European Journal of Medicinal Chemistry 126 (2017) 561–575

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Research paper

Progress in the development of β-lactams as *N*-Acylethanolamine Acid Amidase (NAAA) inhibitors: Synthesis and *SAR* study of new, potent *N*-*O*-substituted derivatives

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ARTICLE INFO

Article history:
Received 12 October 2016
Received in revised form
18 November 2016
Accepted 19 November 2016
Available online 23 November 2016

Keywords:
N-Acylethanolamine acid amidase
Cysteine hydrolase
N-O-Substituted β-lactams
Inhibitors
Structure–activity relationships (SAR)

ABSTRACT

The anti-inflammatory effects resulting from raising the levels of palmitoylethanolamide (PEA), an endogenous bioactive lipid, led to envisage *N*-Acylethanolamine Acid Amidase (NAAA), the cysteine hydrolase mainly responsible for PEA degradation, as an attractive target for small molecule inhibitors. Previous work in our group identified serine-derived β-lactams as potent and systemically active inhibitors of NAAA activity. Aiming to expand the SAR study around this class of compounds, we investigated the effect of the substitution on the endocyclic nitrogen by designing and synthesizing a series of *N*-substituted β-lactams. The present work describes the synthesis of new *N*-*O*-alkyl and *N*-*O*-aryl substituted β-lactams and reports the results of the structure activity relationship (*SAR*) study leading to the discovery of a novel, single-digit nanomolar NAAA inhibitor (**37**). Compound **37** was shown *in vitro* to inhibit human NAAA via *S*-acylation of the catalytic cysteine, and to display very good selectivity vs. human Acid Ceramidase, a cysteine amidase structurally related to NAAA. Preliminary *in vivo* studies showed that compound **37**, administered topically, reduced paw edema and heat hyperalgesia in a carrageenan-induced inflammation mouse model. The high *in vitro* potency of **37** as NAAA inhibitor, and its encouraging *in vivo* activity qualify this compound as a new tool for the study of the role of NAAA in inflammatory and pain states.

1. Introduction

Palmitoylethanolamide (PEA), a member of the Fatty Acid Ethanolamides (FAEs) family, is an endogenous bioactive lipid able to inhibit peripheral inflammation and mast cell degranulation by activating the peroxisome proliferator-activated receptor-α (PPAR-α) [1,2] as well as to exert antinociceptive effects in rat and mouse models of acute and chronic pain [3–5]. The anti-inflammatory effects resulting from raising local levels of PEA led to envisage *N*-Acylethanolamine Acid Amidase (NAAA), the lysosomal cysteine hydrolase mainly involved in PEA degradation [6], as an attractive target for small molecule inhibitors for the treatment of inflammatory and pain conditions [7]. *N*-Acylethanolamine Acid Amidase (NAAA) is activated by autoproteolysis at acidic pH generating a catalytically competent subunit of the enzyme bearing a cysteine (Cys131 in mice, Cys126 in humans) as the nucleophile residue responsible for FAEs hydrolysis [8–10].

Compounds bearing electrophilic chemical functions, such as an α-amino β-lactone moiety, were described in literature to covalently react with NAAA catalytic cysteine [11–15]. Among them, the threonine-derived β-lactone **1** (**ARN077**, Fig. 1), was reported to efficiently inhibit *rat* and *human* NAAA *in vitro* activity (*r*-NAAA IC_{50} = 0.050 μM; *h*-NAAA IC_{50} = 0.007 μM) [16], and to display *in vivo* activity by topical administration in rodent models of

Abbreviations: NAAA, *N*-acylethanolamine acid amidase; FAEs, fatty acid ethanolamides; OEA, oleoylethanolamide; PEA, palmitoylethanolamide; FAAH, fatty acid amide hydrolase; AC, acid ceramidase; PPAR–α, peroxisome proliferator-activated receptor–α; PAMCA, *N*-(4-methyl-2-oxo-chromen-7-yl)-hexadecanamide; EDC HCl, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; 2-DPC, di-2-pyridyl carbonate; HOBt, 1-hydroxybenzotriazole; DEAD, diethyl azadicarboxylate; TCEP, tris(2-carboxyethyl)phosphine; TBTA, tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine; THF, tetrahydrofurane; DCM, dichloromethane; DIPEA, diisopropylethylamine; PPh3, triphenylphosphine.

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http://dx.doi.org/10.1016/j.ejmech.2016.11.039
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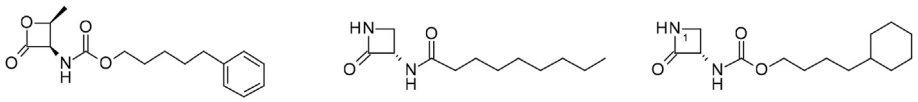


1 (ARN077) 2 3 (ARN0726)

Fig. 1. Potent β-lactone (**1**) and β-lactam amide and carbamate (**2**, **3**) NAAA inhibitors reported in literature.

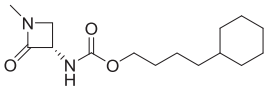
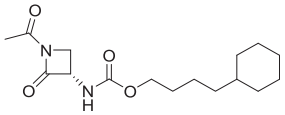
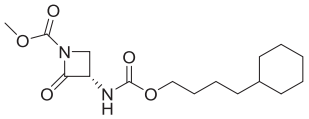
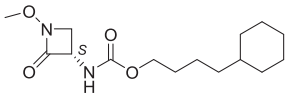
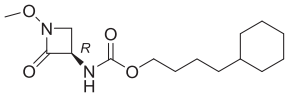
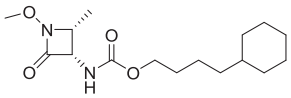
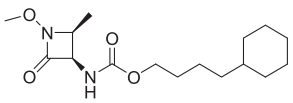
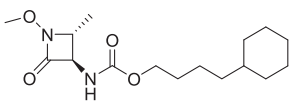
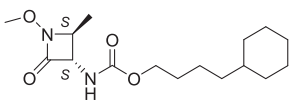
hyperalgesia and allodynia caused by inflammation or nerve damage [17]. The administration of **ARN077** attenuated, in a dose-dependent manner, hyperalgesia and mechanical allodynia induced in mice by carrageenan injection or sciatic nerve ligation, reversed the allodynia induced by ultraviolet B (UVB) radiation in rats, and normalized FAE levels in inflamed mouse skin [16].

Although potent towards our target enzyme, β-lactone inhibitors showed a limited plasma stability ($t_{½}$ < 10 s), thus preventing their potential use as systemic drugs [16,18,19]. Aiming to further explore the therapeutic utility of NAAA inhibitors, the replacement of the β-lactone core with a structurally similar β-lactam moiety was envisioned, leading to a first series of serine-derived β-lactam amides [20]. Among the different amide analogues, the *N*-[(*S*)-2-oxoazetidin-3-yl]nonanamide (**2,** Fig. 1) showed good inhibitory potency *in vitro* against *human* NAAA (*h*-NAAA IC_{50} = 0.34 μM) and an acceptable *mouse* plasma stability ($t_{½}$ = 130 min). After intravenous and oral administration in rat, compound **2** exhibited a good oral bioavailability (*F* = 67%), supporting the use of such class of compounds for systemic administration [20]. Additionally, as demonstrated by a recently published *SAR* study, the functionalization of the exocyclic amino group of the β-lactam scaffold as a carbamate afforded compounds with potent NAAA inhibitory activity [21]. Notably, compound **3** (**ARN0726**, Fig. 1) showed a good inhibitory activity *in vitro*, on both *rat* and *human* NAAA (*r*-NAAA IC_{50} = 0.028 μM, *h*-NAAA IC_{50} = 0.073 μM) and was recently reported to exert anti-inflammatory effects in mouse models of inflammation and suppress LPS-stimulated inflammatory reactions in human macrophages [22].

Up to the present, no crystallographic structure of the target protein (NAAA) is available. Therefore, although potent enzyme inhibitors have been identified, the precise interactions between those molecules and the amino acidic residues of the catalytic binding site cannot be described in detail. In order to collect additional information on the NAAA binding pocket, novel β-lactam derivatives substituted on the endocyclic nitrogen (*N1*) were designed. The chemical growth of those compounds on their left-hand side is expected to shed additional light on the stereo-electronic requirements of the NAAA catalytic site. To this purpose, the extensively characterized **ARN0726** was selected as starting point for our *SAR* study. With the aim to maintain fixed the chemical functions essential for NAAA inhibition (i.e. the β-lactam core and the exocyclic nitrogen derivatization as carbamate) as well as to further explore the available chemical space on the β-lactam scaffold, the functionalization of the endocyclic nitrogen (*N1*) with different substituents was envisaged. To this purpose, a few synthetically accessible *N1*-substituted derivatives were designed, synthesized and tested against *h*-NAAA. Notably, among the possible chemical modifications on *N1*, the corresponding *N*-*O*-alkylation was clearly envisaged as the most beneficial to retain significant *h*-NAAA inhibition (Table 1).

The aim of the present work was to investigate the effect of the *N*-*O*-substitution on *h*-NAAA inhibitory activity as well as to evaluate the available space in the enzyme active site by designing and synthesizing a novel class of *N*-*O*-substituted β-lactams. A conventional *SAR* study was performed to uncover the chemical features beneficial for NAAA inhibition, leading to the discovery of new potent β-lactam inhibitors that were further characterized also in term of stability, selectivity and *in vivo* activity.

Table 1
Inhibitory potency (IC_{50}) of *N*-substituted α-amino β-methyl β-lactams **4**–**12** on *h*-NAAA.[a]

Compounds	Structure	IC_{50} (μM)[a] ± SD
4		1.983 ± 0.095
5		Not active
6		7.18 ± 1.34
7		0.016 ± 0.002
8		0.056 ± 0.010
9		Not active
10		5.07 ± 0.002
11		15.7 ± 0.008
12		0.069 ± 0.013

[a] IC_{50} value (μM) obtained as an average value of three different determinations using a *h*-NAAA fluorogenic assay.

2. Chemistry

A slight modification of the reported synthetic pathway to unprotected serine-derived β-lactams [21] provided an efficient procedure to easily prepare the new *N*-substituted analogues. Based-catalyzed nucleophilic substitutions using **3** (**ARN0726**) and the corresponding methyl iodide, acetyl chloride and methyl-chloroformate afforded compounds **4**, **5**, and **6**, respectively, in

moderate yield (Scheme 1).

Concerning the *N*-*O*-methyl substituted serine- and threonine-derived stereoisomers, the final compounds were obtained starting from the corresponding enantiomerically pure amino acid in a five-step synthetic sequence, as already described in a previous work (see Supporting Information, Schemes S1-3) [21].

The novel *N*-*O*-substituted α-amino β-methyl β-lactams were prepared by the enantioselective synthetic pathway reported in Scheme 2. Except for the *N*-*O*-*tert*-butyl derivative **13** (see Supporting Information, Scheme S4), all the *N*-*O*-alkyl and aryl derivatives were obtained by installation of the corresponding substituent onto the endocyclic nitrogen of the common building block **18.** Two different series of *N*-*O*-substituted analogues were synthesized.

Starting from the enantiomerically pure Boc-L-threonine (**14**), the first step consisted in the conversion of the free carboxylic acid into the benzyloxy amide derivative, followed by transformation of the secondary alcohol into the corresponding mesylate (**15**). An intramolecular base-catalyzed (potassium carbonate) cyclization reaction in acetone afforded the corresponding *N*-*O*-benzyl α-amino β-methyl β-lactam core **16** with inversion of stereochemistry at C3. The insertion of the desired carbamic acid side chain on the exocyclic amino group was accomplished as previously reported for the synthesis of other β-lactam derivatives [21]. First, compound **16** was Boc-deprotected and the obtained amine transformed into the tosylate salt. The carbamoylation reaction was carried out with a mixture (1.6:1 isomeric ratio) of 2-pyridyl carbonate (**S18$_1$**) and 2-oxopyridine-1-carboxylate (**S18$_2$**) derivatives, previously obtained by activation of 4-cyclohexylbutan-1-ol with 2-DPC in the presence of catalytic DMAP (see Supporting Information, Scheme S1) [21,23], to afford the 4-cyclohexylbutyl derivative **17**. As last step, the hydrogenolytic removal of the benzyl group of **17** afforded the *N*-hydroxy substituted β-lactam key intermediate **18** in high yield. The yields of the single steps were generally good, varying from 50% in the carbamoylation reaction to quantitative for the salt preparation.

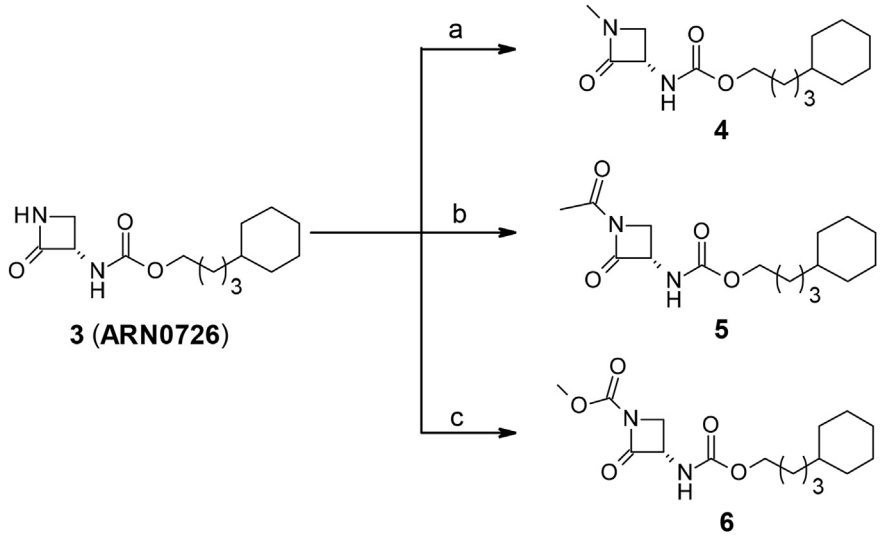
The final step to achieve the *N*-*O*-substituted α-amino β-methyl β-lactam analogues started from **18** which was either submitted to a classic Mitsunobu reaction [24,25] with various alcohols to obtain the *N*-*O*-alkyl derivatives (**19**–**29**), or to a copper catalyzed coupling reaction [26] with arylboronic acids to afford the corresponding *N*-*O*-aryl β-lactams (**30**–**39**) (Scheme 2).

3. Results and discussion

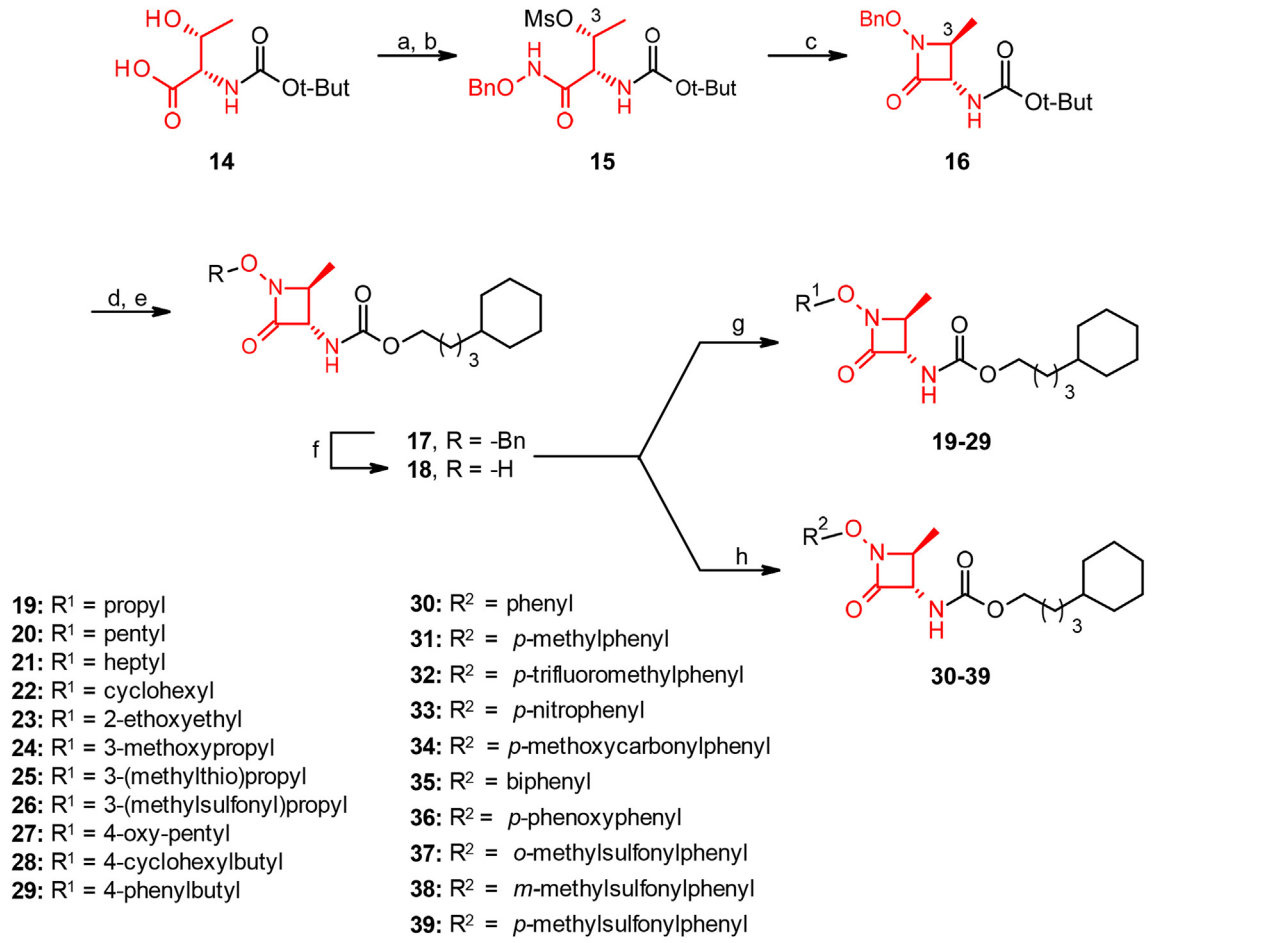
As the first step, in our study we investigated the possible substitutions on the endocyclic nitrogen (*N1*) of β-lactam derivative **3** (**ARN0726**), compatible with the NAAA inhibitory activity of this chemical class. We synthesized a small set of derivatives (Table 1) and tested their ability to inhibit the hydrolysis of *N*-(4-methyl-2-oxo-chromen-7-yl)-hexadecanamide (PAMCA) by recombinant *h*-NAAA heterologously expressed in HEK293 cells (See Supporting Info) [21].

Previous works on β-lactams carried out by our research group reported compound **3** as a potent, double-digit nanomolar NAAA inhibitor with pronounced *in vivo* anti-inflammatory properties in mouse models [21,22]. In this context, a strong stereorecognition by the target enzyme was highlighted, being the (*R*)-enantiomer around 40-fold less active than the (*S*)-enantiomer *in vitro* and failing to show anti-inflammatory activity *in vivo*.

Data reported in Table 1 showed that, while the insertion of a methyl (**4**), an acetyl (**5**) or a methylcarbamoyl (**6**) moiety resulted in decrease or loss of activity compared to **ARN0726**, the substitution of the hydrogen on the endocyclic nitrogen with a methoxy group, as in compound **7**, produced a significant improvement in the *h*-NAAA inhibitory potency (IC_{50} = 0.016 μM). Surprisingly, the (*R*)-serine-derived compound **8**, the enantiomer of **7**, showed inhibitory potency in the low nanomolar range (IC_{50} = 0.056 μM), leading to a loss of the stereorecognition previously observed in similar derivatives [21]. Furthermore, based on previous findings, we asked whether the introduction of a β-methyl substitution on the β-lactam ring would recover such stereorecognition. This modification was earlier reported to restore the effect of stereochemistry on the activity of isomeric threonine-derived analogues [21], and also to beneficially affect the selectivity towards *human* acid ceramidase (*h*-AC), a cysteine amidase that exhibits 33% amino acid identity with NAAA [27,28]. To answer this question, the corresponding four stereoisomeric *N*-*O*-methyl substituted threonine-derived analogues (**9**–**12**) were synthesized. Interestingly, while stereoisomers **9**–**11** resulted to be poorly active against *h*-NAAA (Table 1), the β-lactam **12**, bearing the (2*S*,3*S*) configuration, retained a good potency on the target enzyme (IC_{50} = 0.069 μM). Derivative **12** not only showed promising activity on *h*-NAAA, but also a considerably high selectivity versus *h*-AC, displaying *ca.* 50%



Scheme 1. Synthesis of *N*-substituted β-lactam derivatives of **3** (**ARN0726**).
Reagents and conditions: a) NaH (60% in mineral oil), Methyl iodide, dry THF, 0 °C, 1 h then room temperature, 4 h; b) Acetyl chloride, pyridine, dry DCM, room temperature, 15 h; c) Methyl chloroformate, pyridine, dry DCM, room temperature, 15 h.



Scheme 2. Synthesis of *N-O*-alkyl/aryl α-amino β-methyl β-lactams.
Reagents and conditions: a) O-Benzyl-hydroxylamine, EDC HCl, HOBt, THF, room temperature, 5 h; b) Mesyl chloride, pyridine, 0 °C to room temperature, 15 h; c) K_2CO_3, acetone, reflux, 3 h; d) *p*-Toluensulfonic acid, TFA, room temperature, 15 min; e) Mixture (1.6:1 ratio) of 4-cyclohexylbutyl pyridin-2-yl carbonate (**S18$_1$**) and 4-cyclohexylbutyl 2-oxopyridine-1(*2H*)-carboxylate (**S18$_2$**), DIPEA, dry DCM, room temperature, 15 h; f) Cyclohexene, 10% palladium on activated carbon (Pd/C), EtOH, room temperature, 2 h; g) R^1OH, PPh_3, DEAD, dry THF, 0 °C to room temperature, 5–12 h; h) $R^2B(OH)_2$, CuCl, pyridine, dry DCM, room temperature, 2–10 h.

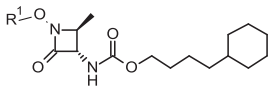
inhibition at 50 μM (data not reported).

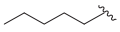
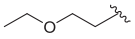
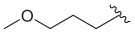
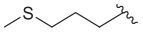
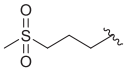
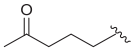
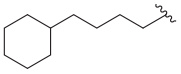
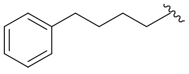
The beneficial effect of the insertion of a methyl group in β-position both in terms of stereoselectivity towards *h*-NAAA and increased selectivity versus *h*-AC, led us to identify carbamate **12** as the starting point (hit compound) for a further structure-activity relationship (*SAR*) expansion of this class of *N-O*-substituted β-lactam analogues.

Based on these findings, while maintaining fixed the (2*S*,3*S*)-α-amino β-methyl β-lactam core and the 4-cyclohexylbutyl side chain, the effect on *h*-NAAA inhibition of the endocyclic nitrogen substitution in terms of size and shape of the substituents was explored. Two different sets of *N-O*-alkyl and *N-O*-aryl substituted α-amino β-methyl β-lactams were designed, synthesized and tested. Table 2 and Table 3 report the outcomes of this investigation in terms of IC_{50} values.

Taking into consideration the importance of *N-O*-substitution on NAAA inhibition, as demonstrated by the modest inhibition (IC_{50} = 47.69 μM) of *h*-NAAA activity of the *N*-hydroxy derivative **18**, the first objective in our *SAR* exploration was the evaluation of *O*-alkyl substituents on the endocyclic nitrogen. A first series of β-lactam derivatives bearing alkyl chains with different bulkiness and length were synthesized and tested (Table 2). First, the optimal length of a linear alkyl chain was evaluated (**12**, **19**–**21**). Increasing the length from a simple methyl group (**12**, IC_{50} = 0.069 μM, Table 2) up to longer alkyl chains led to a progressive decrease in potency. Whereas a good inhibitory activity was retained up to a C5 linear alkyl moiety (**20**, IC_{50} = 0.387 μM), a remarkable drop was observed for compound **21** (IC_{50} = 2.23 μM) bearing a longer alkyl chain (C7). Notably, both the cyclohexyl (**22**, IC_{50} = 3.01 μM) and the *tert*-butyl substitutions (**13**, IC_{50} > 50 μM) were detrimental for enzyme inhibitory activity, probably due to steric hindrance in close proximity of the catalytic active site. Based on these findings, aiming to get additional structural information around the catalytic site, compound **20** was selected as a starting point for the synthesis of a small set of derivatives (**23**–**29**) bearing different substitutions on the alkyl chain. As shown in Table 2, the insertion of a terminal cyclohexyl (**28**) as well as of a phenyl (**29**) moiety on the C5 linear chain led to a decrease in potency (*h*-NAAA IC_{50} = 3.47 μM and 1.24 μM, respectively), suggesting a limited space in this portion of enzyme catalytic site. Interestingly, the derivatives **23**–**27**, bearing an ether (**23**, **24**), a thioether (**25**), a methylsulfonyl (**26**), and a ketone (**27**) moiety respectively, displayed good inhibitory potencies with IC_{50} values ranging between 0.049 and 0.187 μM. When the oxygen was moved from the 4 (compound **24**) to the 3 position (compound **23**) of the alkyl chain, a slight drop in potency was observed (**24**: IC_{50} = 0.088 μM and **23**: IC_{50} = 0.187 μM). These results showed how the insertion of a heteroatom on the linear chain in the *N-O*-substituent could lead to a significant modulation

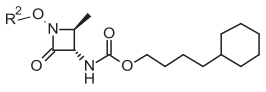
Table 2
Inhibitory potency (IC_{50}) of *N*-*O*-alkyl substituted α-amino β-methyl β-lactams **12**, **13**, **18**–**29** on *h*-NAAA activity.[a]

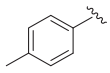
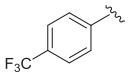
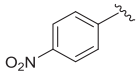
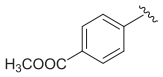
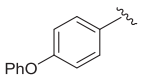
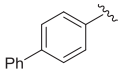
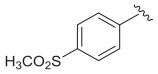
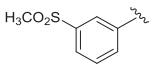


Compound	R^1	IC_{50} (μM)[a] ± SD
18	H	47.69 ± 13.67
12		0.069 ± 0.013
19		0.133 ± 0.056
20		0.387 ± 0.039
21		2.23 ± 0.321
13		n.a.[b]
22		3.01 ± 0.477
23		0.187 ± 0.019
24		0.088 ± 0.009
25		0.167 ± 0.049
26		0.049 ± 0.001
27		0.075 ± 0.019
28		3.47 ± 1.56
29		1.24 ± 0.35

[a] IC_{50} value (μM) obtained as an average value of three different determinations using a *h*-NAAA fluorogenic assay.
[b] n.a.: not active (IC_{50} > 50μM).

Table 3
Inhibitory potency (IC_{50}) of *N*-*O*-aryl substituted α-amino β-methyl β-lactams **30**–**39** on *h*-NAAA activity.[a]



Compound	R^2	IC_{50} (μM)[a] ± SD
30		0.023 ± 0.005
31		0.021 ± 0.009
32		0.031 ± 0.016
33		0.017 ± 0.005
34		0.017 ± 0.001
35		0.048 ± 0.006
36		0.039 ± 0.008
37		0.006 ± 0.001
38		0.010 ± 0.004
39		0.006 ± 0.002

[a] IC_{50} value (μM) obtained as an average value of three different determinations using a *h*-NAAA fluorogenic assay.

of the *h*-NAAA inhibitory activity. The introduction of a methylsulfonyl group (**26**: IC_{50} = 0.049 μM) on the left hand side of the molecule led to an increase in potency, suggesting the beneficial effect of a di-oxygenated substituent on NAAA inhibition.

We next turned our attention to the *N*-*O*-aryl β–lactams and investigated the role of the substituted phenyl rings on NAAA inhibition (Table 3). In general, all *N*-*O*-aryl derivatives showed a very good inhibitory potency, higher than that of the alkyl analogues, with IC_{50}s in the low nanomolar range. We speculated that the higher potency of these derivatives could be explained by an extended conjugation of the phenoxy residue with the β-lactam amidic bond, making the carbonyl more prone to nucleophilic attack by the catalytic cysteine of NAAA.

The unsubstituted phenyl derivative **30** showed a three-fold improvement in IC_{50} compared to the methyl derivative **12** (IC_{50} = 0.023 μM). A similar inhibitory potency (IC_{50} = 0.017–0.031 μM) was also displayed by compounds **31**–**34**, bearing electron-donating (**31**) or electron-withdrawing (**32**–**34**) substituents on the phenyl ring. The apparent lack of correlation between the stereoelectronic properties of the phenyl substitution and the inhibitory activity on the enzyme remains to be explained. Despite the increased bulkiness, the *para*-phenyloxy (**35**) and *para*-phenyl (**36**) substitutions on the phenyl ring demonstrated to be well tolerated by the enzyme (IC_{50} = 0.048 μM and 0.039 μM, respectively). At this point, considering the beneficial effect on activity of a methylsulfonyl substitution on the *N*-*O*-alkyl substituted analogues, we explored such modification also on the aryl substituted β-lactam derivatives. Noteworthy, a significant increase in potency with respect to phenyl compound (**30**) was observed with the *para*-methylsulfonyl derivative **37** (IC_{50} = 0.006 μM), suggesting a possible electrostatic interaction of the two oxygen atoms with the amino acid residues of NAAA active site. To better investigate the effect of this substitution on the phenyl ring, the *meta*- (**38**) and *ortho*- (**39**) methylsulfonyl derivatives were also synthesized. As shown by the IC_{50} values (*meta-:* IC_{50} = 0.010 μM and *ortho*-: IC_{50} = 0.006 μM) both substituted compounds resulted to be equally active to the *para* analogue **37**, suggesting this type of substitution to be beneficial for a potent NAAA inhibition regardless of the substituent position on the phenyl ring.

In this context, we also synthesized and tested few *N*-*O*-aryl β-lactams bearing a substitution in *meta*- or *ortho* position. As seen for methylsulfonyl substituted compounds (**37**–**39**), the position of

the substituent on the phenyl ring did not affect the compounds' NAAA inhibitory activity (data not reported).

A selection of structurally relevant *N*-*O*-substituted α-amino β-methyl β-lactams (**12**, **26**, **30**, **37**) identified in our *SAR* study was also tested for selectivity against *human* Acid Ceramidase (*h*-AC) [27], and *human* FAAH (*h*-FAAH), a serine hydrolase which can cleave FAEs [29] (Table 4).

The activity data on *h*-AC inhibition showed in the case of *N*-*O*-alkyl derivatives **12** and **26** a very high selectivity (*h*-NAAA/*h*-AC > 1000 fold), which drastically decreased when the endocyclic nitrogen was substituted with a phenyloxy moiety (**30**), showing only a 13-fold difference between the activity of the two cysteine hydrolases. The selectivity on *h*-NAAA was recovered when a methyl sulfonyl group was inserted in *para* position (**37**, *h*-AC/*h*-NAAA = ca. 340 fold).

At the tested concentration (10 μM), none of the selected *N*-*O*-substituted β-lactam carbamates inhibited *h*-FAAH in a significant manner (Table 4).

To investigate the physicochemical properties of this new β-lactam class, the same subset of *N*-*O*-substituted α-amino β-methyl β-lactams was also evaluated for the chemical stability in buffer (PBS) at pH 7.4 and for the *mouse* and *rat* plasma stability (Table 4).

The tested compounds showed an overall high chemical stability to hydrolytic cleavage, with half-life of over 24 h (**12**, **26, 30**: $t_{½}$> 1440 min). A lower but still acceptable half-life ($t_{½}$) value was observed for the *para*-methylsulfonyl derivative **37** ($t_{½}$ = 727 min).

With respect to the plasma stability, all selected compounds displayed very low half-life ($t_{½}$< 5 min) either in *rat* and *mouse* plasma, due to a fast hydrolytic cleavage.

In contrast with previously described *β*-lactam derivatives [21], the low plasma stability displayed by *N*-*O*-substituted analogues clearly prevents the potential use of this new class of NAAA inhibitors for systemic administration. However, this limitation (i.e. low plasma stability) may be well turned into an advantage when administering these compounds by topical applications, thus restricting NAAA inhibition to the site of administration and limiting their potential side effects. To assess the *in vivo* activity of a representative compound of this series, we tested compound **37** by topical administration in a mouse model of carrageenan-induced inflammation (see Supporting Information). After induction of paw edema and heat hyperalgesia, a fresh suspension of **37** was given by intraplantar administration.

A single dosing of **37** (0.5–50 μg/paw) was able to significantly attenuate both responses (Fig. 2A and 2B), and when **37** was administered at the highest dose (50 μg/paw), the effect was still statistically significant 24 h after treatment (Fig. 2A, $P < 0.01$).

The same compound **37** was also used as a tool to evaluate the mechanism of NAAA inhibition by this new class of β-lactams. As previously shown for other β-lactam NAAA inhibitors [16,21], high resolution liquid chromatography mass spectrometry (HRLC/MS-MS) studies proved the formation of a covalent thioester adduct with the *N*-terminal cysteine of the active form of *h*-NAAA (see Supporting Information).

The covalent nature of NAAA inhibition by this class of compound was also confirmed by a competitive Activity Based Protein Profiling (ABPP) study using **ARN14686**, a recently published probe for NAAA [30]. We used the lysosomal enriched fraction from HEK-293 cells stably overexpressing NAAA (NAAA HEK-293) as source of the target enzyme. This fraction was pre-incubated with compound **37** (20 μM) for one hour and then a five-fold higher concentration of **ARN14686** was added after 15 min or 4 h. Subsequent click chemistry reaction with ARN14686 allowed the introduction of a fluorophore (i.e., rhodamine) to detect the catalytically active portion of the enzyme. The SDS-PAGE analysis (Fig. 3) clearly showed that β-lactam **37** was able to rapidly and fully inhibit NAAA, as demonstrated by the absence of any fluorescent signals after 15 min incubation with **ARN14686**. The same result was obtained after 4 h incubation with **ARN14686**, thus indicating that NAAA inhibition by compound **37** is irreversible, at least under this experimental conditions.

4. Conclusions

The present study expanded the previous *SAR* on serine- and threonine-derived β-lactams as NAAA inhibitors by investigating the influence of the endocyclic nitrogen *O*-substitution on the activity. First, we explored the effect of a β-methyl substitution on the β-lactam ring: as already seen with other analogues, a strong recognition towards the (2*S*,3*S*) configuration (compound **12**) was confirmed also for this class. Then, *N*-*O*-alkyl and *N*-*O*-aryl substituted compounds were investigated, disclosing a significant preference of the enzyme towards aryl and polar substituents. The expansion of the aryl series, starting from the phenyl derivative **30**, led us to compound **37**, a single-digit nanomolar NAAA inhibitor (*h*-NAAA IC_{50} = 0.006 μM). Surprisingly, similar IC_{50} values were observed for all *para*-substituted phenyl compounds, indicating that the stereo-electronic properties of the substituent did not affect NAAA inhibitory potency. Furthermore, the four-fold increased potency displayed by the methylsulfonyl derivative **37** compared to compound **30**, suggested the importance of oxygen-containing substituents for the interaction with the enzyme. The results of our SAR study showed that β-lactam NAAA inhibitors could be substituted at the endocyclic nitrogen with *O*-alkyl and *O*-aryl groups, besides the exocyclic nitrogen of the α-amino group. This results in linearly extended compounds where the cysteine warhead is no longer at one end of the molecule, as is the case in β-lactones or β-lactams, like compounds **2** and **3**. The enzyme appears therefore able to accommodate relatively extended small molecules at the active site. The introduction of properly substituted *O*-aryl moieties at the endocyclic nitrogen was particularly beneficial in terms of NAAA inhibitory activity, and offered the possibility of modulating the substituent at the exocyclic nitrogen in order to

Table 4
Inhibitory potency (IC_{50}) or % inhibition of selected *N*-*O*-substituted β-lactams (**12**, **26**, **30**, **37**) on *h*-NAAA, *h*-AC, *h*-FAAH, and chemical and *mouse* and *rat* plasma stability.

Compound	*h*-NAAA IC_{50} (μM) ± SD[a]	*h*-AC IC_{50} (μM) ± SD[a]	*h*-FAAH % Inhib. at 10 μM	Chem. stab. (PBS) pH 7.4 $t_{½}$ (min)[b], (%)[c]	*m*/*r*-plasma stab. $t_{½}$ (min)[d]
12	0.069 ± 0.018	n.a.[e]	n.a.[e]	>1440 (*97%*)	<5
26	0.049 ± 0.001	n.a.[e]	n.a.[e]	>1440 (*67%*)	<5
30	0.023 ± 0.005	0.3 ± 0.002	n.a.[e]	>1440 (*63%*)	<5
37	0.006 ± 0.002	2.02 ± 0.002	n.a.[e]	727 ± 59	<5

[a] IC_{50} value (μM) obtained as an average value of three different determinations using a *h*-NAAA or *h*-AC fluorogenic assay.
[b] Measured after 24 h of incubation at 37 °C in 0.01 M PBS +10% MeCN.
[c] Compound remaining (%) after 24 h (1440 min).
[d] Measured in *mouse*/*rat* plasma +5% DMSO.
[e] n.a.: not active (>50% inhibition @50 μM).

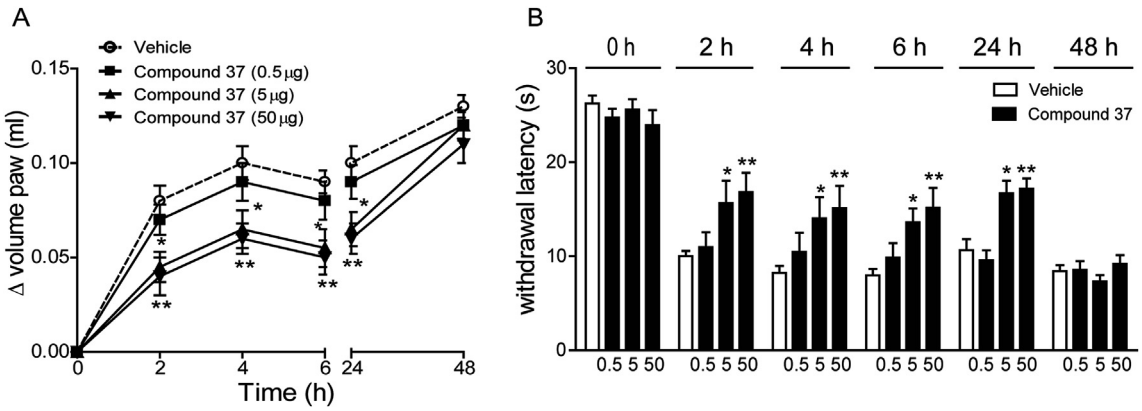


Fig. 2. Effects of topical application of **37** on carrageenan induced edema and hyperalgesia. Administration of **37** (in 80% sterile saline solution/10% PEG-400/10% Tween 80) attenuated paw edema (Figure A) and heat hyperalgesia (Figure B) measured just after carrageenan injection and at various time points following compound administration.

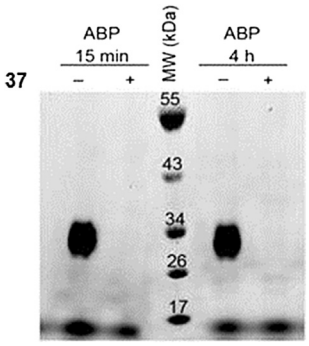


Fig. 3. Competitive ABPP analysis: NAAA-HEK293 lysosomal enrichment was incubated with **37** (+) or DMSO (−) for 1 h before probe (**ARN14686**) addition (for 15 min or 4 h). A rhodamine fluorophore was inserted by click chemistry and in-gel fluorescence analysis was performed.

access further β-lactams with improved NAAA inhibitory activity and desired chemical functionalities.

Based on the interesting outcomes reported for the first NAAA activity-based probe [29], *N-O*-substituted β-lactams could also be exploited for the design of novel ABPs. This would allow the development of additional pharmacological tools for the *in vitro* and *in vivo* detection of the catalytically active form of NAAA, thus contributing to improving our understanding of the enzyme's physiological roles.

A set of this new *β*-lactams was also evaluated for hydrolytic and plasma stability. While all selected compounds showed a good chemical stability at pH 7.4 in PBS, upon incubation with *rat* and *mouse* plasma they were rapidly hydrolyzed, suggesting their possible use as *soft drugs* for skin diseases. In line with these data, a preliminary *in vivo* study with compound **37** in a carragenaan-induced inflammation mouse model demonstrated the promising activity of such compound when administered topically. This encouraging result lends support to its possible use as *soft drug* for skin diseases.

5. Experimental section

5.1. Chemistry

5.1.1. Chemicals, materials and methods

All the commercial 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. Automated column chromatography purifications were performed using a Teledyne ISCO apparatus (CombiFlash® Rf) with pre-packed silica gel columns of different sizes (from 4 g up to 120 g). Mixtures of increasing polarity of cyclohexane (Cy) and ethyl acetate (EtOAc) or cyclohexane and methyl *tert*-butyl ether (MTBE) were used as eluents. 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 dimethylsulfoxide (DMSO-d_6) as solvent. 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 BEH C_{18} column (100 × 2.1mmID, particle size 1.7 mm) with a VanGuard BEH C_{18} precolumn (5 × 2.1 mmID, particle size 1.7 mm). 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. Electrospray ionization in positive and negative mode was applied. 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. HPLC system included a 2747 sample manager, 2545 binary gradient module, system fluidic organizer and 515 HPLC pump. PDA range was 210–400 nm. Purifications were performed on a XBridge™ Prep C_{18} OBD column (100 × 19 mmID, particle size 5 mm) with a XBridge™Prep C_{18} (10 × 19 mmID, particle size 5 mm) guard cartridge. Mobile phase was 10 mM NH_4OAc in MeCN-H_2O (95:5) at pH 5. Electrospray ionization in positive and negative mode was used. 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 $CHCl_3$ as a solvent and a 1 dm cell. All tested compounds (**4**–**13**, **18**–**39**) showed ≥ 95% purity by NMR and UPLC/MS analysis.

5.1.2. Synthesis of the N-substituted serine-derived β-lactams (4–6)

Experimental procedure and ^{1}H NMR relative to **3** (**ARN0726**) are according to previously reported literature [21].

5.1.2.1. 4-Cyclohexylbutyl-(S)-(1-methyl-2-oxoazetidin-3-yl)carbamate (4). Under nitrogen atmosphere, to a stirred solution of **3** (0.02 g, 0.074 mmol) in dry THF (1.5 mL), Cs_2CO_3 (0.026 g, 0.081 mmol) and methyl iodide (0.005 mL, 0.081 mmol) were subsequently added. After stirring 15 h at 50 °C the solution was

filtered over a celite pad, diluted with DCM and evaporated to dryness. The crude product was absorbed over silica gel and purified by column chromatography using a Teledyne ISCO apparatus, eluting with Cy/EtOAc (from 100:0 to 20:80) to afford a crude product, which was submitted to a preparative HPLC/MS using a Waters Autopurification system, affording the title compound (0.018 g, 15%) as a colorless oil. MS (ESI) *m/z*: 283.3 $[M-H]^+$, 300.3 $[M-NH_4]^+$. $[\alpha]_{D20}$ (c = 0.1, $CHCl_3$): +19.48. ^1H NMR (DMSO-d_6): δ 7.76 (d, *J* = 8.65 Hz, 1H), 4.70–4.54 (m, 1H), 3.94 (td, *J* = 6.76, 1.6 Hz, 2H), 3.45 (t, *J* = 5.13 Hz, 1H), 3.12 (dd, *J* = 5.13, 2.52 Hz, 1H), 2.72 (s, 3H), 1.72–1.56 (m, 5H), 1.50 (p, *J* = 6.76 Hz, 2H), 1.33–1.22 (m, 2H), 1.22–1.06 (m, 6H), 0.91–0.76 (m, 2H) ppm. ^{13}C NMR (DMSO-d_6): δ 171.1, 168.7, 65.2, 64.8, 47.6, 49.5, 37.0, 33.6, 29.6, 28.5, 26.6, 22.9 ppm.

5.1.2.2. 4-Cyclohexylbutyl-(S)-(1-acetyl-2-oxoazetidin-3-yl)carbamate (5). Under nitrogen atmosphere, to a stirred solution of **3** (0.05 g, 0.18 mmol) in dry DCM (3.0 mL), pyridine (0.020 mL, 0.025 mmol) and acetyl chloride (0.018 mL, 0.025 mmol) were subsequently added. After stirring 15 h at room temperature the solution was diluted with DCM (15 mL) and washed with a saturated $NaHCO_3$ aqueous solution (3 × 5.0 mL); the organic phase was dried over $NaSO_4$, filtered and evaporated to dryness. The crude product was absorbed over silica gel and purified by column chromatography using a Teledyne ISCO apparatus, eluting with Cy/EtOAc (from 100:0 to 70:30) to afford the title compound (0.01 g, 18%), as white solid. MS (ESI) *m/z*: 311.2 $[M-H]^+$, 328.3 $[M-NH_4]^+$, 309.2 $[M-H]^-$. $[\alpha]_{D20}$ (c = 0.03, $CHCl_3$): −1.64. ^1H NMR (DMSO-d_6): δ 7.91 (d, *J* = 7.79 Hz, 1H), 4.76 (td, *J* = 7.79, 3.80 Hz, 1H), 3.97 (t, *J* = 6.78 Hz, 2H), 3.75 (t, *J* = 6.88 Hz, 1H), 3.45 (dd, *J* = 6.88, 3.80 Hz, 1H), 2.26 (s, 3H), 1.76–1.56 (m, 5H), 1.52 (p, *J* = 6.78 Hz, 2H), 1.35–1.20 (m, 2H), 1.21–1.05 (m, 6H), 0.93–0.75 (m, 2H) ppm. ^{13}C NMR (DMSO-d_6): δ 171.1, 168.7, 65.2, 58.6, 45.1, 37.0, 29.5, 29.3, 27.9, 26.8, 23.9, 21.7 ppm.

5.1.2.3. Methyl-(S)-3-(((4-cyclohexylbutoxy)carbonyl)amino)-2-oxoazetidine-1-carboxylate (6). Under nitrogen atmosphere, to a stirred solution of **3** (0.05 g, 0.18 mmol) in dry DCM (3.0 mL), triethylamine (0.056 mL, 0.409 mmol) and methyl-carbonyl chloride (0.017 mL, 0.223 mmol) were slowly added at 0 °C. After stirring 15 h at room temperature (still starting material left) the solution evaporated to dryness. The crude product was absorbed over silica gel and purified by column chromatography using a Teledyne ISCO apparatus, eluting with Cy/EtOAc (from 100:0 to 40:60) to afford a crude product, which was submitted to a preparative HPLC/MS using a Waters Autopurification system, affording the title compound (0.037 g, 62%) as a colorless oil. MS (ESI) *m/z*: 327.3 $[M-H]^+$, 344.3 $[M-NH_4]^+$. $[\alpha]_{D20}$ (c = 0.08, $CHCl_3$): −4.62. ^1H NMR (DMSO-d_6): δ 7.88 (d, *J* = 8.17 Hz, 1H), 4.79–4.70 (m, 1H), 3.96 (t, *J* = 6.76 Hz, 2H), 3.79 (t, *J* = 6.34 Hz, 1H), 3.73 (s, 3H), 3.53 (dd, *J* = 3.81, 6.34 Hz, 1H), 1.72–1.56 (m, 5H), 1.51 (p, *J* = 6.76 Hz, 2H), 1.36–1.25 (m, 2H), 1.24–1.06 (m, 6H), 0.91–0.76 (m, 2H). ppm. ^{13}C NMR (DMSO-d_6): δ 171.1, 168.7, 65.0, 57.4, 53.2, 46.1, 37.0, 33.4, 33.1, 33.2, 29.3, 26.4, 23.1 ppm.

5.1.3. 4-Cyclohexylbutyl-N-[(S)-1-methoxy-2-oxo-azetidin-3-yl] carbamate (7)

Step 1 to 4. See Supporting Information.

Step 5. Under nitrogen atmosphere, a solution of **S13** (0.082 g, 0.463 mmol) in dry CH_2Cl_2 (7.0 mL) was treated with DIPEA (0.092 mL, 0.55 mmol). A second solution containing a mixture (ratio 1.6:1) of 4-cyclohexylbutyl 2-pyridyl carbonate (**S18$_1$**) and 4-cyclohexylbutyl 2-oxopyridine-1-carboxylate (**S18$_2$**) (0.352 g, 1.27 mmol) in dry CH_2Cl_2 (2.0 mL) was then added. The reaction was left to stir under nitrogen atmosphere at room temperature for 15 h. The solvent was evaporated and the crude product was dissolved in EtOAc (10 mL), washed first with a saturated NH_4Cl aqueous solution (20.0 mL), subsequently with a saturated $NaHCO_3$ (3 × 20 mL) and NaCl (20 mL) aqueous solutions. The organic layer was dried over Na_2SO_4, filtered and evaporated to dryness. The crude was absorbed over silica gel and purified by column chromatography using a Teledyne ISCO apparatus, eluting with Cy/TBME (from 100:0 to 30:70) to afford the title compound (0.02 g, 31%), as a colorless oil. MS (ESI) *m/z*: 299.3 $[M-H]^+$, 316.3 $[M-NH_4]^+$. $[\alpha]_{D20}$ (c = 0.1, $CHCl_3$): −8.14. ^1H NMR (DMSO-d_6): δ 7.82 (d, *J* = 8.35 Hz, 1H), 4.58–4.45 (m, 1H), 3.96 (t, *J* = 7.0 Hz, 2H), 3.78 (dd, *J* = 5.53, 4.15 Hz, 1H), 3.70 (s, 3H), 3.48–3.40 (m, 1H), 1.73–1.57 (m, 5H), 1.52 (p, *J* = 6.86 Hz, 2H), 1.38–1.26 (m, 2H), 1.26–1.06 (m, 6H), 0.93–0.78 (m, 2H) ppm. ^{13}C NMR (DMSO-d_6): δ 156.8, 154.3, 64.6, 62.6, 53.1, 50.5, 36.8, 33.4, 33.3, 29.5, 26.5, 26.3, 23.1 ppm.

5.1.4. 4-Cyclohexylbutyl-N-[(R)-1-methoxy-2-oxo-azetidin-3-yl] carbamate (8)

Step 1 to 3. See Supporting Information.

Step 4. To a refluxing (90 °C) slurry of powdered K_2CO_3 (0.14 g, 1.02 mmol) in acetone (5.0 mL), a solution of **S20** (0.1 g, 0.254 mmol) in acetone (3.0 mL) was added. The resulting suspension was stirred at 100 °C for 3 h. Upon cooling, the thick slurry was filtered through celite and the collected solid was extracted with EtOAc (20.0 mL). The organic layers were washed sequentially with a 1 N HCl aqueous solution (10.0 mL), a saturated $NaHCO_3$ solution (20.0 mL) and a saturated NaCl solution (10.0 mL), dried over Na_2SO_4, filtered and concentrated to dryness. The crude product was absorbed over silica gel and purified by column chromatography using a Teledyne ISCO apparatus, eluting with Cy/TBME (from 100:0 to 70:30) to afford the title compound (0.042 g, 55%), as white solid. MS (ESI) *m/z*: 299.2 $[M-H]^+$, 297.2 $[M-H]^-$. $[\alpha]_{D20}$ (c = 0.1, $CHCl_3$): −18.29. ^1H NMR (DMSO-d_6): δ 7.82 (d, *J* = 8.32 Hz, 1H), 4.57–4.44 (m, 1H), 3.96 (t, *J* = 6.72 Hz, 2H), 3.77 (dd, *J* = 4.18, 5.34 Hz, 1H), 3.70 (s, 3H), 3.48–3.40 (m, 1H), 1.74–1.56 (m, 5H), 1.51 (p, *J* = 6.72 Hz, 2H), 1.37–1.25 (m, 2H), 1.25–1.07 (m, 6H), 0.95–0.76 (m, 2H) ppm. ^{13}C NMR (DMSO-d_6): δ 156.8, 154.3, 64.3, 62.7, 53.2, 50.1, 37.8, 36.5, 33.3, 33.4, 28.8, 26.2, 22.5 ppm.

5.1.5. 4-Cyclohexylbutyl-N-[(2R,3S)-1-methoxy-2-methyl-4-oxo-azetidin-3-yl]carbamate (9)

Step 1. See Supporting Information.

Step 2. The reaction was carried out following the general procedure GP_S1 employing **S14** (0.188 g, 0.62 mmol), a mixture (1.6:1 ratio) of **S18$_1$** and **S18$_2$** (0.516 g, 1.86 mmol) and DIPEA (0.108 mL, 0.62). Purification by column chromatography using a Teledyne ISCO apparatus, eluting with Cy/EtOAc (from 100:0 to 60:40) afforded the title compound (0.11 g, 57%), as a white powder. MS (ESI) *m/z*: 313.1 $[M-H]^+$, 330.2 $[M-NH_4]^+$. $[\alpha]_{D20}$ (c = 0.1, $CHCl_3$): +44.11. ^1H NMR (DMSO-d_6): δ 7.88 (d, *J* = 9.13 Hz, 1H), 4.69 (dd, *J* = 9.13, 5.10 Hz, 1H), 4.22–4.11 (m, 1H), 3.97 (td, *J* = 6.70, 1.63 Hz, 2H), 3.71 (s, 3H), 1.71–1.57 (m, 5H), 1.57–1.47 (m, 2H), 1.37–1.25 (m, 2H), 1.25–1.07 (m, 6H), 1.14 (d, *J* = 6.08 Hz, 3H), 0.93–0.68 (m, 2H) ppm. ^{13}C NMR (DMSO-d_6): δ 161.7, 156.5, 64.6, 63.5, 58.1, 56.3, 37.2, 36.7, 33.0, 29.1, 26.4, 26.1, 22.8, 13.1 ppm.

5.1.6. 4-Cyclohexylbutyl-N-[(2S,3R)-1-methoxy-2-methyl-4-oxo-azetidin-3-yl]carbamate (10)

Step 1. See Supporting Information.

Step 2. The reaction was carried out following the general procedure GP_S1 employing **S15** (0.188 g, 0.62 mmol), a mixture of 4-cyclohexylbutyl 2-pyridyl carbonate (**S18$_1$**) and 4-cyclohexylbutyl 2-oxopyridine-1-carboxylate (**S18$_2$**) (0.516 g, 1.86 mmol) and DIPEA (0.108 mL, 0.62). Purification by column chromatography using a Teledyne ISCO apparatus, eluting with Cy/TBME (from

100:0 to 50:50) afforded the title compound (0.093 g, 48%), as a colorless oil. MS (ESI) *m/z*: 313.2 $[M-H]^+$, 330.2 $[M-NH_4]^+$, 311.3 $[M-H]^-$. $[\alpha]_{D20}$ (c = 0.1, $CHCl_3$): −53.21. ^1H NMR (DMSO-d_6): δ 7.88 (d, *J* = 9.16 Hz, 1H), 4.70 (dd, *J* = 9.16, 5.11 Hz, 1H), 4.22–4.13 (m, 1H), 3.98 (td, *J* = 1.81, 6.73 Hz, 2H), 3.72 (s, 3H), 1.76–1.57 (m, 5H), 1.52 (p, *J* = 6.73 Hz, 2H), 1.37–1.26 (m, 2H), 1.25–1.16 (m, 6H), 1.15 (d, *J* = 6.21 Hz, 3H), 0.95–0.79 (m, 2H) ppm. ^{13}C NMR (DMSO-d_6): δ 161.4, 156.2, 64.3, 63.2, 57.8, 56.0, 36.9, 36.5, 32.8, 28.8, 26.2, 25.8, 22.5, 12.8 ppm.

5.1.7. 4-Cyclohexylbutyl-N-[(2R,3R)-1-methoxy-2-methyl-4-oxo-azetidin-3-yl]carbamate (11)

Step 1. See Supporting Information.

Step 2. The reaction was carried out following the general procedure GP_S1 employing **S16** (0.193 g, 0.64 mmol), a mixture of 4-cyclohexylbutyl 2-pyridyl carbonate (**S18$_1$**) and 4-cyclohexylbutyl 2-oxopyridine-1-carboxylate (**S18$_2$**) (0.532 g, 1.92 mmol) and DIPEA (0.112 mL, 0.64). Purification by column chromatography using a Teledyne ISCO apparatus, eluting with Cy/EtOAc (from 100:0 to 60:40) afforded the title compound (0.099 g, 50%), as a white powder. MS (ESI) *m/z*: 313.2 $[M-H]^+$, 330.2 $[M-NH_4]^+$. $[\alpha]_{D20}$ (c = 0.1, $CHCl_3$): −10.81. ^1H NMR (DMSO-d_6): δ 7.82 (d, *J* = 8.07 Hz, 1H), 4.06–4.00 (m, 1H), 3.95 (td, *J* = 6.66, 2.51 Hz, 2H), 3.90–3.83 (m, 1H), 3.72 (s, 3H), 1.72–1.55 (m, 5H), 1.56–1.46 (m, 2H), 1.33 (d, *J* = 6.10 Hz, 3H), 1.31–1.25 (m, 2H), 1.25–1.08 (m, 6H), 0.93–0.77 (m, 2H) ppm. ^{13}C NMR (DMSO-d_6): δ 161.6, 155.7, 64.3, 63.2, 59.8, 59.4, 37.0, 36.5, 32.8, 28.9, 26.2, 25.9, 22.6, 16.0 ppm.

5.1.8. 4-Cyclohexylbutyl-N-[(2S,3S)-1-methoxy-2-methyl-4-oxo-azetidin-3-yl]carbamate (12)

Step 1. See Supporting Information.

Step 2. The reaction was carried out following the general procedure GP_S1 employing **S17** (0.290 g, 0.96 mmol), a mixture of 4-cyclohexylbutyl 2-pyridyl carbonate (**S18$_1$**) and 4-cyclohexylbutyl 2-oxopyridine-1-carboxylate (**S18$_2$**) (0.798 g, 2.88 mmol) and DIPEA (0.167 mL, 0.96). Purification by column chromatography using a Teledyne ISCO apparatus, eluting with Cy/EtOAc (from 100:0 to 60:40) afforded the title compound (0.17 g, 57%), as a colorless oil. MS (ESI) *m/z*: 313.4 $[M-H]^+$, 330.2 $[M-NH_4]^+$. $[\alpha]_{D20}$ (c = 0.1, $CHCl_3$): +16.94. ^1H NMR (DMSO-d_6): δ 7.83 (d, *J* = 8.15 Hz, 1H), 4.04 (dd, *J* = 8.15, 3.74 Hz, 1H), 4.00–3.93 (m, 2H), 3.87 (dt, *J* = 6.89, 3.74 Hz, 1H), 3.73 (s, 3H), 1.72–1.58 (m, 5H), 1.52 (p, *J* = 6.82 Hz, 2H), 1.34 (d, *J* = 6.89 Hz, 3H), 1.32–1.25 (m, 2H), 1.25–1.07 (m, 6H), 0.92–0.78 (m, 2H). ppm. ^{13}C NMR (DMSO-d_6): δ 161.6, 155.7, 64.3, 63.1, 59.5, 59.3, 36.9, 36.5, 32.8, 28.8, 26.1, 25.8, 22.6, 16.0 ppm.

5.1.9. 4-Cyclohexylbutyl-((2S,3S)-1-(tert-butoxy)-2-methyl-4-oxoazetidin-3-yl)carbamate (13)

Step 1 to 4. See Supporting Information.

Step 5. 4-Cyclohexylbutyl-((2*S*,3*S*)-1-(*tert*-butoxy)-2-methyl-4-oxoazetidin-3-yl)carbamate (**13**).

Under nitrogen atmosphere, a solution of (2*S*,3*S*)-1-(*tert*-butoxy)-2-methyl-4-oxoazetidin-3-ammonium tosylate salt **S23** (0.130 g, 0.37 mmol) in dry CH_2Cl_2 (3.0 mL) was treated with DIPEA (0.074 mL, 0.45 mmol). A second solution containing a mixture (ratio 1.6:1) of 4-cyclohexylbutyl 2-pyridyl carbonate (**S18$_1$**) and 4-cyclohexylbutyl 2-oxopyridine-1-carboxylate (**S18$_2$**) (0.418 g, 1.51 mmol) in dry CH_2Cl_2 (2.0 mL) was then added. The reaction was left to stir under nitrogen atmosphere at room temperature for 15 h. The solvent was evaporated and the crude product was dissolved in EtOAc (10 mL), washed first with a saturated NH_4Cl aqueous solution (20.0 mL), subsequently with a saturated $NaHCO_3$ (3 × 20 mL) and NaCl (20 mL) aqueous solutions. The organic layer was dried over Na_2SO_4, filtered and evaporated to dryness. The crude was absorbed over silica gel and purified by column chromatography using a Teledyne ISCO apparatus, eluting with Cy/TBME (from 100:0 to 30:70) to afford the title compound (0.077 g, 0.21 mmol, 57%), as colorless oil. MS (ESI) *m/z*: 355.2 $[M-H]^+$, 372.3 $[M-NH_4]^+$, 353.2 $[M-H]^-$. $[\alpha]_{D20}$ (c = 0.1, $CHCl_3$): −259.6. ^1H NMR (DMSO-d_6): δ 7.89 (d, *J* = 8.19 Hz, 1H), 4.04 (dd, *J* = 8.19, 2.18 Hz, 1H), 3.96 (td, *J* = 6.69, 2.65 Hz, 2H), 3.82–3.71 (m, 1H), 1.72–1.57 (m, 5H), 1.52 (p, *J* = 6.69 Hz, 2H), 1.31–1.36 (m, 2H), 1.29 (d, *J* = 6.13 Hz, 3H), 1.24 (s, 9H), 1.23–1.05 (m, 6H), 0.95–0.76 (m, 2H) ppm. ^{13}C NMR (DMSO-d_6): δ 164.4, 155.9, 83.5, 64.4, 62.9, 60.3, 37.2, 36.7, 33.1, 29.1, 27.1, 26.4, 26.1, 22.8, 15.4.

5.1.10. 4-Cyclohexylbutyl-((2S,3S)-1-hydroxy-2-methyl-4-oxoazetidin-3-yl)carbamate (18)

Step 1. tert-Butyl-((2*S*,3*R*)-1-((benzyloxy)amino)-3-hydroxy-1-oxobutan-2-yl)carbamate.

To a solution of commercially available *N*-(*tert*-butoxycarbonyl)-L-threonine **14** (3.0 g, 13.7 mmol) in THF (50 mL), *O*-benzylhydroxylamine as free base (1.85 g, 15.07 mmol) and HOBt (2.29 g, 15.07 mmol) were added. EDC HCl (2.89 g, 15.07 mmol) was added portion wise in 30 min and the reaction was vigorously stirred for 5 h at room temperature. After solvent evaporation the crude mixture was extracted with EtOAc (20.0 mL) and washed with a saturated $NaHCO_3$ aqueous solution (3 × 10 mL); the organic phase was dried over Na_2SO_4, filtered and the solvent removed *under vacuum* to yield the title compound (3.37 g, 76%) as colorless oil, which was used in the next step without any further purification. MS (ESI) *m/z*: 325.2 $[M-H]^+$, 323.2 $[M-H]^-$. ^1H NMR (DMSO-d_6): δ 7.48–7.29 (m, 5H), 6.34 (d, *J* = 8.52 Hz, 1H), 4.85–4.73 (m, 2H), 3.90–3.77 (m, 1H), 3.71 (dd, *J* = 8.52, 4.94 Hz, 1H), 1.39 (s, 9H), 1.01 (d, *J* = 6.45 Hz, 3H) ppm.

Step 2. (2*R*,3*S*)-4-((Benzyloxy)amino)-3-((*tert*-butoxycarbonyl)amino)-4-oxobutan-2-yl methanesulfonate (**15**).

To a cooled (0 °C) solution of *tert*-butyl ((2*S*,3*R*)-1-((benzyloxy)amino)-3-hydroxy-1-oxobutan-2-yl)carbamate (3.37 g, 10.39 mmol) in dry pyridine (41 mL), methanesulfonyl chloride (1.61 mL, 20.78 mmol) was added dropwise over 10 min. The reaction was left to stir under nitrogen atmosphere for 3 h. The mixture was poured into 20 mL of iced water; the solution was then acidified with 2 N HCl until reaching pH 4. The aqueous phase was extracted with EtOAc (20.0 mL) and the collected organic layers were dried over Na_2SO_4, filtered and evaporated to dryness. The resulting crude product was absorbed over silica gel and purified by column chromatography using a Teledyne ISCO apparatus, eluting with Cy/EtOAc (from 100:0 to 0:100 for 10 min then only EtOAc) to afford the title compound (4.09 g, 10.5 mmol, 98%), as white solid. MS (ESI) *m/z*: 403.1 $[M-H]^+$, 420.1 $[M-NH_4]^+$, 401.1 $[M-H]^-$. ^1H NMR (DMSO-d_6): δ 7.25–7.49 (m, 5H), 7.09 (d, *J* = 9.47 Hz, 1H), 4.71–4.84 (m, 3H), 4.08 (qd, *J* = 9.47, 6.32 Hz, 1H), 3.11 (s, 3H), 1.38 (s, 9H), 1.24 (d, *J* = 6.32 Hz, 3H), ppm.

Step 3. tert-Butyl-((2*S*,3*S*)-1-(benzyloxy)-2-methyl-4-oxoazetidin-3-yl)carbamate (**16**).

To a refluxing (90 °C) slurry of powdered K_2CO_3 (4.35 g, 31.5 mmol) in acetone (160 mL), a solution of **15** (4.09 g, 10.5 mmol) in acetone (80 mL) was added. The resulting suspension was stirred at 100 °C for 3 h. Upon cooling, the thick slurry was filtered through celite and the collected solid was extracted with EtOAc (30.0 mL). The organic layers were washed sequentially with a 1 N HCl aqueous solution (20.0 mL), a saturated $NaHCO_3$ solution (20.0 mL) and a saturated NaCl solution (20.0 mL), dried over Na_2SO_4, filtered and concentrated to dryness. The crude product was absorbed over silica gel and purified by column chromatography using a Teledyne ISCO apparatus, eluting with Cy/TBME (from 100:0 to 70:30) to afford the title compound (2.72 g, 85%), as white solid. MS (ESI) *m/z*: 307.2 $[M-H]^+$, 324.2 $[M-NH_4]^+$. ^1H NMR (DMSO-d_6): δ 7.58 (d,

J = 8.25 Hz, 1H), 7.35–7.48 (m, 5H), 4.92 (s, 2H), 3.99 (m, 1H), 3.70 (qd, J = 6.10, 2.08 Hz, 1H), 1.39 (s, 9H), 1.19 (d, J = 6.10 Hz, 3H) ppm.

Step 4. (2*S*,3*S*)-1-(Benzyloxy)-2-methyl-4-oxoazetidin-3-yl ammonium tosylate salt.

In a heart shaped flask purged with nitrogen, **16** (2.72 g, 8.87 mmol) was mixed with *p*-toluensulfonic acid (1.60 g, 9.30 mmol) and the solid mixture was cooled to 0 °C. Subsequently trifluoroacetic acid (36 mL) was added dropwise over 20 min and the reaction was left to stir at 0 °C for 15 min. The solution was rotary evaporated maintaining the bath below 30 °C and the obtained oil was left under high vacuum for 1 h. Et_2O (20.0 mL) was added to the obtained crude and a white precipitate was observed. After washing with Et_2O (5 × 10 mL) the title compound (3.35 g, 8.85 mmol, quantitative) was recovered as white solid. ^{1}H NMR (DMSO-d_6): δ 8.70 (s, 3H), 7.38–7.54 (m, 7H), 7.12 (d, J = 7.85 Hz, 2H), 4.99 (d, J = 2.64 Hz, 2H), 3.98–4.07 (m, 1H), 3.83–3.96 (m, 1H), 2.29 (s, 3H), 1.26 (d, J = 6.10 Hz, 3H) ppm.

Step 5. 4-Cyclohexylbutyl 2-pyridyl carbonate (**S18$_1$**) and 4-cyclohexylbutyl 2-oxopyridine-1-carboxylate (**S18$_2$**).

Under nitrogen atmosphere, to a stirred mixture of 4-cyclohexylbutan-1-ol (4.42 mL, 25.6 mmol) in dry CH_2Cl_2 (20 mL), DMAP (0.305 g, 2.5 mmol) and 2-DPC (6.64 g, 30.72 mmol) were added. The reaction mixture was left to react at room temperature for 15 h, then diluted with CH_2Cl_2 (7.0 mL) and washed first with a saturated NH_4Cl solution (10.0 mL) and subsequently with a saturated $NaHCO_3$ aqueous solution (3 × 10 mL). The organic fraction was dried over Na_2SO_4, filtered and concentrated to dryness to afford a colorless oil (7.10 g, 25.6 mmol, quantitative), as a mixture of 4-cyclohexylbutyl 2-pyridyl carbonate and 4-cyclohexylbutyl 2-oxopyridine-1-carboxylate (ratio 1.6:1). The mixture of isomers was used in the next step without any further purification. MS (ESI) m/z: 277.9 $[M\text{-}H]^+$.

Step 6. 4-Cyclohexylbutyl-((2*S*,3*S*)-1-(benzyloxy)-2-methyl-4-oxoazetidin-3-yl)carbamate (**17**).

Under nitrogen atmosphere, a solution of (2*S*,3*S*)-1-(benzyloxy)-2-methyl-4-oxoazetidin-3-yl ammonium tosylate salt (3.35 g, 8.85 mmol) in dry CH_2Cl_2 (70 mL) was treated with DIPEA (1.53 mL, 8.85 mmol). A solution containing a mixture (ratio 1.6:1) of **S18$_1$** and **S18$_2$** (6.95 g, 25.1 mmol) in dry CH_2Cl_2 (5 mL) was then added. The reaction was left to stir under nitrogen atmosphere at room temperature for 15 h. The solvent was evaporated and the crude product was dissolved in EtOAc (10 mL), washed first with a saturated NH_4Cl aqueous solution (20.0 mL), subsequently with a saturated $NaHCO_3$ (3 × 20 mL) and NaCl (20 mL) aqueous solutions. The organic layer was dried over Na_2SO_4, filtered and evaporated to dryness. The crude was absorbed over silica gel and purified by column chromatography using a Teledyne ISCO apparatus, eluting with Cy/TBME (from 100:0 to 30:70) to afford the title compound (2.06 g, 60%), as colorless oil. MS (ESI) m/z: 389.2 $[M\text{-}H]^+$, 406.3 $[M\text{-}NH_4]^+$, 387.2 $[M\text{-}H]^-$. ^{1}H NMR (DMSO-d_6): δ 7.83 (d, J = 8.03 Hz, 1H), 7.29–7.49 (m, 5H), 4.92 (s, 2H), 4.01 (dd, J = 8.03, 2.25 Hz, 1H), 3.94 (td, J = 6.73, 2.95 Hz, 2H), 3.99–4.07 (m, 1H), 3.91–4.00 (m, 1H), 3.68–3.79 (m, 1H), 1.58–1.71 (m, 5H), 1.52 (p, J = 6.73 Hz, 2H), 1.26–1.36 (m, 2H), 1.20 (d, J = 6.14 Hz, 3H), 1.12–1.19 (m, 6H), 0.76–0.92 (m, 2H) ppm. ^{13}C NMR (DMSO-d_6): δ 162.2, 155.7, 135.3, 129.1, 128.7, 128.4, 77.4, 64.3, 60.5, 59.6, 36.9, 36.5, 32.8, 28.8, 26.2, 25.8, 22.6, 15.8.

Step 7. 4-Cyclohexylbutyl-((2*S*,3*S*)-1-hydroxy-2-methyl-4-oxoazetidin-3-yl)carbamate (**18**).

A solution of **17** (2.06 g, 5.31 mmol) in absolute EtOH (100 mL) was submitted to ten cycles of vacuum and argon. Subsequently cyclohexadiene (5.02 mL, 53.0 mmol) and palladium on activated charcoal (2.06 g) were added to the solution and three more cycles of vacuum and argon were made. The reaction was left to stir under argon atmosphere for 4 h at room temperature. The palladium was removed over celite and the crude product was extracted with EtOAc. The solvent was evaporated under reduced pressure and the crude product was absorbed over silica gel and purified by column chromatography using a Teledyne ISCO apparatus, eluting with Cy/TBME (from 100:0 to 40:60) to afford the title compound (1.23 g, 78%), as white solid. MS (ESI) m/z: 299.2 $[M\text{-}H]^+$, 316.2 $[M\text{-}NH_4]^+$, 297.2 $[M\text{-}H]^-$. ^{1}H NMR (DMSO-d_6): δ 10.13 (s, 1H), 7.83 (d, J = 8.25 Hz, 1H), 3.98–4.06 (m, 1H), 3.95 (td, J = 6.74, 3.35 Hz, 2H), 3.56–3.67 (m, 1H), 1.56–1.72 (m, 5H), 1.51 (p, J = 6.74 Hz, 2H), 1.30–1.37 (m, 2H), 1.26 (d, J = 6.07 Hz, 3H), 1.05–1.23 (m, 6H), 0.76–0.94 (m, 2H) ppm. ^{13}C NMR (DMSO-d_6): δ 162.2, 152.3, 64.7, 61.7, 60.2, 37.4, 36.9, 33.3, 29.3, 26.7, 26.3, 23.1, 16.1 ppm.

5.1.11. General procedure (GP1) for the synthesis of N-O-alkyl β-lactams (19–29)

Under nitrogen atmosphere, to a 0 °C solution of **18** (1.0 equiv.) and triphenylphosphine (1.2 equiv) in dry THF (3.0 mL), desired alcohol (1.2 equiv.) was added. Subsequently DEAD (solution 40% in toluene, 1.2 equiv.) was added dropwise. The mixture was left stirring under nitrogen at 0 °C for 30 min and at room temperature for 3 h. The solvent was removed under reduced pressure, and the resulting crude product extracted with EtOAc (3.0 mL), washed first with a saturated NH_4Cl aqueous solution (3.0 mL) and then with a saturated $NaHCO_3$ aqueous solution (3 × 3.0 mL). The organic fraction was dried over Na_2SO_4, filtered and concentrated to dryness. The crude product was purified either by column chromatography using a Teledyne ISCO apparatus or preparative HPLC to afford the corresponding *N-O*-alkyl substituted β-lactams.

5.1.11.1. 4-Cyclohexylbutyl-((2S,3S)-2-methyl-4-oxo-1-propoxyazetidin-3-yl)carbamate (19). The reaction was carried out following the general procedure GP1 employing **18** (0.042 g, 0.24 mmol), triphenylphosphine (0.058 g, 0.22 mmol), propan-1-ol (0.019 mL, 0.24 mmol) and DEAD (0.110 mL, 0.24 mmol). Purification by column chromatography using a Teledyne ISCO apparatus, eluting with Cy/TBME (from 100:0 to 60:40) afforded the title compound (0.035 g, 25%), as a colorless oil. MS (ESI) m/z: 341.3 $[M\text{-}H]^+$, 358.3 $[M\text{-}NH_4]^+$. $[\alpha]_{D20}$ (c = 0.1, $CHCl_3$): +7.74. ^{1}H NMR (DMSO-d_6): δ 7.83 (d, J = 7.95 Hz, 1H), 4.04 (dd, J = 7.95, 1.96 Hz, 1H), 3.95 (td, J = 6.58, 2.40 Hz, 2H), 3.90–3.79 (m, 3H), 1.71-1-58 (m, 7H), 1.56–1.46 (m, 2H), 1.32 (d, J = 6.07 Hz, 3H), 1.35–1.30 (m, 2H), 1.25–1.07 (m, 6H), 0.91 (t, J = 7.41 Hz, 3H), 0.88–0.78 (m, 2H). ppm. ^{13}C NMR (DMSO-d_6): δ 161.9, 155.7, 76.8, 64.2, 59.9, 59.5, 36.9, 36.5, 32.8, 28.8, 26.2, 25.8, 22.6, 21.1, 15.9, 9.9 ppm.

5.1.11.2. 4-Cyclohexylbutyl-((2S,3S)-2-methyl-4-oxo-1-(pentyloxy) azetidin-3-yl)carbamate (20). The reaction was carried out following the general procedure GP1 employing **18** (0.070 g, 0.23 mmol), triphenylphosphine (0.073 g, 0.28 mmol), pentan-1-ol (0.031 mL, 0.28 mmol) and DEAD (0.120 mL, 0.28 mmol). Purification by column chromatography using a Teledyne ISCO apparatus, eluting with Cy/TBME (from 100:0 to 50:50) afforded the title compound (0.040 g, 48%), as a colorless oil. MS (ESI) m/z: 369.2 $[M\text{-}H]^+$, 386.3 $[M\text{-}NH_4]^+$, 367.2 $[M\text{-}H]^-$. $[\alpha]_{D20}$ (c = 0.1, $CHCl_3$): −3.94. ^{1}H NMR (DMSO-d_6): δ 8.15 (d, J = 8.15 Hz, 1H), 4.00–4.10 (m, 1H), 3.95 (td, J = 6.75, 2.62 Hz, 2H), 3.89 (td, J = 6.55, 4.30 Hz, 2H), 3.83 (qd, J = 6.09, 2.09 Hz, 1H), 1.55–1.72 (m, 7H), 1.52 (p, J = 6.78 Hz, 2H), 1.26–1.37 (m, 4H), 1.32 (d, J = 6.09 Hz, 3H), 1.07–1.26 (m, 8H), 0.79–0.94 (m, 5H) ppm. ^{13}C NMR (DMSO-d_6): δ 161.9, 155.7, 75.3, 64.3, 59.9, 59.5, 36.9, 36.5, 32.8, 28.8, 27.4, 27.3, 26.2, 25.9, 22.6, 21.8, 15.9, 13.9 ppm.

5.1.11.3. 4-Cyclohexylbutyl-((2S,3S)-1-(heptyloxy)-2-methyl-4-oxoazetidin-3-yl)carbamate (21). The reaction was carried out following the general procedure GP1 employing **18** (0.07 g,

0.23 mmol), triphenylphosphine (0.073 g, 0.28 mmol), heptan-1-ol (0.040 mL, 0.28 mmol) and DEAD (0.122 mL, 0.28 mmol). Purification by column chromatography using a Teledyne ISCO apparatus, eluting with Cy/EtOAc (from 100:0 to 70:30) afforded the title compound (0.046 g, 50%), as a colorless oil. MS (ESI) *m/z*: 397.4 $[M-H]^+$, 414.5 $[M-NH_4]^+$, 395.4 $[M-H]^-$. $[\alpha]_{D20}$ (c = 0.1, $CHCl_3$): −4.48. ^{1}H NMR (DMSO-d_6): δ 7.83 (d, *J* = 8.11 Hz, 1H), 4.03 (d, *J* = 8.11 Hz, 1H), 3.95 (td, *J* = 6.60, 2.50, 2H), 3.88 (td, *J* = 4.21, 6.60 Hz, 2H), 3.82 (d, *J* = 6.20 Hz, 1H), 1.74–1.55 (m, 7H), 1.55–1.46 (m, 2H), 1.31 (d, *J* = 6.20 Hz, 3H), 1.37–1.22 (m, 8H), 1.23–1.05 (m, 6H), 0.92–0.76 (m, 5H) ppm. ^{13}C NMR (DMSO-d_6): δ 161.9, 155.7, 76.7, 64.9, 60.3, 60.6, 36.9, 36.5, 32.8, 31.2, 28.8, 28.3, 26.2, 25.3, 25.0, 22.6, 22.0, 13.9 ppm.

5.1.11.4. 4-Cyclohexylbutyl-((2S,3S)-1-(cyclohexyloxy)-2-methyl-4-oxoazetidin-3-yl)carbamate (22). The reaction was carried out following the general procedure GP1 employing **18** (0.08 g, 0.27 mmol), triphenylphosphine (0.084 g, 0.32 mmol), cyclohexanol (0.034 mL, 0.32 mmol) and DEAD (0.14 mL, 0.32 mmol). Purification by column chromatography using a Teledyne ISCO apparatus, eluting with Cy/TBME (from 100:0 to 50:50) afforded the title compound (0.04 g, 48%), as a colorless oil. MS (ESI) *m/z*: 381.3 $[M-H]^+$, 398.3 $[M-NH_4]^+$, 379.2 $[M-H]^-$. $[\alpha]_{D20}$ (c = 0.1, $CHCl_3$): −23.47. ^{1}H NMR (DMSO-d_6): δ 7.85 (d, *J* = 8.23 Hz, 1H), 4.09–4.00 (m, 2H), 3.98–3.93 (m, 2H), 3.93–3.85 (m, 1H), 3.85–3.05 (m, 1H), 1.73–1.56 (m, 7H), 1.52 (p, *J* = 6.90 Hz, 2H), 1.32 (d, *J* = 6.04 Hz, 3H), 1.35–1.30 (m, 5H), 1.27–1.08 (m, 8H), 0.94–0.78 (m, 5H) ppm. ^{13}C NMR (DMSO-d_6): δ 161.9, 155.7, 82.9, 64.9, 61.6, 60.3, 37.1, 33.5, 33.4, 31.1, 31.0, 29.4, 26.8, 23.4, 23.3, 16.3 ppm.

5.1.11.5. 4-Cyclohexylbutyl-((2S,3S)-1-(3-methoxypropoxy)-2-methyl-4-oxoazetidin-3-yl)carbamate (23). The reaction was carried out following the general procedure GP1 employing **18** (0.05 g, 0.17 mmol), triphenylphosphine (0.052 g, 0.20 mmol), 3-methoxypropan-1-ol (0.019 mL, 0.20 mmol) and DEAD (0.087 mL, 0.20 mmol). Purification by column chromatography using a Teledyne ISCO apparatus, eluting with Cy/EtOAc (from 100:0 to 70:30) afforded the title compound (0.025 g, 39%), as a colorless oil. MS (ESI) *m/z*: 371.3 $[M-H]^+$, 388.4 $[M-NH_4]^+$, 369.3 $[M-H]^-$. $[\alpha]_{D20}$ (c = 0.05, $CHCl_3$): −7.67°. ^{1}H NMR (DMSO-d_6): δ 7.84 (d, *J* = 8.23 Hz, 1H), 4.08–4.02 (m, 1H), 4.00–3.87 (m, 4H), 3.86–3.78 (m, 1H), 3.41 (td, *J* = 6.32, 1.28 Hz, 2H), 3.23 (s, 3H), 1.84 (td, *J* = 6.32, 2.26 Hz, 2H), 1.71–1.57 (m, 5H), 1.51 (p, *J* = 6.79 Hz, 2H), 1.31 (d, *J* = 6.12 Hz, 3H), 1.34–1.29 (m, 1H), 1.26–1.02 (m, 7H), 0.91–0.76 (m, 2H) ppm. ^{13}C NMR (DMSO-d_6): δ 162.6, 152.1, 72.9, 68.5, 64.8, 60.0, 60.3, 58.4, 36.9, 33.3, 33.1, 29.2, 28.3, 26.6, 25.1, 22.9, 16.3 ppm.

5.1.11.6. 4-Cyclohexylbutyl-((2S,3S)-2-methyl-1-(3-(methylthio)propoxy)-4-oxoazetidin-3-yl)carbamate (24). The reaction was carried out following the general procedure GP1 employing **18** (0.08 g, 0.27 mmol), triphenylphosphine (0.084 g, 0.32 mmol), 3-methylsulfanylpropan-1-ol (0.033 mL, 0.32 mmol) and DEAD (0.140 mL, 0.32 mmol). Purification by column chromatography using a Teledyne ISCO apparatus, eluting with Cy/TBME (from 100:0 to 50:50) afforded the title compound (0.060 g, 58%), as a colorless oil. MS (ESI) *m/z*: 387.2 $[M-H]^+$, 404.2 $[M-NH_4]^+$, 385.2 $[M-H]^-$. $[\alpha]_{D20}$ (c = 0.05, $CHCl_3$): −247.70. ^{1}H NMR (DMSO-d_6): δ 7.85 (d, *J* = 8.14 Hz, 1H), 4.04 (dd, *J* = 8.14, 2.97 Hz, 1H), 4.00–3.91 (m, 4H), 3.83 (qd, *J* = 6.02, 2.97 Hz, 1H), 2.56 (t, *J* = 7.22 Hz, 2H), 2.05 (s, 3H), 1.96–1.79 (m, 2H), 1.74–1.55 (m, 5H), 1.51 (p, *J* = 6.84 Hz, 2H), 1.32 (d, *J* = 6.02 Hz, 3H), 1.37–1.27 (m, 2H), 1.20–1.06 (m, 6H), 0.91–0.74 (m, 2H) ppm. ^{13}C NMR (DMSO-d_6): δ 162.2, 155.7, 73.7, 64.3, 59.8, 59.5, 36.7, 36.5, 32.8, 29.3, 28.8, 27.3, 26.2, 25.9, 22.6, 15.9, 14.6 ppm.

5.1.11.7. 4-Cyclohexylbutyl-((2S,3S)-2-methyl-1-(3-(methylsulfonyl)propoxy)-4-oxoazetidin-3-yl)carbamate (25). The reaction was carried out following the general procedure GP1 employing **18** (0.1 g, 0.33 mmol), triphenylphosphine (0.104 g, 0.40 mmol), 3-(methylsulfonyl)propan-1-ol (0.055 mL, 0.40 mmol) and DEAD (0.175 mL, 0.40 mmol). Purification by column chromatography using a Teledyne ISCO apparatus, eluting with Cy/EtOAc (from 100:0 to 55:45) afforded the title compound (0.074 g, 54%), as a colorless oil. MS (ESI) *m/z*: 419.2 $[M-H]^+$, 436.2 $[M-NH_4]^+$, 417.2 $[M-H]^-$. $[\alpha]_{D20}$ (c = 0.1, $CHCl_3$): −11.81. ^{1}H NMR (DMSO-d_6): δ 7.86 (d, *J* = 8.11 Hz, 1H), 4.08–4.03 (m, 1H), 4.00 (t, *J* = 6.20 Hz, 2H), 3.95 (dt, *J* = 7.55, 3.47 Hz, 2H), 3.90–3.82 (m, 1H), 3.23 (dt, *J* = 8.93, 6.27 Hz, 2H), 2.98 (s, 3H), 2.16–2.00 (m, 2H), 177–1.56 (m, 5H), 1.51 (p, *J* = 6.80 Hz, 2H), 1.37–1.31 (m, 2H), 1.33 (d, *J* = 6.12 Hz, 2H), 1.29 (d, *J* = 8.08 Hz, 2H), 1.24–1.06 (m, 7H), 0.93–0.78 (m, 2H) ppm. ^{13}C NMR (DMSO-d_6): δ 162.2, 156.1, 73.6, 64.8, 60.3, 59.9, 55.4, 50.7, 37.4, 36.9, 33.3, 29.3, 26.6, 26.3, 23.07, 21.2, 16.3 ppm.

5.1.11.8. 4-Cyclohexylbutyl-((2S,3S)-2-methyl-4-oxo-1-((4-oxopentyl)oxy)azetidin-3-yl)carbamate (26). The reaction was carried out following the general procedure GP1 employing **18** (0.08 g, 0.27 mmol), triphenylphosphine (0.084 g, 0.32 mmol), 5-hydroxypentan-2-one (0.033 mL, 0.32 mmol) and DEAD (0.140 mL, 0.32 mmol). Purification by column chromatography using a Teledyne ISCO apparatus, eluting with Cy/TBME (from 100:0 to 50:50) gave a crude product, which was submitted to a preparative HPLC/MS using a Waters Autopurification system affording the title compound (0.03 g, 30%), as a colorless oil. MS (ESI) *m/z*: 383.2 $[M-H]^+$, 400.3 $[M-NH_4]^+$, 381.2 $[M-H]^-$. $[\alpha]_{D20}$ (c = 0.04, $CHCl_3$): +6.65. ^{1}H NMR (DMSO-d_6): δ 7.84 (d, *J* = 8.13 Hz, 1H), 4.04 (dd, *J* = 8.13, 1.94 Hz, 1H), 3.95 (td, *J* = 6.69, 2.34 Hz, 2H), 3.86 (t, *J* = 6.45 Hz, 2H), 3.83–3.77 (m, 1H), 2.60–2.52 (m, 2H), 2.09 (s, 3H), 1.83–1.72 (m, 2H), 1.72–1.56 (m, 5H), 1.51 (p, *J* = 6.69 Hz, 2H), 1.35–1.24 (m, 2H), 1.31 (d, *J* = 6.12 Hz, 3H), 1.25–1.05 (m, 6H), 0.93–0.76 (m, 2H) ppm. ^{13}C NMR (DMSO-d_6): δ 162.2, 156.1, 74.9, 64.8, 60.4, 60.0, 39.2, 37.0, 33.4, 33.3, 33.2, 29.3, 29.4, 27.9, 26.7, 23.1, 22.5, 16.3 ppm.

5.1.11.9. 4-Cyclohexylbutyl-((2S,3S)-1-(2-ethoxyethoxy)-2-methyl-4-oxoazetidin-3-yl)carbamate (27). The reaction was carried out following the general procedure GP1 employing **18** (0.1 g, 0.33 mmol), triphenylphosphine (0.102 g, 0.39 mmol), 2-ethoxyethan-1-ol (0.037 mL, 0.39 mmol) and DEAD (0.17 mL, 0.39 mmol). Purification by column chromatography using a Teledyne ISCO apparatus, eluting with Cy/EtOAc (from 100:0 to 70:30) gave a crude product, which was submitted to a preparative HPLC/MS using a Waters Autopurification system affording the title compound (0.02 g, 16%), as a colorless oil. MS (ESI) *m/z*: 371.3 $[M-H]^+$, 388.3 $[M-NH_4]^+$, 369.2 $[M-H]^-$. $[\alpha]_{D20}$ (c = 0.1, $CHCl_3$): +9.93. ^{1}H NMR (DMSO-d_6): δ 7.83 (d, *J* = 8.22 Hz, 1H), 4.06–3.98 (m, 3H), 3.95 (dt, *J* = 6.71, 3.66 Hz, 2H), 3.88–3.78 (m, 1H), 3.60–3.56 (m, 2H), 3.46 (q, *J* = 6.99 Hz, 2H), 1.72–1.56 (m, 5H), 1.51 (p, *J* = 6.71 Hz, 2H), 1.32 (d, *J* = 6.09 Hz, 3H), 1.35–1.25 (m, 1H), 1.25–1.15 (m, 7H), 1.12 (t, *J* = 6.99 Hz, 3H), 0.91–0.78 (m, 2H). ppm. ^{13}C NMR (DMSO-d_6): δ 162.5, 156.1, 75.5, 67.8, 66.1, 64.4, 60.8, 59.7, 36.8, 32.8, 28.9, 28.8, 26.3, 26.4, 22.7, 15.9, 15.6 ppm.

5.1.11.10. 4-Cyclohexylbutyl-((2S,3S)-1-(4-cyclohexylbutoxy)-2-methyl-4-oxoazetidin-3-yl)carbamate (28). The reaction was carried out following the general procedure GP1 employing **18** (0.09 g, 0.30 mmol), triphenylphosphine (0.094 g, 0.36 mmol), 4-cyclohexylbutan-1-ol (0.062 mL, 0.36 mmol) and DEAD (0.157 mL, 0.36 mmol). Purification by column chromatography using a Teledyne ISCO apparatus, eluting with Cy/TBME (from 100:0 to 50:50) afforded the title compound (0.054 g, 41%), as a colorless oil. MS

(ESI) *m/z*: no ionization profile. $[\alpha]_{D20}$ (c = 0.1, $CHCl_3$): −90.56. ^{1}H NMR (DMSO-d_6): δ 7.82 (d, *J* = 8.12 Hz, 1H), 4.06–3.99 (m, 1H), 3.95 (td, *J* = 6.65, 2.55 Hz, 2H), 3.91–3.84 (m, 2H), 3.84–3.77 (m, 1H), 1.71–1.45 (m, 13H), 1.38–1.25 (m, 4H), 1.31 (d, *J* = 6.16 Hz, 3H), 1.24–1.04 (m, 13H), 0.91–0.77 (m, 4H). ppm. ^{13}C NMR (DMSO-d_6): δ 161.9, 155.7, 75.3, 64.3, 59.8, 59.5, 36.96, 36.93, 36.5, 36.4, 32.8, 28.8, 27.9, 26.2, 25.8, 22.6, 22.3, 15.9 ppm.

5.1.11.11. 4-Cyclohexylbutyl-((2S,3S)-2-methyl-4-oxo-1-(4-phenylbutoxy)azetidin-3-yl)carbamate (29). The reaction was carried out following the general procedure GP1 employing **18** (0.08 g, 0.27 mmol), triphenylphosphine (0.044 g, 0.32 mmol), 4-cyclohexylbutan-1-ol (0.044 g, 0.32 mmol) and DEAD (0.14 mL, 0.32 mmol). Purification by column chromatography using a Teledyne ISCO apparatus, eluting with Cy/TBME (from 100:0 to 50:50) afforded the title compound (0.029 g, 32%), as a colorless oil. MS (ESI) *m/z*: 431.4 $[M-H]^+$, 448.4 $[M-NH_4]^+$, 429.4 $[M-H]^-$. $[\alpha]_{D20}$ (c = 0.1, $CHCl_3$): −980.7. ^{1}H NMR (DMSO-d_6): δ 7.83 (d, *J* = 8.13 Hz, 1H), 7.31–7.23 (m, 2H), 7.22–7.12 (m, 3H), 4.07–4.00 (m, 1H), 4.00–3.85 (m, 4H), 3.84–3.76 (m, 1H), 2.60 (t, *J* = 7.15 Hz, 2H), 1.74–1.59 (m, 8H), 1.57–1.42 (m, 2H), 1.34–1.27 (m, 3H), 1.30 (d, *J* = 6.08 Hz, 2H), 1.27–1.02 (m, 7H), 0.96–0.72 (m, 2H) ppm. ^{13}C NMR (DMSO-d_6): δ 161.9, 141.9, 128.2, 125.7, 75.1, 64.3, 59.5, 36.9, 36.5, 34.7, 32.8, 28.8, 27.3, 27.0, 26.1, 25.8, 22.6, 15.9 ppm.

5.1.12. General procedure (GP2) for the synthesis on N-O-aryl β-lactams (30–39)

Under nitrogen atmosphere, a round bottom flask was charged with freshly activated 4 Å molecular sieves (0.15 g), copper chloride (1.0 equiv.), **18** (1.0 equiv.) and the desired boronic acid (2.0 equiv.) in dry DCM (3.0 mL). The suspension was left to stir 20 min at room temperature. Subsequently pyridine (1.1 equiv.) was added dropwise and immediately the solution turned into a brown coloration. The mixture was left to stir at open air 5 h at room temperature. The crude product was absorbed over silica gel and purified either by column chromatography using a Teledyne ISCO apparatus or preparative HPLC to afford the corresponding *N-O*-aryl substituted β-lactams.

5.1.12.1. 4-Cyclohexylbutyl-((2S,3S)-2-methyl-4-oxo-1-phenoxyazetidin-3-yl)carbamate (30). The reaction was carried out following the general procedure GP2 employing **18** (0.1 g, 0.33 mmol), copper chloride (0.033 g, 0.33 mmol), phenylboronic acid (0.08 g, 0.66 mmol) and pyridine (0.029 mL, 0.36 mmol). Purification by column chromatography using a Teledyne ISCO apparatus, eluting with Cy/TBME (from 100:0 to 80:20) afforded the title compound (0.038 g, 43%), as a white powder. MS (ESI) *m/z*: 375.3 $[M-H]^+$, 392.3 $[M-NH_4]^+$. $[\alpha]_{D20}$ (c = 0.1, $CHCl_3$): −102.89. ^{1}H NMR (DMSO-d_6): δ 8.03 (d, *J* = 8.21 Hz, 1H), 7.38 (dd, *J* = 7.27, 8.40 Hz, 2H), 7.23 (d, *J* = 8.40 Hz, 2H), 7.18–7.07 (m, 1H), 4.33 (dd, *J* = 8.12, 2.28 Hz, 1H), 4.06 (dd, *J* = 2.28, 6.25 Hz, 1H), 4.04–3.97 (m, 2H), 1.73–1.60 (m, 5H), 1.59–1.47 (m, 2H), 1.35 (d, *J* = 6.25 Hz, 3H), 1.33–1.25 (m, 2H), 1.27–1.02 (m, 6H), 0.96–0.77 (m, 2H) ppm. ^{13}C NMR (DMSO-d_6): δ 163.4, 158.2, 155.7, 129.7, 123.5, 113.4, 64.4, 61.1, 59.9, 36.9, 36.5, 32.8, 28.8, 26.2, 25.8, 22.6, 15.4 ppm.

5.1.12.2. 4-Cyclohexylbutyl-((2S,3S)-2-methyl-4-oxo-1-(p-tolyloxy) azetidin-3-yl)carbamate (31). The reaction was carried out following the general procedure GP2 employing **18** (0.1 g, 0.33 mmol), copper chloride (0.033 g, 0.33 mmol), *p*-tolylboronic acid (0.089 g, 0.66 mmol) and pyridine (0.029 mL, 0.36 mmol). Purification by column chromatography using a Teledyne ISCO apparatus, eluting with Cy/TBME (from 100:0 to 80:20) afforded a crude product, which was submitted to a preparative HPLC/MS using a Waters Autopurification system affording the title compound (0.028 g, 22%), as a white powder. MS (ESI) *m/z*: 389.3 $[M-H]^+$, 406.3 $[M-NH_4]^+$, 387.3 $[M-H]^-$. $[\alpha]_{D20}$ (c = 0.1, $CHCl_3$): −9.52. ^{1}H NMR (DMSO-d_6): δ 8.02 (d, *J* = 8.20 Hz, 1H), 7.21–7.15 (m, 2H), 7.12 (d, *J* = 8.42 Hz, 2H), 4.35–4.29 (m, 1H), 4.03–3.95 (m, 3H), 2.28 (s, 3H), 1.73–1.60 (m, 5H), 1.58–1.49 (m, 2H), 1.34 (d, *J* = 6.09 Hz, 3H), 1.37–1.32 (m, 2H), 1.22–1.10 (m, 6H), 0.93–0.78 (m, 2H) ppm. ^{13}C NMR (DMSO-d_6): δ 163.3, 156.2, 155.7, 132.6, 130.0, 113.4, 64.4, 61.0, 59.9, 59.7, 36.9, 36.5, 32.8, 28.8, 26.2, 25.8, 22.6, 15.5 ppm.

5.1.12.3. 4-Cyclohexylbutyl-((2S,3S)-2-methyl-4-oxo-1-(4-(trifluoromethyl)phenoxy)azetidin-3-yl)carbamate (32). The reaction was carried out following the general procedure GP2 employing **18** (0.1 g, 0.33 mmol), copper chloride (0.033 g, 0.33 mmol), (4-(trifluoromethyl)phenyl)boronic acid (0.125 g, 0.66 mmol) and pyridine (0.029 mL, 0.36 mmol). Purification by column chromatography using a Teledyne ISCO apparatus, eluting with Cy/TBME (from 100:0 to 80:20) afforded the title compound (0.082 g, 52%), as a white powder. MS (ESI) *m/z*: 443.3 $[M-H]^+$, 460.3 $[M-NH_4]^+$, 441.3 $[M-H]^-$. $[\alpha]_{D20}$ (c = 0.1, $CHCl_3$): −30.33. ^{1}H NMR (DMSO-d_6): δ 8.04 (d, *J* = 8.23 Hz, 1H), 7.76 (d, *J* = 8.55 Hz, 2H), 7.46 (d, *J* = 8.55 Hz, 2H), 4.38 (dd, *J* = 8.23, 3.03 Hz, 1H), 4.12 (dt, *J* = 6.72, 3.03 Hz, 1H), 4.01 (t, *J* = 6.57 Hz, 2H), 1.74–1.60 (m, 5H), 1.60–1.48 (m, 2H), 1.38 (d, *J* = 6.72 Hz, 3H), 1.37–1.27 (m, 2H), 1.27–1.03 (m, 6H), 0.99–0.76 (m, 2H) ppm. ^{13}C NMR (DMSO-d_6): δ 164.1, 161.1, 155.7, 127.4, 125.4, 99.5, 114.0, 64.5, 61.2, 59.9, 36.9, 36.5, 32.8, 28.8, 26.2, 25.8, 22.6, 15.40 ppm.

5.1.12.4. 4-Cyclohexylbutyl-((2S,3S)-2-methyl-1-(4-nitrophenoxy)-4-oxoazetidin-3-yl)carbamate (33). The reaction was carried out following the general procedure GP2 employing **18** (0.1 g, 0.33 mmol), copper chloride (0.033 g, 0.33 mmol), (4-nitrophenyl) boronic acid (0.110 g, 0.66 mmol) and pyridine (0.029 mL, 0.36 mmol) Purification by column chromatography using a Teledyne ISCO apparatus, eluting with Cy/TBME (from 100:0 to 80:20) afforded the title compound (0.080 g, 58%), as a white powder. MS (ESI) *m/z*: no ionization profile. $[\alpha]_{D20}$ (c = 0.015, $CHCl_3$): −27.43. ^{1}H NMR (DMSO-d_6): δ 8.28 (d, *J* = 9.23 Hz, 2H), 8.04 (d, *J* = 8.18 Hz, 1H), 7.50 (d, *J* = 9.23 Hz, 2H), 4.41 (dd, *J* = 8.18, 2.42 Hz, 1H), 4.15 (dd, *J* = 6.13, 2.42 Hz, 1H), 4.01 (t, *J* = 6.55 Hz, 2H), 1.72–1.59 (m, 5H), 1.59–1.50 (m, 2H), 1.39 (d, *J* = 6.13 Hz, 3H), 1.37–1.28 (m, 2H), 1.26–1.07 (m, 6H), 0.95–0.77 (m, 2H) ppm. ^{13}C NMR (DMSO-d_6): δ 164.4, 163.2, 143.2, 126.0, 114.1, 64.5, 61.4, 59.9, 36.9, 36.5, 32.8, 28.8, 26.2, 25.8, 22.6, 15.4 ppm.

5.1.12.5. Methyl 4-(((2S,3S)-3-(((4-cyclohexylbutoxy)carbonyl) amino)-2-methyl-4-oxoazetidin-1-yl)oxy)benzoate (34). The reaction was carried out following the general procedure GP2 employing **18** (0.1 g, 0.33 mmol), copper chloride (0.033 g, 0.33 mmol), (4-(methoxycarbonyl)phenyl)boronic acid (0.118 g, 0.66 mmol) and pyridine (0.029 mL, 0.36 mmol). Purification by column chromatography using a Teledyne ISCO apparatus, eluting with Cy/TBME (from 100:0 to 60:40) affording the title compound (0.032 g, 23%), as a white powder. MS (ESI) *m/z*: 433.3 $[M-H]^+$, 450.3 $[M-NH_4]^+$. $[\alpha]_{D20}$ (c = 0.1, $CHCl_3$): −131.71. ^{1}H NMR (DMSO-d_6): 8.04 (d, *J* = 8.19 Hz, 1H), 7.98 (d, *J* = 8.68 Hz, 2H), 7.38 (d, *J* = 8.68 Hz, 2H), 4.38 (dd, *J* = 8.19, 2.28 Hz, 1H), 4.11 (d, *J* = 6.67 Hz, 1H), 4.06–3.92 (m, 2H), 3.84 (s, 3H), 1.72–1.62 (m, 5H), 1.55 (p, *J* = 6.80 Hz, 2H), 1.38 (d, *J* = 6.67 Hz, 3H), 1.36–1.27 (m, 2H), 1.28–1.05 (m, 6H), 0.96–0.73 (m, 2H). ppm. ^{13}C NMR (DMSO-d_6): δ 164.0, 162.0, 131.4, 124.9, 113.5, 65.1, 61.6, 60.6, 52.5, 36.9, 36.5, 32.8, 30.7, 28.8, 26.2, 25.8, 22.6, 15.4 ppm.

5.1.12.6. 4-Cyclohexylbutyl-((2S,3S)-2-methyl-4-oxo-1-(4-phenoxyphenoxy)azetidin-3-yl)carbamate (35). The reaction was

carried out following the general procedure GP2 employing **18** (0.05 g, 0.16 mmol), copper chloride (0.016 g, 0.16 mmol), (4-phenoxyphenyl)boronic acid (0.068 g, 0.32 mmol) and pyridine (0.013 mL, 0.17 mmol). Purification by column chromatography using a Teledyne ISCO apparatus, eluting with Cy/TBME (from 100:0 to 60:40) afforded the title compound (0.025 g, 34%), as a colorless oil. MS (ESI) *m/z*: 467.2 $[M-H]^+$, 484.2 $[M-NH_4]^+$, 465.2 $[M-H]^-$. ^{1}H NMR (DMSO-d_6): δ 8.03 (d, J = 8.18 Hz, 1H), 7.42–7.32 (m, 2H), 7.27 (d, J = 8.78 Hz, 2H), 7.16–7.08 (m, 1H), 7.07–7.00 (m, 2H), 7.00–6.92 (m, 2H), 4.38–4.28 (m, 1H), 4.13–3.89 (m, 3H), 1.74–1.57 (m, 3H), 1.57–1.45 (m, 2H), 1.37 (d, J = 6.13 Hz, 3H), 1.35–1.25 (m, 2H), 1.22–1.07 (m, 6H), 0.93–0.73 (m, 2H) ppm. ^{13}C NMR (DMSO-d_6): δ 164.4, 163.2, 130.5, 123.3, 120.8, 118.1, 115.6, 64.9, 61.6, 60.6, 36.9, 33.4, 33.3, 29.3, 26.6, 25.3, 23.2, 16.3 ppm.

5.1.12.7. 4-Cyclohexylbutyl-((2S,3S)-1-([1,1'-biphenyl]-4-yloxy)-2-methyl-4-oxoazetidin-3-yl)carbamate (36). The reaction was carried out following the general procedure GP2 employing **18** (0.1 g, 0.33 mmol), copper chloride (0.033 g, 0.33 mmol), [1,1'-biphenyl]-4-ylboronic acid (0.13 g, 0.66 mmol) and pyridine (0.029 mL, 0.36 mmol). Purification by column chromatography using a Teledyne ISCO apparatus, eluting with Cy/EtOAc (from 100:0 to 60:40) afforded the title compound (0.036 g, 24%), as a white powder. MS (ESI) *m/z*: 451.3 $[M-H]^+$, 468.3 $[M-NH_4]^+$. $[\alpha]_{D20}$ (c = 0.1, $CHCl_3$): −10.48. ^{1}H NMR (DMSO-d_6): δ 8.05 (d, J = 8.14 Hz, 1H), 7.70–7.56 (m, 4H), 7.50–7.39 (m, 2H), 7.39–7.26 (m, 3H), 4.40–4.32 (m, 1H), 4.10 (d, J = 5.39 Hz, 1H), 4.05–3.97 (m, 2H), 1.71–1.60 (m, 5H), 1.60–1.50 (m, 2H), 1.39 (d, J = 5.39 Hz, 3H), 1.36–1.28 (m, 2H), 1.23–1.03 (m, 6H), 0.90–0.78 (m, 2H). ppm. ^{13}C NMR (DMSO-d_6): δ 164.4, 163.2, 129.1, 128.1, 127.5, 126.9, 114.5, 64.9, 61.5, 60.5, 36.9, 33.4, 33.3, 29.7, 26.8, 26.7, 23.0, 15.9 ppm.

5.1.12.8. 4-Cyclohexylbutyl-((2S,3S)-2-methyl-1-(4-(methylsulfonyl)phenoxy)-4-oxoazetidin-3-yl)carbamate (37). The reaction was carried out following the general procedure GP2 employing **18** (0.1 g, 0.33 mmol), copper chloride (0.036 g, 0.33 mmol), (4-(methylsulfonyl)phenyl)boronic acid (0.132 g, 0.66 mmol) and pyridine (0.029 mL, 0.36 mmol). Purification by column chromatography using a Teledyne ISCO apparatus, eluting with Cy/TBME (from 100:0 to 20:80) afforded a crude product, which was submitted to a preparative HPLC/MS using a Waters Autopurification system affording the title compound (0.054 g, 37%), as a white powder. MS (ESI) *m/z*: 453.2 $[M-H]^+$, 470.2 $[M-NH_4]^+$, 451.2 $[M-H]^-$. $[\alpha]_{D20}$ (c = 0.1, $CHCl_3$): −151.66. ^{1}H NMR (DMSO-d_6): δ 8.04 (d, J = 8.21 Hz, 1H), 7.93 (d, J = 8.81 Hz, 2H), 7.50 (d, J = 8.81 Hz, 2H), 4.39 (dd, J = 8.21, 2.34 Hz, 1H), 4.17–4.09 (m, 1H), 4.00 (t, J = 6.67 Hz, 2H), 3.21 (s, 3H), 1.73–1.61 (m, 5H), 1.54 (dt, J = 6.67, 14.40 Hz, 2H), 1.38 (d, J = 6.10 Hz, 3H), 1.36–1.27 (m, 2H), 1.26–1.04 (m, 6H), 0.94–0.77 (m, 2H). ppm. ^{13}C NMR (DMSO-d_6): δ 164.0, 162.0, 135.9, 129.5, 113.9, 43.7, 36.9, 36.5, 32.8, 28.8, 26.2, 25.9, 22.6, 15.40 ppm.

5.1.12.9. 4-Cyclohexylbutyl-((2S,3S)-2-methyl-1-(3-(methylsulfonyl)phenoxy)-4-oxoazetidin-3-yl)carbamate (38). The reaction was carried out following the general procedure GP2 employing **18** (0.08 g, 0.27 mmol), copper chloride (0.027 g, 0.27 mmol), (3-(methylsulfonyl)phenyl)boronic acid (0.108 g, 0.54 mmol) and pyridine (0.023 mL, 0.29 mmol). Purification by column chromatography using a Teledyne ISCO apparatus, eluting with Cy/TBME (from 100:0 to 60:40) afforded the title compound (0.060 g, 49%), as a white powder. MS (ESI) *m/z*: 453.2 $[M-H]^+$, 470.2 $[M-NH_4]^+$, 451.2 $[M-H]^-$. $[\alpha]D_{20}$ (c = 0.1, $CHCl_3$): −222.34. ^{1}H NMR (DMSO-d_6): δ 8.05 (d, J = 8.09 Hz, 1H), 7.79–7.71 (m, 1H), 7.68 (dd, J = 4.99, 1.10 Hz, 2H), 7.67–7.58 (m, 1H), 4.39 (dd, J = 8.09, 2.37 Hz, 1H), 4.13 (dd, J = 6.15, 2.39 Hz, 1H), 4.00 (t, J = 6.25 Hz, 2H), 3.28 (s, 3H), 1.74–1.61 (m, 5H), 1.55 (td, J = 14.64, 6.25 Hz, 2H), 1.38 (d, J = 6.15 Hz, 3H), 1.36–1.27 (m, 2H), 1.25–1.08 (m, 6H), 0.93–0.76 (m, 2H) ppm. ^{13}C NMR (DMSO-d_6): δ 164.2, 158.7, 155.6, 142.6, 131.1, 121.8, 118.6, 111.8, 64.5, 61.1, 59.94, 43.0, 36.9, 32.8, 28.8, 26.2, 22.6, 15.43 ppm.

5.1.12.10. 4-Cyclohexylbutyl-((2S,3S)-2-methyl-1-(2-(methylsulfonyl)phenoxy)-4-oxoazetidin-3-yl)carbamate (39). The reaction was carried out following the general procedure GP2 employing **18** (0.08 g, 0.27 mmol), copper chloride (0.027 g, 0.27 mmol), (2-(methylsulfonyl)phenyl)boronic acid (0.108 g, 0.54 mmol) and pyridine (0.023 mL, 0.29 mmol). Purification by column chromatography using a Teledyne ISCO apparatus, eluting with Cy/TBME (from 100:0 to 50:50) afforded the title compound (0.006 g, 5%), as a colorless oil. MS (ESI) *m/z*: 451.3 $[M-H]^-$. $[\alpha]_{D20}$ (c = 0.1, $CHCl_3$): −208.76. ^{1}H NMR (DMSO-d_6): δ 8.04 (d, J = 8.24 Hz, 1H), 7.86 (dd, J = 7.84, 1.56 Hz, 1H), 7.83–7.68 (m, 2H), 7.43–7.33 (m, 1H), 4.42 (dd, J = 8.24, 2.35 Hz, 1H), 4.20–4.10 (m, 1H), 4.00 (t, J = 6.86 Hz, 2H), 1.70–1.56 (m, 5H), 1.55 (dd, J 2H). ppm. ^{13}C NMR (DMSO-d_6): δ 164.2, 158.7, 155.6, 142.6, 129.8, 124.8, 115.7, 65.2, 62.2, 60.6, 43.7, 37.8, 33.3, 29.5, 26.4, 22.9, 15.8 ppm.

5.2. In vitro chemical and mouse and rat plasma stability

Studies were performed as described in the Supporting Information [14].

5.3. Pharmacology

5.3.1. h-NAAA, h-AC and h-FAAH in vitro assays

Screenings were performed as described in the See Supporting Information [20,21,31,32].

5.3.2. In vivo carragenaan-induced inflammation experiment [17]

Animals. We used male CD1 mice (25–30 g; Charles River, Calco, Italy). All procedures were performed in accordance with the Ethical Guidelines of the International Association for the Study of Pain, Italian regulations on the protection of animals used for experimental and other scientific purposes (D.M. 116192) and European Economic Community regulations (O.J. of E.C. L 358/1 12/18/1986). Animals were group-housed in ventilated cages and had free access to food and water. They were maintained under a 12 h light/dark cycle (lights on at 8:00 a.m.) at controlled temperature (21 ± 1 °C) and relative humidity (55 ± 10%). Behavioral testing was performed during the light cycle (between 9:00 a.m. and 5:00 p.m.). Animals were killed by cervical dislocation under anesthesia. Experimenters were unaware of the treatment protocol at the time of the test (blind procedure).

Carrageenan-induced inflammation. We induced paw edema by injecting λ-carrageenan (1% weight/vol in sterile water, 50 μl) into the left hind paw of lightly restrained adult male CD1 mice. Edema was measured with a plethysmometer (Ugo Basile, Comerio, Italy). Fresh suspension of compound **37** was prepared and given by intraplantar injection (in 80% sterile saline solution/10% PEG-400/10% Tween 80, 10 μl per animal), just after carrageenan injection.

Behavioral tests. All experiments were performed in a quiet room, and experimenters were blinded to the treatment protocol at the time of the test. Heat hyperalgesia was assessed by the method of Hargreaves et al. [33] measuring the latency to withdraw the hind paw from a focused beam of radiant heat (thermal intensity: infrared 3.0) applied to the plantar surface in a plantar test apparatus (Ugo Basile). The cutoff time was set at 30 s.

5.4. LC-MS/MS study for the evaluation of mechanism of action

5.4.1. h-NAAA purification and activation

h-NAAA was produced and purified from *h*-NAAA

overexpressing HEK293 cell line as described [16]. The purified enzyme was incubated in activation buffer [100 mM Sodium Phosphate/Sodium Citrate Buffer, 3 mM DL-dithiothreitol (DTT), 0.1% Triton ×100, pH 4.5] for 3 h at 37 °C and the enzyme activation was checked by SDS-PAGE and Coomassie blue staining.

5.4.2. Analysis of covalent adducts by LC-MS/MS

The presence of possible inhibitor covalent adducts on *h*-NAAA was investigated by high-resolution nano LC-MS/MS analysis. A solution of purified *h*-NAAA (2.5 μM) was incubated with compound **37** (25 μM in 5% DMSO, final concentration). A no-inhibitor control (DMSO 5%) was also included. After the reaction time, samples were precipitated with 10 vol of cold acetone and centrifuged 10 min at 12000 × g. The pellets were re-suspended in 50 μl of 50 mM NH_4HCO_3 (pH 8) and trypsin (1:50 w/w) was added for 16 h at 37 °C. The pellets were resuspended in 50 μl of NH_4HCO_3 (pH 8) and proteomic grade trypsin (1:50 w/w) was added for 16 h at 37 °C. The resulting peptides were analyzed on a UPLC chromatographic system equipped with a BEH C18 reversed phase column (1 × 100 mm). Peptides were eluted with a linear gradient of CH_3CN in water (both added with 0.1% formic acid) from 3 to 50% in 8 min. Flow rate was set to 0.09 mL/min. Eluted peptides were analyzed in positive ion mode by high-resolution tandem mass spectrometry on a Synapt G2 qTOF mass spectrometer (UPLC, column and qTOF instrument were purchased from Waters, Milford MA, USA). A linear ramp of the collision energy from 15 to 45 eV was used to induce backbone fragmentation of the eluting peptides. 500 nM gluco-fibrino peptide infused at 500 nL/min was used as lock spray mass. MS/MS data were analyzed using the BioLynx software embedded in the MassLynx software suite. MassLynx and ProteinLynx software (Waters, USA) were used for the interpretation of LC-MS data.

5.4.3. Competitive Activity Based Protein Profiling (ABPP)

For competitive ABPP, 50 μl of lysosomal enrichment (0.5 mg/mL) from of *h*-NAAA-overexpressing HEK293 cell line were incubated 1 h at 37 °C with compound **37** at a final concentration of 10 μM (DMSO 2%). Following the incubation time, the activity based probe **ARN14686** [30] was added at 10 μM for 15 min or for 3.5 h at 37 °C. Next, click chemistry reaction was performed by adding the following reagents at the indicated final concentrations: 100 μM Azide-PEG3-Alexa Fluor 545 (CLK-AZ109, Jena Bioscience), 1 mM tris(2-carboxyethyl)phosphine (TCEP) hydrochloride, 100 μM tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA), 1 mM $CuSO_4 5H_2O$ [34]. TBTA was first dissolved in DMSO at 83.5 mM and then diluted with four volumes of *tert*-butanol. The reaction was mixed by vortexing and incubated 2 h at 25 °C. Samples (10 μl) were analyzed by SDS-PAGE and gel florescence was scanned at 532 nm wavelength (Fuji Film FLA-9000 instrument).

Author contributions

R.P. and S.P. designed and synthesized compounds; S.M.B. performed chemical and project; R.P., T.B. and F.B. wrote the manuscript. plasma stability tests; O.S. performed *in vivo* studies; D.P. ideated and coordinated the project; R.P., T.B. and F.B. wrote the manuscript.

Acknowledgments

The authors wish to thank Debora Russo and Ilaria Penna for *h*-NAAA, *h*-AC and *h*-FAAH-1 screenings, Elisa Romeo for competitive ABPP study, Giuliana Ottonello and Andrea Armirotti for bioanalytical support, Silvia Venzano for compounds handling and Luca Goldoni for NMR support.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2016.11.039.

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