **26c** having half-lives >5 min (5.5 and 6.5 min, respectively). An analogous *tert*-butyl substitution effect was also highlighted for *cis*-amide **27c**, which showed a $t_{1/2}$ of 8.3 min, as a sole compound in this subset.

Although all the other *cis*-amides featuring a 6-phenylhexyl side chain were found to be quite unstable ($t_{1/2} < 5$ min), *cis*-benzamide analogues **29a,c** and **35h** showed improved plasma stability with significant half-life values ($t_{1/2} = 6.3$–10.3 min) for this class of compounds. The reason for this improvement in stability of compounds having a 4-benzyloxyphenyl chain is unclear at the moment and needs further investigation to be properly elucidated.

Conclusions

Herein we report on a novel series of β-lactone inhibitors of NAAA. NAAA is a promising target for the treatment of pain and inflammation, but few classes of inhibitors of this enzyme have been identified thus far. Among them, α-amino-β-lactones have emerged as potential candidates in this respect, as they can covalently and reversibly bind the catalytic cysteine residue of NAAA. In this study, we described SARs of two series of β-lactones: the carbamic acid ester and the amide series, which differed in the nature of the chain at the α-position and the aliphatic substituent at the β-position. We showed that derivatives of the carbamate series are generally more potent than analogues belonging to the amide series. In addition, we found that bulky substituents at the β-position of the β-lactone ring have a negative effect on NAAA inhibitory potency.

We next focused our attention on the hydrolytic stability of this class of compounds both under physiological (pH 7.4) and acidic (pH 5.0) conditions, and in plasma. Indeed, it is known that β-lactones have intrinsically low chemical stability due to their propensity toward hydrolysis into the corresponding β-hydroxy acids. We measured half-life values of several of the present lactones, and laid down initial SSRs for these compounds. We first noticed that molecules of the amide series were generally less stable than carbamates. Second, in the carbamate series, the stability was related to the size of the alkyl substituent and relative stereochemistry at the β-position. This was particularly clear for compound **23c**, carrying a β-*tert*-butyl group in *cis*-configuration with respect to the chain at the α-position. Finally, DFT calculations showed that in the case of *cis* derivatives with a bulky substituent, one of the two possible pathways of attack by a water molecule was energetically disfavored, providing a possible explanation for the improved stability of **23c** over other derivatives. Most of the α-amino-β-lactones were unstable in rat plasma. However, a few of them showed measurable half-life values on the order of a few minutes. This may be due to the mutual contribution of two effects, that is, increased β-substitution and amino group functionalization.

In summary, we have reported new SARs around α-amino-β-lactones as NAAA inhibitors, along with an investigation on the stability of these compounds in buffer and in plasma. This information may be valuable in the discovery of better NAAA inhibitors, which might help to validate NAAA as a therapeutic target and serve as starting points for the development of novel anti-inflammatory agents.

Experimental Section

Chemistry

Materials and methods: All of the commercially available reagents and solvents were used as purchased from vendors without further purification. Dry solvents (THF, Et_2O, CH_2Cl_2, DMF, DMSO, MeOH) were purchased from Sigma–Aldrich. Optical rotations were measured on a Rudolf Research Analytical Autopol II Automatic polarimeter using a sodium lamp (λ 589 nm) as the light source; concentrations are expressed in g(100 mL)$^{-1}$ using $CHCl_3$ as a solvent and a 1 dm cell. Melting points were determined on a Büchi M-560 melting point apparatus. Automated column chromatography purifications were done using a Teledyne ISCO apparatus (Combi*Flash* R_f) with pre-packed silica gel columns of various sizes (4–120 g). Mixtures of increasing polarity of cyclohexane and ethyl acetate (EtOAc) or cyclohexane and methyl *tert*-butyl ether (MTBE) were used as eluents. 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. Microwave heating was performed using Explorer-48 positions instrument (CEM). 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 di-

methyl 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 single quadrupole detector (SQD) mass spectrometer equipped with an electrospray ionization (ESI) interface and a photodiode array (PDA) detector. The PDA range was λ 210–400 nm. Analyses were performed on an Acquity UPLC BEH C_{18} column (100×2.1 mm ID, particle size: 1.7 μm) with a VanGuard BEH C_{18} pre-column (5×2.1 mm ID, particle size: 1.7 μm). Mobile phase was 10 mM NH_4OAc in H_2O at pH 5 adjusted with CH_3COOH (A) and 10 mM NH_4OAc in CH_3CN/H_2O (95:5) at pH 5.0. ESI in positive and negative mode was applied. Accurate mass measurement (HRMS) was performed on a Synapt G2 Quadrupole-ToF Instrument (Waters, USA), equipped with an ESI ion source. All tested compounds (**6**, **7**, **23a–c**–**30a–c**, **35g**,**h** and **38d**,**e**) showed ≥95% purity by NMR and UPLC–MS analysis. Compounds **6**,[19c,28b] **7**,[28b] **35g**,[28a] **38d**,[28b] and activated alcohols **41**–**44**[28b] were synthesized by following published procedures; the synthesis of compounds **35h** and **38e** are described in the Supporting Information.

General procedure for the synthesis of *cis*- and *trans*-α-amino-β-lactone carbamic acid ester derivatives (23a–c–26a–c; Scheme 1).

Preparation of β-lactones 23a–c–26a–c. To a stirred mixture of carbamates **15a–c**–**18a–c** (1.0 equiv) in dry CH_2Cl_2, under nitrogen atmosphere, at 0 °C, were added Et_3N (3.0 equiv) and subsequently TBTU (1.2 equiv). The mixture was left stirring at 0 °C for 1 h and at room temperature for 15 h. Upon full conversion of the starting material, the organics were removed under reduced pressure, and the resulting crude product absorbed over silica gel and purified by column chromatography, eluting with cyclohexane/EtOAc (from 90:10 to 60:40) to afford final products **23a–c**–**26a–c**.

5-Phenylpentyl-*N*-[(2*S,3*R**)-2-ethyl-4-oxo-oxetan-3-yl]carbamate (23a)**. The reaction was carried out with **15a** (0.070 g, 0.22 mmol), dry CH_2Cl_2 (10 mL), Et_3N (0.091 mL, 0.66 mmol), and TBTU (0.083 g, 0.26 mmol) to give, after purification, pure **23a** (0.043 g, 65%) as a white solid: mp: 63.8–64.7 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 0.89 (t, J = 7.4 Hz, 3H), 1.27–1.41 (m, 2H), 1.45–1.64 (m, 4H), 1.64–1.95 (m, 2H), 2.58 (t, J = 7.7 Hz, 2H), 4.01 (t, J = 6.5 Hz, 2H), 4.60 (dt, J = 6.0, 8.0 Hz, 1H), 5.45 (dd, J = 6.0, 9.4 Hz, 1H), 7.05–7.38 (m, 5H), 8.23 ppm (d, J = 9.4 Hz, 1H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 8.9, 21.9, 24.9, 28.3, 30.6, 35.1, 59.5, 64.6, 79.1, 125.6, 128.2 (2C), 128.2 (2C), 142.1, 155.8, 169.9 ppm; MS (ESI+) m/z (%): 323 (100) [M+NH$_4$]$^+$; (ESI−) m/z (%): 304 (100) [M−H]$^-$; HRMS-ESI: m/z [M+H]$^+$ calcd for $C_{17}H_{24}NO_4$: 306.1705, found: 306.1691.

5-Phenylpentyl-*N*-[(2*S,3*R**)-2-isopropyl-4-oxo-oxetan-3-yl]carbamate (23b)**. The reaction was carried out using **15b** (0.14 g, 0.43 mmol), dry CH_2Cl_2 (20 mL), Et_3N (0.18 mL, 1.30 mmol), and TBTU (0.17 g, 0.55 mmol) to give, after purification, pure **23b** (0.052 g, 40%) as a white solid: mp: 82.6–83.1 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 0.75 (d, J = 6.5 Hz, 3H), 0.96 (d, J = 6.5 Hz, 3H), 1.25–1.39 (m, 2H), 1.52–1.65 (m, 4H), 2.03 (dt, J = 6.5, 10.8 Hz, 1H), 2.57 (t, J = 7.7 Hz, 2H), 4.01 (t, J = 6.7 Hz, 2H), 4.24 (dd, J = 5.9, 10.8 Hz, 1H), 5.44 (dd, J = 5.9, 9.5 Hz, 1H), 7.13–7.34 (m, 5H), 8.27 ppm (d, J = 9.5 Hz, 1H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 17.1, 18.8, 25.4, 27.7, 28.8, 31.1, 35.5, 59.6, 65.1, 82.7, 126.1, 128.7 (4C), 142.5, 156.2, 170.3 ppm; MS (ESI+) m/z (%): 337 (100) [M+NH$_4$]$^+$; (ESI−) m/z (%): 318 (100) [M−H]$^-$; HRMS-ESI: m/z [M+NH$_4$]$^+$ calcd for $C_{18}H_{29}N_2O_4$: 337.2127, found: 337.2143.

5-Phenylpentyl-*N*-[(2*S,3*R**)-2-*tert*-butyl-4-oxo-oxetan-3-yl]carbamate (23c)**. The reaction was carried out using **15c** (0.346 g, 0.98 mmol), dry CH_2Cl_2 (44 mL), Et_3N (0.41 mL, 2.95 mmol), and TBTU (0.38 g, 1.20 mmol) to give, after purification, pure **23c** (0.125 g, 38%) as a colorless oil: ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 0.93 (s, 9H), 1.34 (m, 2H), 1.53–1.65 (m, 4H), 2.57 (t, J = 7.8 Hz, 2H), 4.02 (q, J = 6.2 Hz, 2H), 4.36 (d, J = 6.3 Hz, 1H), 5.56 (dd, J = 6.3, 8.5 Hz, 1H), 7.07–7.34 (m, 5H), 8.38 ppm (d, J = 8.5 Hz, 1H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 25.3 (3C), 25.4, 28.9, 31.1, 32.9, 35.5, 58.9, 65.1, 84.2, 126.1, 128.7 (4C), 142.5, 156.2, 169.8 ppm; MS (ESI+) m/z (%): 351 (100) [M+NH$_4$]$^+$; (ESI−) m/z (%): 332 (100) [M−H]$^-$; HRMS-ESI: m/z [M+H]$^+$ calcd for $C_{19}H_{28}NO_4$: 334.2018, found: 334.2001.

(4-Phenylphenyl)methyl-*N*-[(2*S,3*R**)-2-ethyl-4-oxo-oxetan-3-yl]-carbamate (25a)**. The reaction was carried out using **16a** (0.150 g, 0.44 mmol), dry CH_2Cl_2 (20 mL), Et_3N (0.183 mL, 1.31 mmol), and TBTU (0.168 g, 0.52 mmol) to give, after purification, pure **25a** (0.054 g, 38%) as a white solid: mp: 128.8–129.2 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 0.88 (t, J = 7.4 Hz, 3H), 1.56–1.88 (m, 2H), 4.61 (dt, J = 6.0, 8.1 Hz, 1H), 5.11 (d, J = 12.9 Hz, 1H), 5.15 (d, J = 12.9 Hz, 1H), 5.49 (dd, J = 6.0, 9.4 Hz, 1H), 7.29–7.57 (m, 5H), 7.60–7.78 (m, 4H), 8.41 ppm (d, J = 9.4 Hz, 1H); ^{13}C NMR (75 MHz, [D$_6$]DMSO): δ = 8.9, 21.9, 59.6, 65.9, 79.1, 126.7 (2C), 126.7 (2C), 127.5, 128.5 (2C), 128.9 (2C), 135.7, 139.7, 139.9, 155.6, 169.8 ppm; MS (ESI+) m/z (%): 343 (100) [M+NH$_4$]$^+$; (ESI−) m/z (%): 324 (100) [M−H]$^-$; HRMS-ESI: m/z [M+NH$_4$]$^+$ calcd for $C_{19}H_{23}N_2O_4$: 343.1658, found: 343.1660.

(4-Phenylphenyl)methyl-*N*-[(2*S,3*R**)-2-isopropyl-4-oxo-oxetan-3-yl]carbamate (25b)**. The reaction was carried out using **16b** (0.193 g, 0.54 mmol), dry CH_2Cl_2 (25.0 mL), Et_3N (0.225 mL, 1.65 mmol), and TBTU (0.21 g, 0.65 mmol) to give, after purification, pure **25b** (0.041 g, 22%) as a white solid: mp: 149.7–150.7 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 0.76 (d, J = 6.6 Hz, 3H), 0.95 (d, J = 6.5 Hz, 3H), 1.93–2.13 (m, 1H), 4.25 (dd, J = 5.9, 10.9 Hz, 1H), 5.14 (s, 2H), 5.48 (dd, J = 5.9, 9.5 Hz, 1H), 7.34–7.52 (m, 5H), 7.61–7.70 (m, 4H), 8.46 ppm (d, J = 9.5 Hz, 1H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 16.8, 18.5, 27.4, 59.3, 65.9, 82.4, 126.8 (2C), 126.8 (2C), 127.7, 128.6 (2C), 129.1 (2C), 135.8, 139.7, 140.0, 155.7, 169.8 ppm; MS (ESI+) m/z (%): 357 (100) [M+NH$_4$]$^+$; (ESI−) m/z (%): 338 (100) [M−H]$^-$; HRMS-ESI: m/z [M+NH$_4$]$^+$ calcd for $C_{20}H_{25}N_2O_5$: 357.1814, found: 357.1832.

(4-Phenylphenyl)methyl-*N*-[(2*S,3*R**)-2-*tert*-butyl-4-oxo-oxetan-3-yl]carbamate (25c)**. The reaction was carried using **16c** (0.18 g, 0.48 mmol), dry CH_2Cl_2 (22 mL), Et_3N (0.203 mL, 1.45 mmol), and TBTU (0.186 g, 0.6 mmol) to give, after purification, pure **25c** (0.017 g, 10%, over two steps) as a white solid: mp: 83.3–87.7 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 0.93 (s, 9H), 4.38 (d, J = 6.2 Hz, 1H), 5.15 (d, J = 2.8 Hz, 2H), 5.61 (dd, J = 6.2, 8.5 Hz, 1H), 7.23–7.81 (m, 9H), 8.58 ppm (d, J = 8.5 Hz, 1H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 24.9 (3C), 32.6, 58.7, 66.1, 83.9, 126.8 (4C), 127.7, 128.6 (2C), 129.1 (2C), 135.9, 139.9, 140.1, 155.8, 169.5 ppm; MS (ESI+) m/z (%): 371(100) [M+NH$_4$]$^+$; (ESI−) m/z (%): 352 (100) [M−H]$^-$; HRMS-ESI: m/z [M+NH$_4$]$^+$ calcd for $C_{21}H_{27}N_2O_4$: 371.1971, found: 371.1960.

5-Phenylpentyl-*N*-[(2*R,3*R**)-2-ethyl-4-oxo-oxetan-3-yl]carbamate (24a)**. The reaction was carried out using **17a** (0.155 g, 0.48 mmol) as a 8:2 *threo*/*erythro* diastereomeric mixture, dry CH_2Cl_2 (15 mL), Et_3N (0.200 mL, 1.44 mmol), and TBTU (0.185 g, 0.58 mmol) to give, after purification, **24a** (0.75 g, 57%) as a pure *trans* diastereomer, as a white solid: mp: 64.1–64.8 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 0.90 (t, J = 7.4 Hz, 3H), 1.26–1.38 (m, 2H), 1.58 (m, 4H), 1.67–1.90 (m, 2H), 2.57 (t, J = 7.6 Hz, 2H), 3.98 (t, J = 6.6 Hz, 2H), 4.51 (td, J = 4.3, 6.8 Hz, 1H), 4.69 (dd, J = 4.3, 8.1 Hz, 1H), 7.09–7.32 (m, 5H), 8.06 ppm (d, J = 8.1 Hz, 1H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 8.7, 24.9, 25.2, 28.3, 30.6, 35.0, 62.0, 64.6, 79.6, 125.6, 128.2 (2C), 128.2

(2C), 142.1, 155.5, 169.1 ppm; MS (ESI+) *m/z* (%): 323 (100) $[M+NH_4]^+$; (ESI−) *m/z*: 304 (100) $[M-H]^-$; HRMS-ESI: *m/z* $[M+NH_4]^+$ calcd for $C_{17}H_{27}N_2O_4$: 323.1970, found: 323.1971.

5-Phenylpentyl-*N*-[(2*R,3*R**)-2-isopropyl-4-oxo-oxetan-3-yl]carbamate (24b)**. The reaction was carried out using **17b** (0.076 g, 0.24 mmol) as a 8:2 *threo/erythro* diastereomeric mixture, dry CH_2Cl_2 (7.5 mL), Et_3N (0.100 mL, 0.72 mmol), and TBTU (0.091 g, 0.29 mmol) to give, after purification, **24b** (0.405 g, 56%) as a pure *trans* diastereomer, as a white solid: mp: 63.7–64.6 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 0.87 (d, *J* = 6.6 Hz, 3H), 0.95 (d, *J* = 6.6 Hz, 3H), 1.27–1.43 (m, 2H), 1.49–1.69 (m, 4H), 1.87–2.08 (m, 1H), 2.57 (t, *J* = 7.7 Hz, 2H), 3.99 (t, *J* = 6.6 Hz, 2H), 4.22 (dd, *J* = 4.4, 9.1 Hz, 1H), 4.73 (dd, *J* = 4.4, 8.2 Hz, 1H), 7.10–7.35 (m, 5H), 8.06 ppm (d, *J* = 8.2 Hz, 1H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 16.3, 17.9, 24.9, 28.3, 30.6, 30.7, 35.0, 60.9, 64.6, 83.2, 125.6, 128.2 (2C), 128.2 (2C), 142.1, 155.5, 168.9 ppm; MS (ESI+) *m/z* (%): 320 (100) $[M+NH_4]^+$; (ESI−) *m/z* (%): 318 (100) $[M-H]^-$; HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{18}H_{26}NO_4$: 320.1862, found: 320.1869.

5-Phenylpentyl-*N*-[(2*R,3*R**)-2-*tert*-butyl-4-oxo-oxetan-3-yl]carbamate (24c)**. The reaction was carried out using **17c** (0.325 g, 0.92 mmol) as a 9:1 *threo/erythro* diastereomeric mixture, dry CH_2Cl_2 (29 mL), Et_3N (0.387 mL, 2.77 mmol), and TBTU (0.356 g, 1.11 mmol) to give, after purification, **24c** (0.165 g, 54%) as a pure *trans* diastereomer, as a white solid: mp: 59.7–62.3 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 0.94 (s, 9H), 1.29–1.39 (m, 2H), 1.52–1.65 (m, 4H), 2.58 (t, *J* = 7.6 Hz, 2H), 4.01 (t, *J* = 6.6 Hz, 2H), 4.33 (d, *J* = 4.7 Hz, 1H), 4.76 (dd, *J* = 4.7, 8.2 Hz, 1H), 7.11–7.36 (m, 5H), 8.05 ppm (d, *J* = 8.2 Hz, 1H); ^{13}C NMR (75 MHz, [D$_6$]DMSO): δ = 24.37 (3C), 25.47, 28.78, 31.07, 32.34, 35.53, 58.52, 65.14, 85.55, 126.10, 128.68 (2C), 128.72 (2C), 142.56, 155.99, 169.18 ppm; MS (ESI+) *m/z* (%): 351 (100) $[M+NH_4]^+$; (ESI−) *m/z* (%): 332 (100) $[M-H]^-$; HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{19}H_{28}NO_4$: 334.2018, found: 334.2004.

(4-Phenylphenyl)methyl-*N*-[(2*R,3*R**)-2-ethyl-4-oxo-oxetan-3-yl]-carbamate (26a)**. The reaction was carried out using **18a** (0.193 g, 0.56 mmol) as a 8:2 *threo/erythro* diastereomeric mixture, dry CH_2Cl_2 (17.5 mL), Et_3N (0.235 mL, 1.69 mmol), and TBTU (0.217 g, 0.67 mmol) to give, after purification, **26a** (0.089 g, 49%) as a pure *trans* diastereomer, as a white solid: mp: 131.6–133.2 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 0.92 (t, *J* = 7.4 Hz, 3H), 1.68–1.94 (m, 2H), 4.49–4.60 (m, 1H), 4.76 (dd, *J* = 4.3, 8.1 Hz, 1H), 5.12 (s, 2H), 7.33–7.42 (m, 1H), 7.42–7.53 (m, 4H), 7.62–7.76 (m, 4H), 8.25 ppm (d, *J* = 8.1 Hz, 1H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 8.7, 25.2, 62.0, 65.8, 79.6, 126.6 (2C), 126.7 (2C), 127.5, 128.6 (2C), 128.9 (2C), 135.5, 139.6, 139.9, 155.3, 168.9 ppm; MS (ESI+) *m/z* (%): 343 (100) $[M+NH_4]^+$; (ESI−) *m/z* (%): 324 (100) $[M-H]^-$; HRMS-ESI: *m/z* $[M+NH_4]^+$ calcd for $C_{19}H_{23}N_2O_4$: 342.1658, found: 343.1662.

(4-Phenylphenyl)methyl-*N*-[(2*R,3*R**)-2-isopropyl-4-oxo-oxetan-3-yl]carbamate (26b)**. The reaction was carried out using **18b** (0.059 g, 0.17 mmol) as a 8:2 *threo/erythro* diastereomeric mixture, dry CH_2Cl_2 (5.3 mL), Et_3N (0.070 mL, 0.51 mmol), and TBTU (0.065 g, 0.20 mmol) to give, after purification, **26b** (0.010 g, 17%) as a pure *trans* diastereomer, as a white solid: mp: 127.5–129.5 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 0.89 (d, *J* = 6.7 Hz, 3H), 0.97 (d, *J* = 6.7 Hz, 3H), 1.81–2.12 (m, 1H), 4.26 (dd, *J* = 4.4, 9.1 Hz, 1H), 4.80 (dd, *J* = 4.4, 8.2 Hz, 1H), 5.12 (s, 2H), 7.29–7.54 (m, 5H), 7.63–7.77 (m, 4H), 8.26 ppm (d, *J* = 8.2 Hz, 1H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 16.3, 17.9, 30.6, 61.0, 65.9, 83.2, 126.7 (2C), 126.7 (2C), 127.5, 128.6 (2C), 128.9 (2C), 135.5, 139.7, 139.9, 155.3, 168.7 ppm; MS (ESI+) *m/z* (%): 357 (100) $[M+NH_4]^+$; (ESI) *m/z* (%): 338 (100) $[M-H]^-$; HRMS-ESI: *m/z* $[M+NH_4]^+$ calcd for $C_{20}H_{25}N_2O_4$: 357.1814, found: 357.1825.

(4-Phenylphenyl)methyl-*N*-[(2*R,3*R**)-2-*tert*-butyl-4-oxo-oxetan-3-yl]carbamate (26c)**. The reaction was carried out using **18c** (0.203 g, 0.55 mmol) as a 9:1 *threo/erythro* diastereomeric mixture, dry CH_2Cl_2 (17 mL), Et_3N (0.230 mL, 1.65 mmol), and TBTU (0.210 g, 0.66 mmol) to give, after purification, **26c** (0.093 g, 48%) as a pure *trans* diastereomer, as a white solid: mp: 147.1–147.8 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 0.95 (s, 9H), 4.37 (d, *J* = 4.6 Hz, 1H), 4.83 (dd, *J* = 4.6, 8.2 Hz, 1H), 5.14 (s, 2H), 7.32–7.56 (m, 5H), 7.60–7.75 (m, 4H), 8.25 ppm (d, *J* = 8.2 Hz, 1H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 24.4 (3C), 32.4, 58.6, 66.4, 85.6, 127.1 (2C), 127.2 (2C), 128.0, 129.1 (2C), 129.4 (2C), 136.0, 140.6, 140.4, 155.8, 169.1 ppm; (ESI+) *m/z* (%): 371 (100) $[M+NH_4]^+$; (ESI−) *m/z* (%): 352 (100) $[M-H]^-$; HRMS-ESI: *m/z* $[M+NH_4]^+$ calcd for $C_{21}H_{27}N_2O_4$: 371.1971, found: 371.1971.

General procedure for the synthesis of *cis*- and *trans*-α-amino β-lactone amide derivatives (27a–c–30a–c; Scheme 1).

Preparation of β-lactone amides 27a–c–28a–c. In a pear flask, under nitrogen atmosphere, at room temperature, to a stirred mixture of 7-phenylheptanoic acid (1.1 equiv) in dry CH_2Cl_2 was added TBTU (1.1 equiv). The reaction mixture was left stirring at room temperature for 15 min. Subsequently was added a solution of **21a–c–22a–c** (1.0 equiv) and Et_3N (2.2 equiv) in dry CH_2Cl_2. The reaction mixture was left stirring at room temperature for 16 h. The solvent was rotary evaporated and the crude product was purified by column chromatography, using a Teledyne ISCO apparatus, eluting with cyclohexane/EtOAc (from 100:0 to 70:30) to afford compounds **27a–c–28a–c**.

***N*-[(2*S**,3*R**)-2-Ethyl-4-oxo-oxetan-3-yl]-7-phenylheptanamide (27a)**. The reaction was carried out using **21a** (0.095 g, 0.33 mmol), Et_3N (0.097 mL, 0.73 mmol) in dry CH_2Cl_2 (4.5 mL), and 7-phenylheptanoic acid (0.075 mL, 0.37 mmol), TBTU (0.117 g, 0.37 mmol) in dry CH_2Cl_2 (4.5 mL) to give, after purification, pure **27a** (0.045 g, 44%) as a white solid: mp: 70.1–72.6 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 0.87 (t, *J* = 7.4 Hz, 3H), 1.29 (p, *J* = 3.6 Hz, 4H), 1.44–1.68 (m, 5H), 1.67–1.82 (m, 1H), 2.16 (t, *J* = 7.3 Hz, 2H), 2.54–2.63 (m, 2H), 4.59 (dt, *J* = 6.0, 7.9 Hz, 1H), 5.59 (dd, *J* = 6.0, 8.8 Hz, 1H), 7.12–7.23 (m, 3H), 7.24–7.33 (m, 2H), 8.77 ppm (d, *J* = 8.8 Hz, 1H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 9.4, 22.6, 25.4, 28.8 (2C), 31.3, 35.3, 35.5, 58.1, 79.3, 126.1, 128.6 (4C), 142.7, 170.6, 172.9 ppm; MS (ESI+) *m/z* (%): 304 (100) $[M+H]^+$; (ESI−) *m/z* (%): 302 (100) $[M-H]^-$; HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{18}H_{26}NO_3$: 304.1913, found: 304.1930.

***N*-[(2*S**,3*R**)-2-Isopropyl-4-oxo-oxetan-3-yl]-7-phenylheptanamide (27b)**. The reaction was carried out using **21b** (0.100 g, 0.33 mmol), Et_3N (0.097 mL, 0.73 mmol) in dry CH_2Cl_2 (4.5 mL), and 7-phenylheptanoic acid (0.075 mL, 0.37 mmol), TBTU (0.117 g, 0.37 mmol) in dry CH_2Cl_2 (4.5 mL) to give, after purification, pure **27b** (0.032 g, 30%) as a white solid: mp: 91.8–92.8 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 0.72 (d, *J* = 6.6 Hz, 3H), 0.95 (d, *J* = 6.6 Hz, 3H), 1.18–1.38 (m, 4H), 1.40–1.64 (m, 4H), 1.98 (dp, *J* = 6.6, 10.7 Hz, 1H), 2.15 (t, *J* = 7.4 Hz, 2H), 2.53–2.61 (m, 2H), 4.23 (dd, *J* = 5.9, 10.7 Hz, 1H), 5.58 (dd, *J* = 5.9, 9.0 Hz, 1H), 7.08–7.34 (m, 5H), 8.81 ppm (d, *J* = 9.0 Hz, 1H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 17.2, 18.7, 25.4, 27.9, 28.7, 28.8, 31.3, 35.4, 35.5, 57.6, 82.5, 126.1, 128.6 (4C), 142.7, 170.6, 172.8 ppm; MS (ESI+) *m/z* (%): 318 (100) $[M+H]^+$; (ESI) *m/z*: 316 (100) $[M-H]^-$; HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{19}H_{28}NO_3$: 318.2069, found: 318.2083.

***N*-[(2*S**,3*R**)-2-*tert*-Butyl-4-oxo-oxetan-3-yl]-7-phenylheptanamide (27 c)**. The reaction was carried out using **21 c** (0.100 g, 0.32 mmol), Et_3N (0.093 mL, 0.70 mmol) in dry CH_2Cl_2 (4.5 mL), and 7-phenylheptanoic acid (74 mL, 0.36 mmol), TBTU (0.116 g, 0.36 mmol) in dry CH_2Cl_2 (4.5 mL) to give, after purification, pure **27 c** (0.040 g, 33%) as a colorless oil: ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 0.91 (s, 9H), 1.21–1.33 (m, 4H), 1.42–1.59 (m, 4H), 2.09–2.28 (m, 2H), 2.55 (t, J = 7.7 Hz, 2H), 4.37 (d, J = 6.2 Hz, 1H), 5.73 (dd, J = 6.2, 8.1 Hz, 1H), 7.08–7.54 (m, 5H), 8.90 ppm (d, J = 8.1 Hz, 1H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 25.2 (3C), 25.4, 28.7, 28.9, 31.4, 32.9, 35.1, 35.5, 56.8, 84.2, 126.1, 128.6 (4C), 142.7, 170.6, 172.9 ppm; MS (ESI+) *m/z* (%): 332 (100) $[M+H]^+$; (ESI−) *m/z*: 330 (100) $[M-H]^-$; HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{20}H_{30}NO_3$: 332.2226, found: 332.2218.

***N*-[(2*R**,3*R**)-2-Ethyl-4-oxo-oxetan-3-yl]-7-phenylheptanamide (28 a)**. The reaction was carried out using **22 a** (0.110 g, 0.38 mmol) as a 8:2 *trans*/*cis* diastereomeric mixture, Et_3N (0.117 mL, 0.84 mmol) in dry CH_2Cl_2 (5.3 mL), and 7-phenylheptanoic acid (86 mL, 0.42 mmol), TBTU (0.135 g, 0.42 mmol) in dry CH_2Cl_2 (5.3 mL) to give, after purification, **28 a** (0.040 g, 34%) as a pure *trans* diastereomer, as a white solid: ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 0.91 (t, J = 7.4 Hz, 3H), 1.21–1.39 (m, 4H), 1.46–1.64 (m, 4H), 1.79 (m, 2H), 2.14 (t, J = 7.4 Hz, 2H), 2.57 (m, 2H), 4.53 (m, 1H), 4.75 (dd, J = 4.3, 7.7 Hz, 1H), 7.14–7.22 (m, 3H), 7.28 (m, 2H), 8.65 ppm (d, J = 7.7 Hz, 1H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 9.2, 25.3, 25.8, 28.7, 28.8, 31.4, 35.2, 35.5, 61.3, 79.6, 126.1, 128.7 (2C), 128.7 (2C), 142.7, 169.5, 173.2 ppm; MS (ESI+) *m/z* (%): 304 (100) $[M+H]^+$; (ESI−) *m/z* (%): 302 (100) $[M-H]^-$.

***N*-[(2*R**,3*R**)-2-Isopropyl-4-oxo-oxetan-3-yl]-7-phenylheptanamide (28 b)**. The reaction was carried out using **22 b** (0.100 g, 0.41 mmol) as a 8:2 *trans*/*cis* diastereomeric mixture, Et_3N (0.125 mL, 0.90 mmol) in dry CH_2Cl_2 (5.6 mL), and 7-phenylheptanoic acid (0.092 g, 0.45 mmol), TBTU (0.144 g, 0.45 mmol) in dry CH_2Cl_2 (5.6 mL) to give, after purification, **28 b** (0.06 g, 46%) as a pure *trans* diastereomer, as a white solid: ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 0.87 (d, J = 6.6 Hz, 3H), 0.96 (d, J = 6.6 Hz, 3H), 1.28 (m, 4H), 1.40–1.65 (m, 4H), 1.89–2.04 (m, 1H), 2.14 (t, J = 7.3 Hz, 2H), 2.53–2.61 (m, 2H), 4.24 (dd, J = 4.3, 9.1 Hz, 1H), 4.80 (dd, J = 4.3, 7.8 Hz, 1H), 7.11–7.34 (m, 5H), 8.66 ppm (d, J = 7.8 Hz, 1H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 16.3, 17.9, 24.8, 28.2, 28.3, 30.7, 30.8, 34.7, 35.0, 59.7, 82.6, 125.6, 128.2 (4C), 142.2, 168.8, 172.7 ppm; MS (ESI+) *m/z*: 316 $[M+H]^+$; (ESI−) *m/z*: 318 $[M-H]^-$.

***N*-[(2*R**,3*R**)-2-*tert*-Butyl-4-oxo-oxetan-3-yl]-7-phenylheptanamide (28 c)**. The reaction was carried out using **22 c** (0.075 g, 0.23 mmol) as a 9:1 *trans*/*cis* diastereomeric mixture, Et_3N (0.068 mL, 0.52 mmol) in dry CH_2Cl_2 (3.2 mL), and 7-phenylheptanoic acid (0.052 g, 0.26 mmol), TBTU (0.084 g, 0.26 mmol) in dry CH_2Cl_2 (3.2 mL) to give, after purification, **28 c** (0.035 g, 46%) as a pure *trans* diastereomer, as a white solid: mp: 89.0–91.2 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 0.92 (s, 9H), 1.24–1.32 (m, 4H), 1.45–1.60 (m, 4H), 2.13 (t, J = 7.3 Hz, 2H), 2.55 (t, J = 6.8, 2H), 4.32 (d, J = 4.6 Hz, 1H), 4.84 (dd, J = 4.6, 7.8 Hz, 1H), 7.13–7.19 (m, 3H), 7.24–7.29 (m, 2H), 8.62 ppm (d, J = 7.8 Hz, 1H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 23.9 (3C), 24.9, 28.2, 28.3, 30.8, 31.8, 34.7, 35.1, 56.7, 84.6, 125.6, 128.2 (2C), 128.2 (2C), 142.3, 168.7, 172.8 ppm; MS (ESI+) *m/z* (%): 332 (100) $[M+H]^+$; (ESI−) *m/z* (%): 330 (100) $[M-H]^-$; HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{21}H_{24}NO_4$: 355.1783, found: 355.1772.

Preparation of β-lactone amides 29 a–c–30 a–c. In a round-bottomed flask, at 0 °C, under nitrogen atmosphere, to a stirred mixture of **21 a–c–22 a–c** (1.0 equiv) in dry CH_2Cl_2 was added dropwise Et_3N (2.2 equiv) and the reaction stirred for 15 min. Subsequently, at 0 °C, under nitrogen atmosphere was slowly added a solution of 4-benzyloxybenzoyl chloride (1.1 equiv) in dry CH_2Cl_2, and the reaction stirred at 0 °C for 2 h. Upon full conversion of starting material, the crude mixture was concentrated under reduced pressure, and the crude residue purified by column chromatography, using a Teledyne ISCO apparatus, eluting with cyclohexane/EtOAc (from 100:0 to 70:30) to afford compounds **29 a–c–30 a–c**.

***N*-[(2*S**,3*R**)-2-Ethyl-4-oxo-oxetan-3-yl]-4-benzyloxybenzamide (29 a)**. The reaction was carried out using **21 a** (0.087 g, 0.30 mmol) in dry CH_2Cl_2 (4.2 mL), Et_3N (0.088 mL, 0.67 mmol), and 4-benzyloxybenzoyl chloride (0.082, 0.33 mmol) in dry CH_2Cl_2 (4.2 mL), to give, after purification, **29 a** (0.012 g, 12%) as a white solid: mp: 141.0–144.2 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 0.88 (t, J = 7.4 Hz, 3H), 1.64–1.77 (m, 1H), 1.79–1.93 (m, 1H), 4.68 (dt, J = 5.8, 8.0 Hz, 1H), 5.20 (s, 2H), 5.78 (dd, J = 5.8, 8.5 Hz, 1H), 7.13 (d, J = 8.8 Hz, 2H), 7.32–7.38 (m, 1H), 7.39–7.45 (m, 2H), 7.44–7.51 (m, 2H), 7.88 (d, J = 8.8 Hz, 2H), 9.21 ppm (d, J = 8.5 Hz, 1H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 9.4, 22.5, 58.6, 69.9, 79.6, 115.0, 115.1, 125.7, 128.2 (2C), 128.4, 128.9 (2C), 129.9, 131.8, 137.1, 161.7, 166.3, 170.4 ppm; MS (ESI+) *m/z* (%): 226 (100) $[M+H]^+$; (ESI−) *m/z* (%): 224 $[M-H]^-$; HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{19}H_{20}NO_4$: 326.1392, found: 326.1400.

***N*-[(2*S**,3*R**)-2-Isopropyl-4-oxo-oxetan-3-yl]-4-benzyloxybenzamide (29 b)**. The reaction was carried out using **21 b** (0.100 g, 0.33 mmol) in dry CH_2Cl_2 (4.5 mL), Et_3N (0.097 mL, 0.73 mmol), and 4-benzyloxybenzoyl chloride (0.090 g, 0.37 mmol) in dry CH_2Cl_2 (4.5 mL), to give, after purification, pure **29 b** (0.016 g, 13%) as a white solid: mp: 148.0–149.0 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 0.71 (d, J = 6.6 Hz, 3H), 0.98 (d, J = 6.6 Hz, 3H), 2.07–2.26 (m, 1H), 4.31 (dd, J = 5.9, 10.8 Hz, 1H), 5.19 (s, 2H), 5.78 (dd, J = 5.9, 8.7 Hz, 1H), 7.12 (d, J = 8.5 Hz, 2H), 7.29–7.59 (m, 5H), 7.87 ppm (d, J = 8.5 Hz, 2H), 9.26 (d, J = 8.7 Hz, 1H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 16.8, 18.5, 27.6, 57.8, 69.6, 82.5, 114.8 (2C), 125.4, 127.9 (2C), 128.2, 128.6 (2C), 129.6 (2C), 136.8, 161.4, 165.9, 170.2 ppm; MS (ESI+) *m/z* (%): 340 (100) $[M+H]^+$; (ESI−) *m/z* (%): 338 (100) $[M-H]^-$; HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{20}H_{22}NO_4$: 340.1549, found: 340.1552.

***N*-[(2*S**,3*R**)-2-*tert*-Butyl-4-oxo-oxetan-3-yl]-4-benzyloxybenzamide (29 c)**. The reaction was carried out using **21 c** (0.100 g, 0.32 mmol) in dry CH_2Cl_2 (4.5 mL), Et_3N (0.93 mL, 0.70 mmol), and 4-benzyloxybenzoyl chloride (0.086 g, 0.35 mmol) in dry CH_2Cl_2 (4.5 mL) to give, after purification, pure **29 c** (0.019 g, 17%) as a white solid: mp: 146.7–148.9 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 0.95 (s, 9H), 4.46 (d, J = 6.2 Hz, 1H), 5.19 (s, 2H), 5.95 (dd, J = 6.2, 7.6 Hz, 1H), 7.00–7.16 (m, 2H), 7.29–7.52 (m, 5H), 7.84–8.01 (m, 2H), 9.34 ppm (d, J = 7.6 Hz, 1H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 25.6 (3C), 33.1, 57.8, 70.0, 84.5, 115.1 (2C), 125.8, 128.4 (2C), 128.6, 129,1 (2C), 130.2 (2C), 137.2, 161.8, 166.4, 170.0 ppm; MS (ESI+) *m/z* (%): 354 (100) $[M+H]^+$; (ESI−) *m/z* (%): 352 (100) $[M-H]^-$; HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{21}H_{24}NO_4$: 354.1705, found: 354.1718.

***N*-[(2*R**,3*R**)-2-Ethyl-4-oxo-oxetan-3-yl]-4-benzyloxybenzamide (30 a)**. The reaction was carried out using **22 a** (0.150 g, 0.52 mmol) as a 8:2 *trans*/*cis* diastereomeric mixture, dry CH_2Cl_2 (7.3 mL), Et_3N (0.159 mL, 1.14 mmol), and 4-benzyloxybenzoyl chloride (0.141 g, 0.57 mmol) in dry CH_2Cl_2 (7.3 mL) to give, after purification, **30 a** (0.010 g, 8.5%) as a pure *trans* diastereomer, as a white solid: ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 0.95 (t, J = 7.4 Hz, 3H), 1.75–1.98 (m, 2H), 4.69 (td, J = 4.3, 6.9 Hz, 1H), 4.92 (dd, J = 4.3, 7.6 Hz, 1H), 5.20 (s, 2H), 7.05–7.17 (m, 2H), 7.34–7.48 (m, 5H), 7.90 (m, 2H),

9.18 ppm (d, $J=7.6$ Hz, 1H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta=9.3$, 25.9, 61.8, 69.9, 79.6, 115.1 (2C), 125.5, 128.3 (2C), 128.4, 128.9 (2C), 129.8, 131.8, 137.0, 161.7, 167.4, 169.4 ppm; MS (ESI+) *m/z* (%): 226 (100) $[M-H]^+$; (ESI−) *m/z* (%): 224 (100) $[M-H]^-$.

***N*-[(2*R**,3*R**)-2-Isopropyl-4-oxo-oxetan-3-yl]-4-benzyloxybenzamide (30b)**. The reaction was carried out using **22b** (0.100 g, 0.33 mmol) as a 8:2 *trans*/*cis* diastereomeric mixture, dry CH_2Cl_2 (4.5 mL), Et_3N (0.097 mL, 0.73 mmol) and 4-benzyloxybenzoyl chloride (0.090 g, 0.37 mmol) in dry CH_2Cl_2 (5.0 mL) to give, after purification, **30b** (0.021 g, 19%) as a pure *trans* diastereomer, as a white solid: mp: 119.0–121.0 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta=0.92$ (d, $J=6.7$ Hz, 3H), 0.99 (d, $J=6.7$ Hz, 3H), 1.95–2.09 (m, 1H), 4.39 (dd, $J=4.4$, 9.1 Hz, 1H), 4.95 (dd, $J=4.4$, 7.7 Hz, 1H), 5.19 (s, 2H), 7.09–7.17 (m, 2H), 7.31–7.51 (m, 5H), 7.81–7.90 (m, 2H), 9.19 ppm (d, $J=7.7$ Hz, 1H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta=16.4$, 17.9, 30.8, 60.2, 69.4, 82.7, 114.6 (2C), 124.9, 127.7 (2C), 127.9, 128.4 (2C), 129.3 (2C), 136.5, 161.2, 165.7, 168.8 ppm; MS (ESI+) *m/z* (%): 340 (100) $[M+H]^+$; (ESI−) *m/z* (%): 338 (100) $[M-H]^-$; HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{20}H_{22}NO_4$: 340.1549, found: 340.1551.

***N*-[(2*R**,3*R**)-2-*tert*-Butyl-4-oxo-oxetan-3-yl]-4-benzyloxybenzamide (30c)**. The reaction was carried out using **22c** (0.050 g, 0.16 mmol) as a 9:1 *trans*/*cis* diastereomeric mixture, dry CH_2Cl_2 (2.5 mL), Et_3N (0.049 mL, 0.35 mmol) and 4-benzyloxybenzoyl chloride (0.042 g, 0.17 mmol) in dry CH_2Cl_2 (2.5 mL), to give, after purification, **30c** (0.03 g, 56%) as a pure *trans* diastereomer, as a white solid: mp: 145.2–146.7 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta=0.97$ (s, 9H), 4.48 (d, $J=4.6$ Hz, 1H), 4.99 (dd, $J=4.6$, 7.7 Hz, 1H), 5.19 (s, 2H), 7.13 (d, $J=8.8$ Hz, 2H), 7.29–7.53 (m, 5H), 7.84 (d, $J=8.8$ Hz, 2H), 9.17 ppm (d, $J=7.7$ Hz, 1H); ^{13}C NMR (75 MHz, [D$_6$]DMSO): $\delta=$ 23.93 (3C), 31.92, 57.11, 69.42, 84.57, 99.50, 114.63 (2C), 127.77 (2C), 127.96, 128.45 (2C), 129.28 (2C), 136.54, 161.26, 165.68, 168.67 ppm; MS (ESI) *m/z* (%): 354 (100) $[M+Na]^+$; HRMS-ESI: *m/z* $[M+H]^+$ calcd for $C_{20}H_{30}NO_3$: 333.2304, found: 333.2312.

Pharmacology

Fluorogenic hNAAA assay: HEK293 cells stably transfected with the human NAAA coding sequence cloned from a human spleen cDNA library were used as enzyme source. Recombinant HEK-hNAAA pellets were resuspended in homogenizing buffer, and sonicated. Samples were centrifuged at 800 *g* for 15 min at 4 °C, and the resultant supernatants were then ultracentrifuged at 12000 *g* for 30 min at 4 °C. The pellets were resuspended in PBS pH 7.4 on ice and subjected to a freeze/thaw cycle at −80 °C. The suspension was finally centrifuged at 105000 *g* for 1 h at 4 °C. Protein concentration was measured, and aliquots of samples were stored at −80 °C until use. The assay was run in Optiplate 96-well black plates, in a total reaction volume of 200 μL. NAAA protein preparation (4.0 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 3.0 mM DTT, 0,1% Triton X-100, 0.05% BSA, 150 mM NaCl. *N*-(4-methyl-2-oxochromen-7-yl)hexadecanamide (PAMCA)[14c] was used as substrate (5.0 μM), and the reaction was held for 30 min at 37 °C. The samples were then read in a PerkinElmer Envision plate reader using an excitation wavelength of 360 nm and emission at 460 nm. IC_{50} values were calculated by nonlinear regression analysis of log[concentration]/inhibition curves using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA) applying a standard slope curve fit.

In vitro chemical stability: Chemical stability of selected compounds was evaluated under physiological (0.01 M PBS, pH 7.4) and acidic (0.01 M phosphate buffer, pH 5.0) conditions for up to 24 h. Both buffers were added with 10% CH_3CN. Stock solutions of each compound (10 mM) were prepared freshly in DMSO. Each compound was incubated at a final concentration of 1.0 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 (no shaking). A reference solution of each compound (final concentration: 1.0 μM at 1% DMSO) in preheated CH_3CN (37 °C) was prepared from the stock solutions (10 μM in DMSO) and maintained at 37 °C in the UPLC–MS autosampler during the study (no shaking). The analyses were performed on a Waters Acquity UPLC–MS TQD system consisting of a triple quadrupole detector (TQD) mass spectrometer equipped with an ESI interface and a PDA detector. The 24 h time-course analysis for **29b**, **30b**, **25b**, and **26b** and their corresponding rearrangement/hydrolysis products (Supporting Information, figures S1–S4) was 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 ($R^2>$ 0.999 for all compounds). The concentration of the rearranged and hydrolyzed products of each parent was then calculated on the corresponding calibration curve, assuming no changes in the molar absorptivity (ε) values. To test this assumption, each parent was fully hydrolyzed with 1.0 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 performed on an Acquity UPLC BEH C_{18} 1.7 μm 1.0×100 mm column, kept 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.: 10% B, 0.5–4.0 min.: 10→100% B, 4.0–5.0 min.: 100% B, 5.0–5.1 min.: 100→10% B, 5.1–6.5 min: 10% B at a flow rate of 0.1 mL min^{-1}.

In vitro rat plasma stability:[19c] Compounds were diluted in rat plasma added with 20% PBS (pH 7.4) and 5% DMSO to aid in solubilization. Plasma was preheated at 37 °C (30 min). The final compound concentration was 3.0 μM. At time points (immediately after dilution, 1, 5, 15 and 30 min) a 40 μL aliquot of the incubation solution was diluted in 150 μL cold CH_3CN spiked with Warfarin (200 nM) as internal standard. After vortexing for 30 s, the solution was centrifuged for 15 min at 3500 *g*, and the supernatant transferred for LC–MS analysis. A reference 30 min incubation in PBS (pH 7.4) added with 5% DMSO was also prepared for each compound. Samples were analyzed on a UPLC-Xevo triple quadrupole system (Waters Inc., Milford, USA).

Computational methods

The quantum chemical calculations were performed with the Gaussian 09 (G09) program suite.[41] For all stationary points, both geometry and analytical frequency calculations were carried out at the DFT level of theory by using the B3LYP functional. The employed basis set was Pople's 6-31++G(d,p).[42] All the reported calculations were performed in polarizable conductor calculation model (CPCM),[43] and the chosen solvent was water. To mimic hydrolysis of the α-amino β-lactone ring, we used a water molecule as nucleophile, assisted by a second one. We were able to identify the transition states (TSs) for all the four compounds and for both the two different attacks, and to decipher the nature of the reaction mechanism. In total, eight reactions were studied. The structures of the identified TSs were further investigated: the diagonalized mass-weighted Hessian matrix showed only one negative eigenvalue, revealing a first-order saddle point. To confirm the reac-

tion path, we performed intrinsic reaction coordinate (IRC)[44] calculations in order to follow the reaction starting from the TS reaching the reactants and the products. Finally the end points of the IRC pathway were optimized, and the nature of these points was investigated, confirming that they are energy minima in the potential energy surface.

Acknowledgements

The authors thank Luisa Mengatto (IIT) for hNAAA screenings, Dr. Marco Migliore (IIT) and Luca Goldoni (IIT) for NMR technical support, Silvia Venzano (IIT) for handling compounds, and Dr. Alessandro Fioni, Dr. Federica Vacondio, Prof. Marco Mor (University of Parma) for providing the experimental protocols for chemical and plasma stability studies.

Keywords: inhibitors · *N*-acylethanolamine acid amidase · stability · structure–activity relationships · β-lactones

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[38] For threonine-derived α-amino-β-lactones the reaction has been reported to proceed with inversion of configuration at Cβ and retention at Cα.

[39] We speculate that the behavior observed for the stereomeric β-isopropyl-β-lactones as carbamates and amides could be extended to the other β-alkyl-substituted compounds.
[40] To rule out that variations in chemical stability could be attributed to differing solubility, four representative compounds (**23c**–**26c**) were selected based on clog*D* values and tested at 200 nM at pH 7.4. No significant difference was observed between (pseudo)half-life values at 1.0 μM and 200 nM (see Supporting Information).
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Received: October 18, 2013
Published online on January 8, 2014