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Synthesis and Structure–Activity Relationship (SAR) of 2-Methyl-4-oxo-3-oxetanylcarbamic Acid Esters, a Class of Potent *N*-Acylethanolamine Acid Amidase (NAAA) Inhibitors

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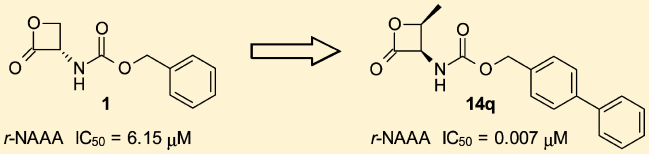
Supporting Information

ABSTRACT: *N*-Acylethanolamine acid amidase (NAAA) is a lysosomal cysteine hydrolase involved in the degradation of saturated and monounsaturated fatty acid ethanolamides (FAEs), a family of endogenous lipid agonists of peroxisome proliferator-activated receptor-α, which include oleoylethanolamide (OEA) and palmitoylethanolamide (PEA). The β-lactone derivatives (*S*)-*N*-(2-oxo-3-oxetanyl)-3-phenylpropionamide (**2**) and (*S*)-*N*-(2-oxo-3-oxetanyl)-biphenyl-4-carboxamide (**3**) inhibit NAAA, prevent FAE hydrolysis in activated inflammatory cells, and reduce tissue reactions to pro-inflammatory stimuli. Recently, our group disclosed ARN077 (**4**), a potent NAAA inhibitor that is active in vivo by topical administration in rodent models of hyperalgesia and allodynia. In the present study, we investigated the structure–activity relationship (SAR) of threonine-derived β-lactone analogues of compound **4**. The main results of this work were an enhancement of the inhibitory potency of β-lactone carbamate derivatives for NAAA and the identification of (4-phenylphenyl)-methyl-*N*-[(2*S*,3*R*)-2-methyl-4-oxo-oxetan-3-yl]carbamate (**14q**) as the first single-digit nanomolar inhibitor of intracellular NAAA activity (IC_{50} = 7 nM on both rat NAAA and human NAAA).



INTRODUCTION

The ethanolamides of long-chain fatty acids, or fatty acid ethanolamides (FAEs), are a class of bioactive lipids that serve important signaling functions in both plants and animals.[1] Polyunsaturated FAEs such as arachidonoylethanolamide (anandamide) are endogenous agonists for G protein-coupled cannabinoid receptors and participate in the control of stress-coping responses and pain initiation.[2] On the other hand, monounsaturated and saturated FAEs, such as oleoylethanolamide (OEA) and palmitoylethanolamide (PEA), regulate energy balance, pain, and inflammation primarily by engaging peroxisome proliferator-activated receptor-α (PPAR-α), a member of the nuclear receptor superfamily.[3−6]

The pharmacology of PEA has been extensively investigated.[7] The compound inhibits peripheral inflammation and mast cell degranulation[8,9] and exerts profound antinociceptive effects in rat and mouse models of acute and chronic pain.[3,9−11] Moreover, it suppresses pain behaviors induced, in mice, by tissue injury, nerve damage, or inflammation.[12] These properties are dependent on PPAR-α activation, because they are absent in PPAR-α-deficient mice and blocked by PPAR-α antagonists.[12,13] The possibility that PEA might attenuate skin inflammation and neuropathic pain in humans heightens the translational value of the results outlined above.[14,15]

Tissue levels of bioactive FAEs are regulated at both the synthesis and degradation levels.[1] These compounds are generated by the action of a selective phospholipase D, which catalyzes the cleavage of the membrane precursor, *N*-acylphosphatidylethanolamine (NAPE),[16] and are deactivated by either of two intracellular lipid amidases: fatty acid amide hydrolase (FAAH) and *N*-acylethanolamine acid amidase (NAAA).[17−19] NAAA preferentially hydrolyzes PEA and OEA over anandamide, whereas FAAH displays an opposite substrate selectivity.[18,20] Whereas several classes of FAAH inhibitors have been reported in the literature, only a few NAAA inhibitors have been identified so far.[21−28]

NAAA is a cysteine hydrolase that belongs to the N-terminal nucleophile (Ntn) family of enzymes[18,19] and bears a significant degree of sequence homology with the choloylglycine hydro-

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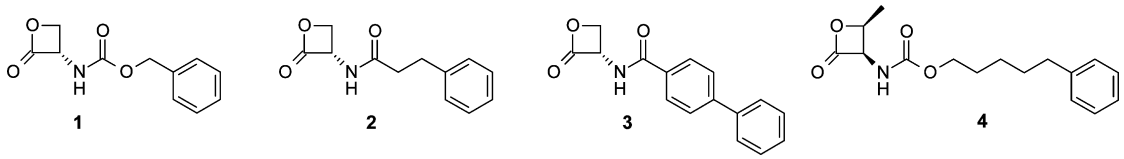
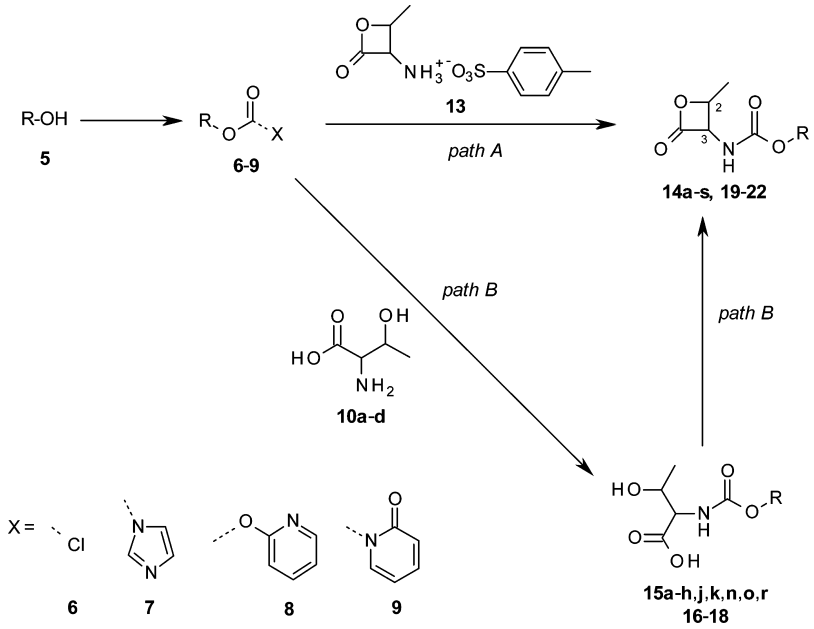


Figure 1. Noncompetitive β-lactone-based inhibitors of intracellular NAAA activity.

Scheme 1. General Synthetic Pathways for the Preparation of 2-Methyl-4-oxo-3-oxetanylcarbamic Acid Esters



lases, which are characterized by the ability to cleave nonpeptide amide bonds.[29] Like other Ntn enzymes, NAAA is activated by autoproteolysis, which occurs at acidic pH and generates a catalytically competent form of the enzyme.[30] Site-directed mutagenesis experiments have unequivocally identified Cys131 (in mice) and Cys126 (in humans) as the catalytic residues responsible for both autoproteolysis and FAE hydrolysis.[23,31−33]

Compounds containing chemical groups susceptible to nucleophilic attack, such as a β-lactone moiety, are known to inhibit enzymes that contain a catalytic cysteine.[34] Within this class of compounds, (*S*)-2-oxo-3-oxetanyl-carbamic acid benzyl ester (**1**, Figure 1), an inhibitor of a viral cysteine hydrolase,[35] was found to inhibit NAAA activity with micromolar potency.[24] Replacement of the carbamic acid benzyl ester function of **1** with a 3-phenylpropionamide moiety led to (*S*)-*N*-(2-oxo-3-oxetanyl)-3-phenylpropionamide [**2**, (*S*)-OOPP, Figure 1], a relatively potent NAAA inhibitor (IC_{50} = 0.42 μM).[24] A pharmacological characterization of **2** showed that this compound prevents FAE hydrolysis in activated inflammatory cells and dampens tissue reactions to various pro-inflammatory stimuli.[24] In addition, compound **2** does not inhibit FAAH or other lipid hydrolases, such as monoacylglycerol lipase and diacylglycerol lipase type-α, a selectivity profile that allows its use as a pharmacological probe. Structure−activity relationship (SAR) studies of serine-derived 2-oxo-3-oxetanyl amides confirmed the key role of the β-lactone ring for NAAA inhibition and identified lipophilic side chains of the carboxamide moiety with optimal size and shape for potent enzyme inhibition. This work led to the identification of the NAAA inhibitor **3** (Figure 1; IC_{50} = 0.115 μM), which prevented carrageenan-induced decrease of FAE levels in vivo with a potency that paralleled its ability to inhibit NAAA in vitro.[25] A first investigation of threonine-derived β-lactone derivatives, which have enhanced chemical stability compared to their serine-derived analogues,[28,35,36] led to the discovery of ARN077 (**4**, Figure 1), a potent NAAA inhibitor[28,37] that is active in vivo by topical administration in rodent models of hyperalgesia and allodynia caused by inflammation or nerve damage.

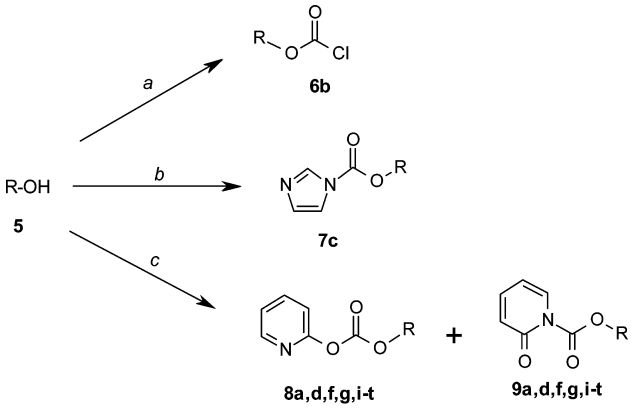
In the present study, we describe a further expansion of the SAR on threonine-derived β-lactones. We synthesized and tested a series of 2-methyl-4-oxo-3-oxetanylcarbamic acid esters to investigate the influence on NAAA inhibition of the size and shape of the carbamic acid ester side chain.

■ CHEMISTRY

The synthesis of the 2-methyl-4-oxo-3-oxetanylcarbamic acid esters **14a−s** and **19−22** was carried out following the synthetic pathways represented in Scheme 1. First, alcohols **5** were converted into the corresponding chloroformates (**6**), imidazole 1-carboxylates (**7**), or 2-pyridyl carbonates (**8**) and 2-oxopyridine-1-carboxylates (**9**). Then, compounds **6−9** were reacted either with (2*S*,3*R*)-2-methyl-4-oxo-3-oxetanylammonium toluene-4-sulfonate (**13**) to give the desired final compounds (path A) or with the commercially available threonines **10a−d** (path B) to furnish the intermediate α-substituted-β-hydroxycarboxylic acids **15a−h,j,k,n,o,r** and **16−18**, which upon cyclization afforded the corresponding 2-methyl-4-oxo-3-oxetanylcarbamic acid esters.

The preparation of compounds **6−9** was accomplished as reported in Scheme 2. Chloroformate **6b** was prepared by

Scheme 2. Synthetic Pathways for the Preparation of Compounds 6–9[a]



a: R = 2-phenylethyl
b: R = 3-phenylpropyl
c: R = 4-phenylbutyl
d: R = 6-phenylhexyl
f: R = *n*-hexyl
g: R = *n*-heptyl
i: R = 3-benzyloxypropyl
j: R = 2-phenethyloxyethyl
k: R = 5-cyclohexylpentyl
l: R = 1,1-dimethyl-5-phenylpentyl
m: R = 1-methyl-5-phenylpentyl
n: R = 1-methyloctyl
o: R = 1-isopropyl-5-phenylpentyl
p: R = (3-phenylphenyl)methyl
q: R = (4-phenylphenyl)methyl
r: R = benzylcyclohexyl
s: R = 4-benzyloxyphenyl
t: R = 5-phenylpentyl

[a]Reagents and conditions: (*a*) **5b**, $(Cl_3CO)_2CO$, pyridine, toluene, room temperature, 16 h; (*b*) **5c**, CDI, DMF, room temperature, 2 h; (*c*) **5a,d,f,g,i–t**, Et_3N or DMAP, DPC, CH_2Cl_2, room temperature, 15 h.

reacting 3-phenylpropan-1-ol (**5b**) with triphosgene in the presence of pyridine. Imidazole 1-carboxylate **7c** was obtained in a straightforward manner by reaction of the corresponding alcohol **5c** with 1,1′-carbonyldiimidazole (CDI).[38] Compound **7c** was not isolated and used in a one-pot procedure in the subsequent step of the synthetic pathway.

Synthesis problems encountered during the preparation of chloroformates **6** and the observed poor reactivity of imidazole 1-carboxylates **7c** with either D-threonine (**10a**) or the tosylate salt **13** (Scheme 1) prompted us to search for alternative carbonic acid derivatives such as pyridyl carbonates deriving from di-2-pyridyl carbonate (DPC), to be reacted with compounds **10a–d** or **13**. DPC is reported to activate primary, secondary, and tertiary alcohols as alkyl 2-pyridyl carbonates and, as described in the literature, the pyridyl moiety makes these mixed carbonates highly reactive species toward amino acids.[39,40]

Alcohols **5a,d,f,g,i–t** were therefore reacted with DPC in the presence of triethylamine to give an isomeric mixture of 2-pyridyl carbonates **8a,d,f,g,i–t** and 2-oxopyridine-1-carboxylates **9a,d,f,g,i–t** (Scheme 2, path c).[41,42] The mixture of isomers was used as such in the following synthesis step, as the limited stability of both compounds to chromatographic purification prevented the isolation of the pure products.

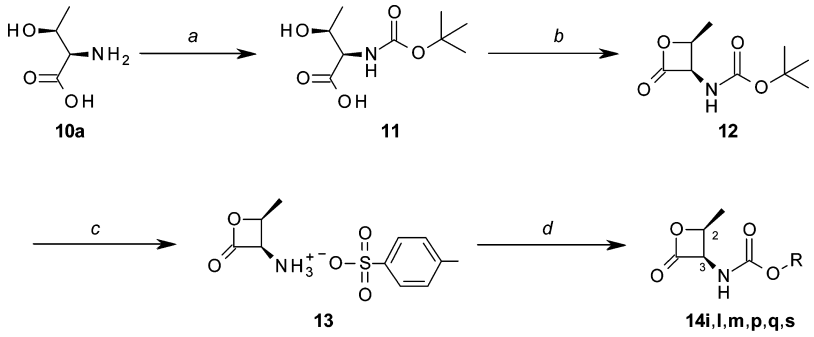
The synthesis of the tosylate salt **13** was carried out with a minor modification of the previously reported procedure (Scheme 3).[25,35] According to path A of Scheme 1, 2-methyl-4-oxo-3-oxetanylcarbamic acid esters were obtained in moderate yields (17–41%) by coupling the tosylate salt **13** with a large excess (3 equiv) of the isomeric mixture containing the suitable 2-pyridyl carbonates **8i,l,m,p,q,s** and the 2-oxopyridine-1-carboxylates **9i,l,m,p,q,s** (Scheme 3, step *d*).

More 2-methyl-4-oxo-3-oxetanylcarbamic acid ester analogues were prepared according to path B of Scheme 1, as detailed in Scheme 4. D-Threonine (**10a**) was reacted with chloroformates **6b,e,h** (procedure *a*), imidazole 1-carboxylates **7c** (procedure *b*), or the isomeric mixtures containing the 2-pyridyl carbonates **8a,d,f,g,j,k,n,o,r** and the 2-oxopyridine-1-carboxylates **9a,d,f,g,j,k,n,o,r** (procedure *c*) to afford the corresponding *α*-substituted *β*-hydroxycarboxylic acids**15a–h,j,k,n,o,r**.

When procedure *c* was applied, a slight excess (1.5 equiv) of the isomeric mixtures containing **8** and **9** was required, leading to intermediates **15** in higher yields (36–97%) compared to that observed in the reaction with **13**.

Compounds **15** were submitted to a cyclization reaction using different coupling reagents [2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HBTU), benzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP), or 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate (TBTU)] to afford the

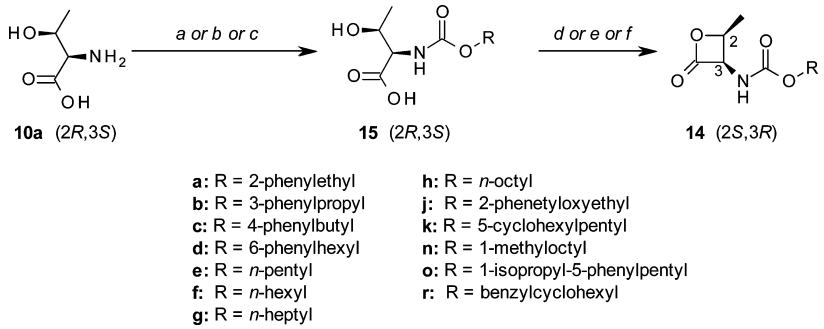
Scheme 3. Synthesis of D-Threonine-Derived *β*-Lactones 14i,l,m,p,q,s via Tosylate Salt 13[a]



i: R = 3-benzyloxypropyl
l: R = 1,1-dimethyl-5-phenylpentyl
m: R = 1-methyl-5-phenylpentyl
p: R = (3-phenylphenyl)methyl
q: R = (4-phenylphenyl)methyl
s: R = 4-benzyloxyphenyl

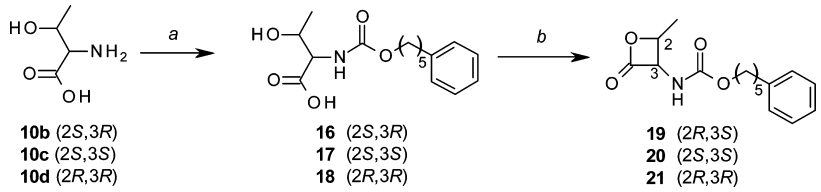
[a]Reagents and conditions: (*a*) Boc_2O, $NaHCO_3$, H_2O, MeOH, room temperature, 36 h; (*b*) PyBOP, Et_3N, CH_2Cl_2, room temperature, 15 h; (*c*) CF_3COOH, *p*-TsOH, 0 °C, 15 min; (*d*) DIPEA, mixture of **8i,l,m,p,q,s** and **9i,l,m,p,q,s**, CH_2Cl_2, room temperature, 15 h.

Scheme 4. Synthesis of 2-Methyl-4-oxo-3-oxetanylcarbamic Acid Esters via β-Hydroxycarboxylic Acids[a]



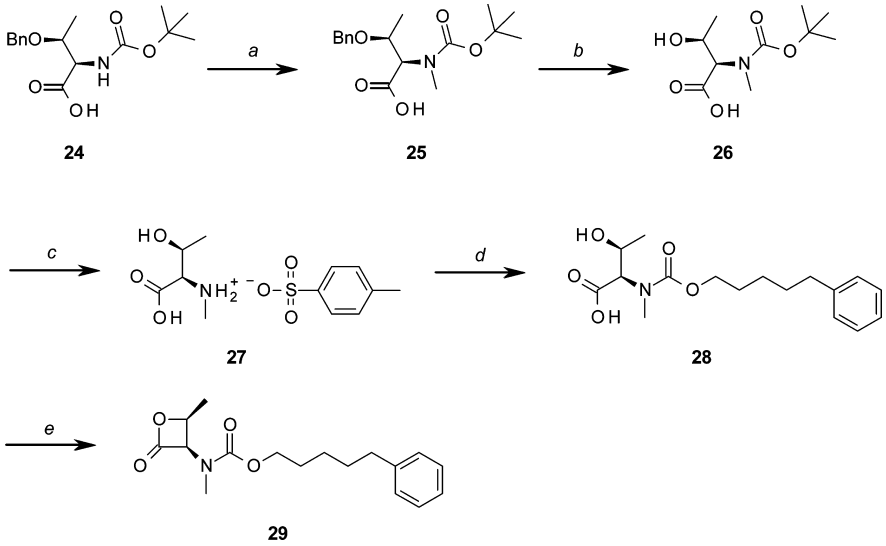
[a]Reagents and conditions: (*a*) $NaHCO_3$, **6b,e,h**, $(n\text{-Bu})_4NBr$, THF/H_2O, room temperature, 18 h; (*b*) Et_3N, **7c**, DMF/H_2O, 50 °C, 16 h; (*c*) $NaHCO_3$, mixture of **8a,d,f,g,j,k,n,o,r** and **9a,d,f,g,j,k,n,o,r**, H_2O/THF, room temperature, 15 h; (*d*) **15b,c,h**, HBTU, Et_3N, CH_2Cl_2, 0 °C, 3 h then room temperature, 3.5−16 h; (*e*) **15 a,d,f,g,j,k,n,o,r**, TBTU, Et_3N, CH_2Cl_2, 0 °C, 1 h then room temperature, 15 h; (*f*) **15e**, PyBOP, Et_3N, CH_2Cl_2, 0 °C, 3 h then room temperature, 3.5 h.

Scheme 5. Synthesis of Isomeric 5-Phenylpentyl-*N*-[(2*S*,3*R*)-2-methyl-4-oxo-oxetan-3-yl]carbamates 19−21 via β-Hydroxycarboxylic Acids[a]



[a]Reagents and conditions: (*a*) $NaHCO_3$, mixture of **8t** and **9t**, H_2O/THF, room temperature, 15 h; (*b*) TBTU, Et_3N, CH_2Cl_2, 0 °C, 1 h then room temperature, 15 h.

Scheme 6. Synthesis of Compound 29[a]

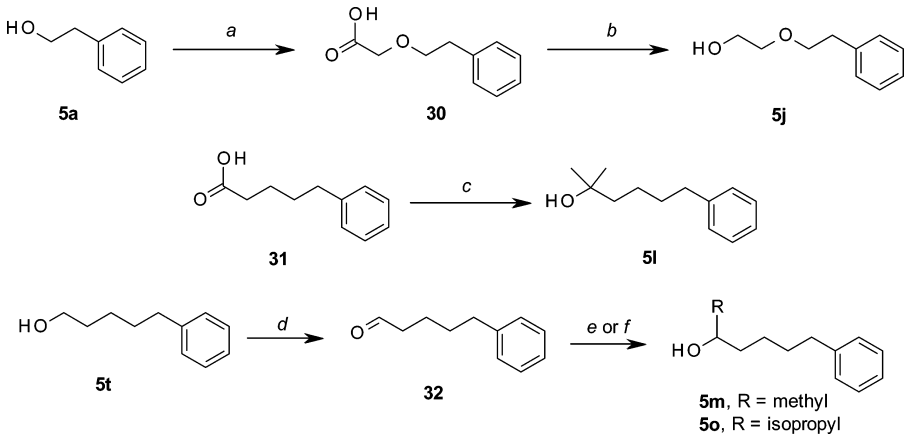


[a]Reagents and conditions: (*a*) NaH, THF, 0 °C, 10 min, then MeI, DMF, 0 °C, 2 h then room temperature, 15 h; (*b*) H_2, 10% Pd/C (H-Cube, EtOH, 60 °C, 1.0 bar); (*c*) CF_3COOH, *p*-TsOH, 0 °C, 15 min; (*d*) $NaHCO_3$, mixture of **8t** and **9t**, H_2O/THF, room temperature, 15 h; (*e*) TBTU, Et_3N, CH_2Cl_2, 0 °C, 1 h, then room temperature, 15 h.

desired 2-methyl-4-oxo-3-oxetanylcarbamic acid esters **14a−h,j,k,n,o,r** in moderate to good yields (37−76%). The difference in the cyclization step yields was not dependent on the coupling reagent but rather on the nature of the α-substituted β-hydroxycarboxylic acids **15a−h,j,k,n,o,r**. The overall yields of final compounds following path *B* of Scheme 1 were always higher than those obtained with path A.

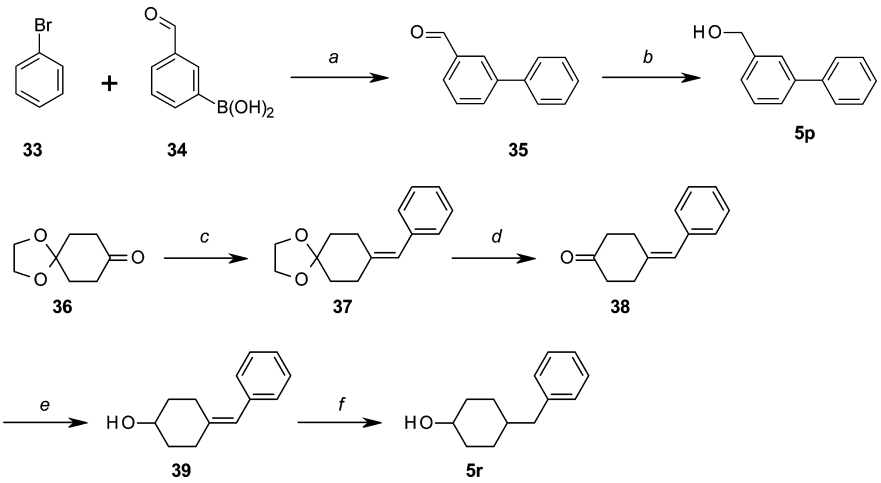
Compounds **19−21**, respectively the enantiomer and the epimers of compound **4**, were synthesized starting from the commercially available L-threonine **10b**, L-*allo*-threonine **10c**, and D-*allo*-threonine **10d**. The amino acids were first reacted with the isomeric mixture of **8t** and **9t** to give the corresponding α-substituted β-hydroxycarboxylic acids **16−18** and subsequently cyclized to provide the desired analogues **19−21** (Scheme 5).

Scheme 7. Syntheses of Alcohols 5j,l,m,o[a]



[a]Reagents and conditions: (*a*) NaH, DMSO, 60 °C, 10 min, then $ClCH_2COOH$, 80 °C, 3 h; (*b*) $LiAlH_4$, Et_2O, room temperature, 4 h; (*c*) MeOH-BF_3, CH_3MgBr, NH_4Cl (aq); (*d*) $(COCl)_2$, DMSO, −78 °C, 2 h; (*e*) CH_3Li, THF, −78 °C, 2 h, then room temperature, 1 h; (*f*) $(CH_3)_2CHMgCl$, Et_2O, −78 °C, 0.5 h, then 0 °C, 2 h.

Scheme 8. Synthesis of Alcohols 5p,r[a]



[a]Reagents and conditions: (*a*) $Pd(PPh_3)_4$, 10% Na_2CO_3, Toluene/EtOH, microwave, 100 °C, 0.5 h; (*b*) $NaBH_4$, MeOH, 0 °C, 1 h; (*c*) NaH, $[PhCH_2P(Ph)_3]Br$, DMSO, room temperature, 0.5 h then 50 °C, 0.5 h, then 16 h, 50 °C after addition of **36**; (*d*) 2:1 mixture acetone/HCl (10% v/v), room temperature, 4 h; (*e*) $NaBH_4$, MeOH, 0 °C, 1 h; (*f*) H_2, 10% Pd/C (H-Cube, EtOAc, 30 °C, 1.0 bar).

The diastereoisomeric mixture **14n** was separated by chiral HPLC to afford the single diastereoisomers **22** and **23** as pure enantiomers (see Table 2). Compound **22** was also synthesized following the same procedure applied to compound **14n**, starting from the commercially available optically pure (*S*)-nonan-2-ol, and used as a reference to assign the correct absolute configuration of diastereoisomer **23** after chiral HPLC purification.

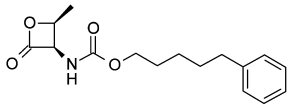
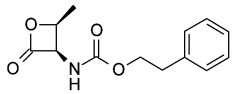
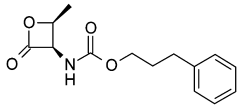
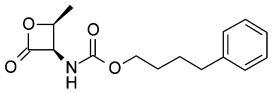
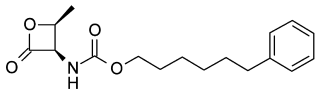
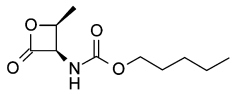
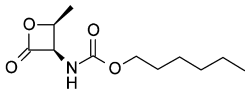
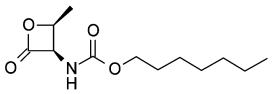
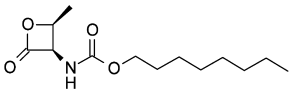
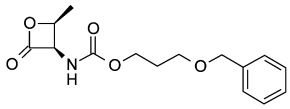
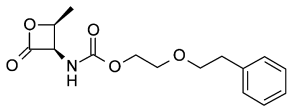
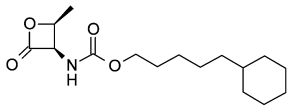
The synthesis of compound **29** (Scheme 6) was carried out starting from the commercially available product **24**, which was N-methylated using methyl iodide and sodium hydride to afford intermediate **25**. The benzyl group was then removed by catalytic hydrogenation and the obtained *N*-methyl-*N*-Boc-D-threonine (**26**) was converted into the corresponding tosylate salt **27**. Reaction of **27** with the isomeric mixture containing the 2-pyridyl carbonate **8t** and the 2-oxopyridine-1-carboxylate **9t** afforded the 5-phenylpentyl derivative **28**, which was finally cyclized to give the desired compound **29**.

The syntheses of alcohols **5j** and **5l** were accomplished according to literature procedures as reported in Scheme 7. The commercially available phenethyl alcohol **5a** was reacted with chloroacetic acid, in the presence of sodium hydride, to afford 2-phenylethoxyacetic acid (**30**), which upon reduction with lithium aluminum hydride furnished the desired alcohol **5j**.[43] Alcohol **5l** was synthesized by addition of methylmagnesium bromide to 5-phenylpentanoic acid methyl ester, which was prepared in situ by treatment of the commercially available compound **31** with BF_3−methanol complex.[44]

Alcohols **5m,o** were prepared in a two-step procedure starting from the commercially available alcohol **5t** (Scheme 7). Swern oxidation to the aldehyde **32** followed by addition of methyl-lithium or isopropylmagnesium bromide furnished the desired compounds.

Alcohols **5p,r** were prepared as reported in Scheme 8. The compound **5p** was obtained via a Suzuki cross-coupling reaction between the commercially available 3-formylphenylboronic acid (**34**) and bromobenzene (**33**) followed by reduction of the intermediate 3-phenylbenzaldehyde (**35**). Alcohol **5r** was prepared via a Wittig reaction between 1,4-dioxaspiro[4.5]decan-8-one (**36**) and benzyltriphenylphospho-

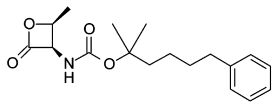
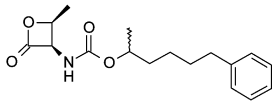
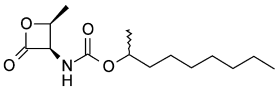
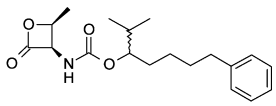
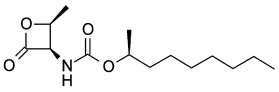
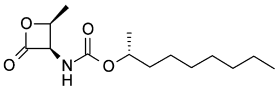
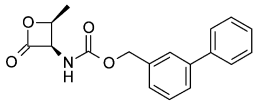
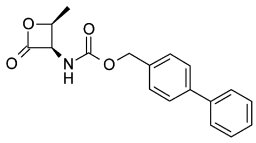
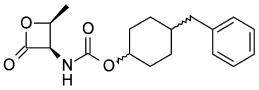
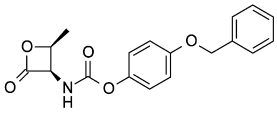
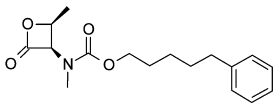
Table 1. Inhibitory Potencies (IC_{50}) of Compounds 4 and 14a–k on Rat NAAA Activity[a]

Compounds	Structure	IC_{50} (μM) ± SD
4		0.05 ± 0.01
14a		2.50 ± 0.85
14b		0.09 ± 0.07
14c		0.39 ± 0.17
14d		1.17 ± 0.40
14e		0.76 ± 0.35
14f		0.27 ± 0.14
14g		0.04 ± 0.02
14h		0.03 ± 0.03
14i		0.05 ± 0.02
14j		0.14 ± 0.05
14k		0.013 ± 0.004

[a] IC_{50} values are reported as mean values of three or more determinations.

nium bromide to afford compound **37**.[45] Removal of the acetal group under acidic conditions and subsequent reduction of ketone **38** furnished the alcohol **39**, which was submitted to catalytic hydrogenation to afford **5r**.

Table 2. Inhibitory Potencies (IC_{50}) of Compounds 14l–s, 22, 23, and 29 on Rat NAAA Activity[a]

Compounds	Structure	IC_{50} (μM) ± SD
14l		0.31 ± 0.14
14m		0.029 ± 0.001
14n		0.06 ± 0.03
14o		0.10 ± 0.05
22		0.016 ± 0.003
23		0.27 ± 0.12
14p		23.0 ± 6.6
14q		0.007 ± 0.001
14r		0.31 ± 0.08
14s		0.29 ± 0.22
29		No inhibition

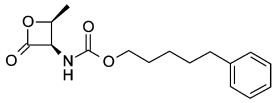
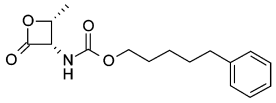
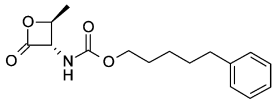
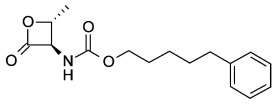
[a] IC_{50} values are reported as mean values of three or more determinations.

RESULTS AND DISCUSSION

Previous pharmacological studies by our group have identified the 2-methyl-4-oxo-3-oxetanylcarbamic acid ester derivative **4** (ARN077) as a potent NAAA inhibitor.[46] In vitro experiments have shown that compound **4** inhibits rat NAAA through a mechanism that is rapid, noncompetitive, and fully reversible after overnight dialysis.[46] Moreover, in rodent models of hyperalgesia and allodynia caused by inflammation or nerve damage, **4** administered by the topical route partially normalized FAE levels in the skin and sciatic nerve, which were reduced by inflammation and surgical ligation, respectively, and attenuated nociception through a mechanism that required PPAR-α activation.[46]

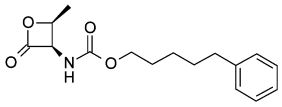
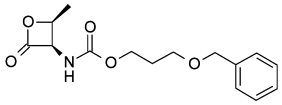
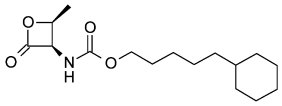
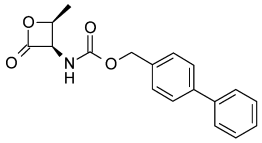
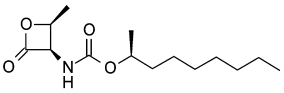
We identified compound **4** starting from the serine-derived 2-oxo-3-oxetanylamide, (*S*)-OOPP (**2**), which inhibits NAAA with a median inhibitory concentration (IC_{50}) of 0.42 μM.[24]

Table 3. Inhibitory Potencies (IC_{50}) of Compounds 4 and 19−21 on Rat NAAA Activity[a]

Compounds	Structure	IC_{50} (μM) ± SD
4		0.05 ± 0.01
19		3.53 ± 2.22
20		0.48 ± 0.26
21		0.02 ± 0.01

[a]IC_{50} values are reported as mean values of three or more determinations.

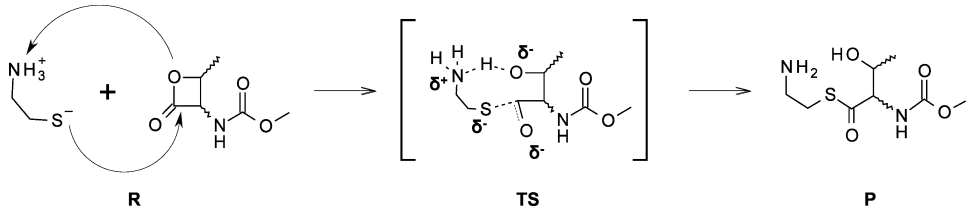
Table 4. Inhibitory Potencies (IC_{50}) on Rat and Human NAAA[a] and Percent Inhibitory Activity on Rat FAAH[b] and Rat AC[b] of Compounds 4, 14i,k,q and 22

Compounds	Structure	*r*-NAAA IC_{50} (μM) ± SD	*h*-NAAA IC_{50} (μM) ± SD	*r*-FAAH (%Inhib.)[c]	*r*-AC (%Inhib.)[c]
4		0.05 ± 0.01	0.007 ±0.001	n.a.	n.a.
14i		0.05 ± 0.02	0.02 ± 0.01	n.a.	n.a.
14k		0.013 ± 0.004	0.005 ± 0.002	n.a.	n.a.
14q		0.007 ± 0.001	0.007 ± 0.002	n.a.	30%
22		0.016 ± 0.003	0.005 ± 0.002	19%	45%

[a]IC_{50} values are reported as mean values of three or more determinations. [b]Inhibitory activities are reported as a % value of two determinations at 10 μM. [c]n.a.= < 10% inhibition

The (*S*)-configuration at position 3 in the *β*-lactone ring of **2** is essential for inhibitory potency, as indicated by the substantially lower activity of its enantiomer, (*R*)-OOPP (IC_{50} = 6.0 μM).[24] The benzyl carbamate **1** was less potent than **2** at inhibiting NAAA (IC_{50} = 2.96 μM),[28] whereas its enantiomer, (*R*)-2-oxo-3-oxetanylbenzyl carbamate, showed potency comparable to that of **2** (IC_{50} = 0.70 μM).[28] This indicated an opposite stereochemical preference of NAAA at position 3 of the *β*-lactone ring in the carbamic acid ester series relative to the amide series. The introduction of a methyl group with (*S*)-stereochemistry at the *β*-position of the *β*-lactone ring of (*R*)-2-oxo-3-oxetanylbenzyl carbamate led to the corresponding threonine-derived *β*-lactone analogue that, although slightly less potent (IC_{50}= 1.0 μM),[28] showed an increased chemical

Scheme 9. Schematic Representation of a Possible NAAA Inhibition Mechanism by β-Lactones



stability with respect to serine-derived β-lactone analogues.[28,35,36] These results prompted us to focus our attention on carbamic acid ester derivatives bearing a methyl substitution at the β-position of the β-lactone ring.

2-Methyl-4-oxo-3-oxetanylcarbamic acid esters were tested for their ability to inhibit the hydrolysis of 10-*cis*-heptadecenoylethanolamide (an unnatural FAE) by either native NAAA prepared from rat lungs (r-NAAA) or recombinant human NAAA heterologously expressed in HEK293 cells (h-NAAA). IC_{50} values are reported in Tables 1−3 (r-NAAA) and Table 4 (h-NAAA). The pharmacological potencies of several test compounds slightly varied between r-NAAA and h-NAAA. We attribute such variations to differences in primary sequence and enzyme preparation.

As a first step in our study, we synthesized a small series of 2-methyl-4-oxo-3-oxetanylcarbamic acid esters where the length of the aliphatic chain of the phenylalkyl alcohol was progressively increased from two to six methylene units (**14a−c**, **4**, **14d**). Compound **4** emerged as the most potent NAAA inhibitor (IC_{50} = 0.05 μM) within this small series. The potency and the promising in vivo efficacy of **4** prompted us to investigate in greater detail the structural determinants for activity of this compound.

To test the role of the phenyl ring, this moiety was replaced either with an unsubstituted aliphatic chain (**14e−h**) or with a cyclohexyl residue (**14k**). Removal of the phenyl ring led to a >10-fold drop in potency (**14e**, IC_{50} = 0.76 μM). However, potency was recovered by increasing the length of the aliphatic chain, with compounds bearing an *n*-heptyl (**14g**, IC_{50} = 0.04 μM) or an *n*-octyl (**14h**, IC_{50} = 0.03 μM) residue showing IC_{50} values comparable to that of compound **4**. Replacement of the phenyl ring with a cyclohexyl moiety, as in compound **14k**, led to a significant improvement in NAAA inhibitory potency (IC_{50} = 0.013 μM). The replacement of a carbon atom with an oxygen in the aliphatic chain of **4** (**14i,j**, Table 1) was tolerated only at a specific position, as shown by the different potencies of compounds **14i** (IC_{50} = 0.05 μM) and **14j** (IC_{50} = 0.14 μM). To evaluate the influence of the shape of the phenylalkyl chain, we synthesized compounds **14l−n** (Table 2), which derive from secondary and tertiary alcohols bearing branched aliphatic chains. Introduction of a *gem*-dimethyl (**14l**) moiety close to the carbamic function was detrimental for potency, indicating a limited space in the region of the enzyme occupied by the carbamic function and the adjacent carbon atom. A single methyl group, however, appeared to be well accommodated in this region because compound **14m** (IC_{50} = 0.029 μM), although a mixture of diastereoisomers, displayed a slight increase in potency compared to compound **4**. The introduction of the more sterically demanding isopropyl group (**14o**) decreased the inhibitory potency relative to **4**.

To determine whether there is any stereochemical preference for a substituent at the carbon atom in the α-position to the carbamic function, we synthesized compounds **14n**, as a diastereomeric mixture, and compound **22**, from optically pure (*S*)-1-methyloctanol. Compound **14n** (IC_{50} = 0.06 μM) displayed a slightly decreased potency compared to the unsubstituted derivative **14h** (IC_{50} = 0.03 μM). The optically pure (*S*)-1-methyloctyl diastereoisomer **22** inhibited NAAA with IC_{50} = 0.016 μM, a value comparable to that of the unsubstituted compound **14h**, whereas the (*R*)-diastereoisomer **23** (IC_{50} = 0.27 μM), obtained by chiral HPLC purification of **14n**, was 10 times less potent than **14h**. These results indicate a stereorecognition among the isomers and, specifically, a preference for the (*S*)-configuration at the carbon atom in position α to the carbamate function, at least for compounds bearing a 1-methyloctyl side chain.[47]

The influence of the flexibility of the aliphatic chain on NAAA inhibition was studied by preparing the series of analogues **14p−s** (Table 2). Compounds bearing a 4-benzylcyclohexyl (**14r**, IC_{50} = 0.31 μM) or a *p*-benzyloxyphenyl (**14s**, IC_{50} = 0.29 μM) moiety were less potent than compound **4**, indicating that some flexibility is required in close proximity to the carbamate function. Of particular interest were the results obtained with the biphenylmethyl derivatives (**14p,q**). The *p*-biphenylmethyl compound **14q** turned out to be a very potent NAAA inhibitor, showing an IC_{50} = 0.007 μM. On the contrary, the *m*-biphenylmethyl analogue **14p** did not effectively inhibit NAAA (IC_{50} = 23 μM). These results show a clear preference for a linear moiety in the carbamic acid ester for optimal enzyme recognition.

The role of the carbamic function was also investigated by preparing compound **29** (Table 2), a derivative in which the carbamate nitrogen is methylated. Highlighting the importance of the N−H group, the compound showed no inhibitory activity on NAAA.

To investigate the effect of the stereochemistry at positions 2 and 3 of the β-lactone ring, we synthesized compound **19**, the enantiomer of compound **4**, and the epimers **20** and **21** (Table 3). A marked drop in inhibitory potency (70-fold) was observed with compound **19**, whereas the (2*S*,3*S*) epimer **20** (IC_{50} = 0.48 μM) showed a 10-fold decrease in potency compared to **4**. By contrast, the (2*R*,3*R*) epimer **21** turned out to be a potent NAAA inhibitor (IC_{50} = 0.02 μM). These findings show that the configuration of the two stereogenic centers is important for potency. Whereas the relative importance of the configuration at position 2 versus 3 remains to be fully explored, our data support a primary role for position 3.

To search for a rational explanation of the different potencies of compounds **4** and **19−21**, we carried out density functional theory (DFT)-based calculations to mimic the nucleophilic attack of the sulfur atom onto the carbonyl group of the β-lactone ring, the first step of the covalent inhibition mechanism hypothesized for the present series of compounds.[33,46] In our simulations, the four compounds were truncated and modeled as methyl carbamate, and the sulfur atom was provided by

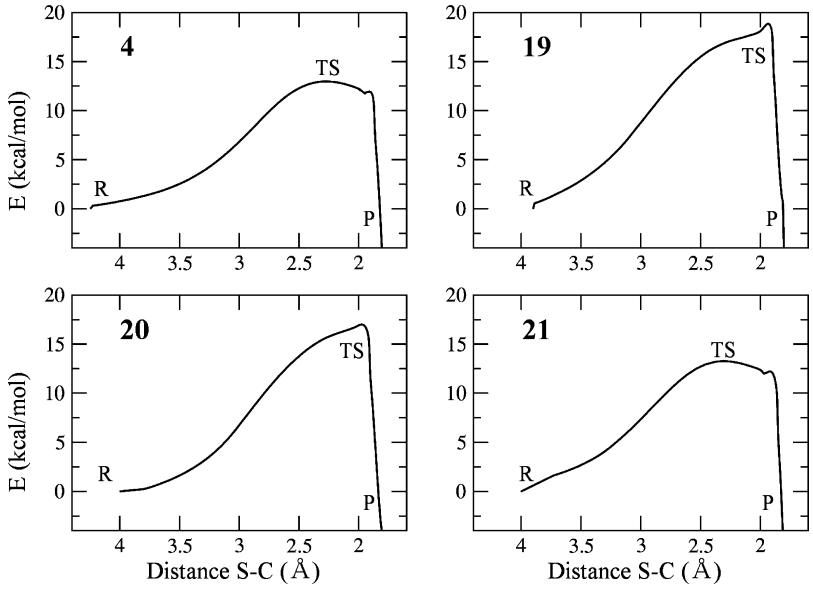


Figure 2. Potential energy surfaces plotted versus the reaction coordinate, which was represented by the distance between the nucleophilic sulfur atom and the carbonylic carbon of the β-lactone ring.

cysteamine (see Computational Methods under Experimental Section). Starting from the noncovalent complex, we performed a scan on the potential energy surface, using as reaction coordinate the distance between the sulfur atom of the cysteamine molecule and the carbon atom of the carbonyl group of the β-lactone ring. These calculations allowed us to identify the transition state (TS) for all four compounds under investigation and to determine the activation energy related to the nucleophilic attack carried by the sulfur atom. In Scheme 9 is reported a possible NAAA inhibition mechanism by β-lactones. Going from the reactants (R) through the transition state (TS) to the product (P), the sulfur atom attacks the carbonyl group of the β-lactone ring, and the protonated amino terminal group donates the proton to the oxygen atom. The nucleophilic attack and the protonation steps are concerted.

In Figure 2, we report the potential energy plotted versus the reaction coordinate, whereas in Figure 3, we show the geometry of the four TSs. The activation energy (E_a) was calculated as the energy difference between TS and R. The E_a values for compounds **4** and **21** were 13.0 and 13.3 kcal/mol, whereas the E_a values for the nucleophilic attack to **19** and **20** were 18.9 and 17.0 kcal/mol. These calculations suggest that compounds **4** and **21** might be more prone to react with NAAA's nucleophilic cysteine, and therefore more potent, than compounds **19** and **20**. This possibility is supported by the experimental IC_{50} values obtained with these compounds (Table 3), which show that **4** and **21** are up to 2 orders of magnitude more potent than **19** and **20**, respectively. The TSs structures (Figure 3) demonstrated that the presence of the carbamic substituent on the lactone ring allowed the formation of a transient intramolecular interaction—between the carbonylic oxygen of the β-lactone and the hydrogen atom of the carbamate—only when the absolute configuration of the carbamate-carrying carbon was (*R*). Conversely, such a stabilizing interaction could not be established when the configuration of the same atom was (*S*). Moreover, in the reactants showing (*S*)-configuration, we observed an intermolecular stabilizing interaction between the sulfur atom of the cysteamine and the hydrogen atom of the carbamate. This interaction lowered the reactant energy, with a

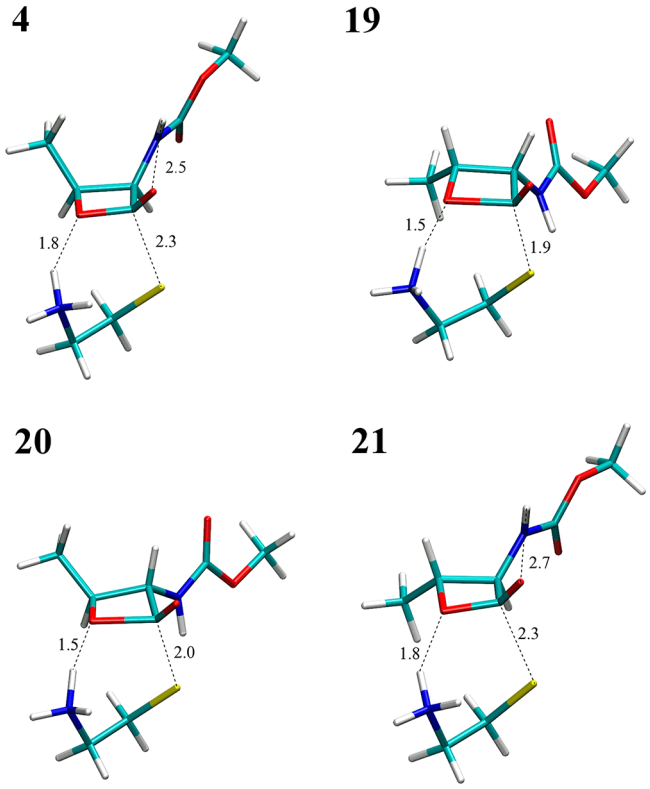


Figure 3. Transition state structures for the nucleophilic attack to **4** and **19**−**21**. In the case of **4** and **21**, the stabilization of the TS might be due to an intramolecular interaction between the oxygen atom of the lactone carbonyl and the hydrogen atom of the carbamate. The mechanism was a concerted asynchronous type, where the nucleophilic attack and the protonation of the carbonyl oxygen occur simultaneously. All distances are in Å.

further increase of E_a for **19** and **20** (see the Supporting Information). We conclude that the different potencies between (*R*)- and (*S*)-configurations of the carbon carrying the carbamate group may be due to both intra- and intermolecular interactions, responsible for markedly different

activation energies for the sulfur nucleophilic attack. However, we cannot exclude that compounds **4** and **19**−**21** might also bind to NAAA in different ways, and therefore, inhibitor−NAAA interactions at the Michaelis complex can also modulate their potency.

Selected 2-methyl-4-oxo-3-oxetanylcarbamic acid esters were tested in a functional assay using h-NAAA (Table 4). A common trend between relative potencies at human and rat NAAA was found. Potencies were usually 2−7-fold greater on human NAAA than on rat NAAA, with a particularly pronounced difference observed with compound **4**. Compound **14q**, the *p*-biphenylmethyl carbamate derivative, proved to be equally active on the two enzyme orthologues, showing very high potency on both h-NAAA (IC_{50} = 0.007 μM) and r-NAAA (IC_{50} = 0.007 μM).

The same compounds were also tested for selectivity against rat brain FAAH (r-FAAH), which can also cleave FAEs,[48] and rat lung acid ceramidase (r-AC), a cysteine amidase that exhibits 33% amino acid identity with r-NAAA (Table 4).[18] At the concentration tested (10 μM), the compounds had little or no effect on the activity of these enzymes, demonstrating a good selectivity toward NAAA.

Finally, we used high-resolution liquid chromatography−mass spectrometry (LC-MS) to probe the mechanism through which **14q**, the most potent among the inhibitors described here, interacts with h-NAAA. We examined whether **14q** might form a covalent adduct with the N-terminal cysteine of the active form of h-NAAA (see the Supporting Information), as previously shown for **4**.[37] Consistent with our hypothesis, incubation of h-NAAA with **14q**, followed by trypsin digestion, revealed the presence of a covalent adduct of **14q** with the N-terminal peptide of h-NAAA (CTSIVAQDSR) (Figure 4). On the basis of the high-resolution MS/MS analysis of the modified peptide, we conclude that **14q** inhibits h-NAAA through S-acylation of catalytic Cys126.

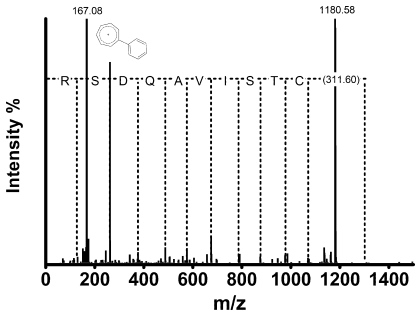


Figure 4. Tandem mass spectrum of peptide CTSIVAQDSR covalently modified by **14q**. The backbone fragment ion series perfectly matches the primary sequence of the peptide, clearly indicating that the mass increase (+311 Da) is carried by the N-terminal cysteine residue. A very intense tropylium ion (167.08 *m*/*z*) and a side-chain loss of the biphenyl group and CO_2 (1180.58 *m*/*z*) are also clearly visible in the spectrum.

■ CONCLUSIONS

The present work expanded our previous SAR studies on threonine-derived 2-methyl-4-oxo-3-oxetanylcarbamic acid esters as NAAA inhibitors. Those studies had led to the discovery of 5-phenylpentyl-*N*-[(2*S*,3*R*)-2-methyl-4-oxo-oxetan-3-yl]-carbamate **4**, a potent inhibitor of intracellular NAAA activity. We modified compound **4** with the aim of clarifying the relevant structural determinants for NAAA inhibition by threonine-derived β-lactones. Our new investigations highlight the importance of the configuration of the stereogenic centers at positions 2 and 3 of compound **4** for potency. Although the relative importance of the configuration at position 2 versus 3 remains to be fully explored, our data support a primary role for position 3.

As previously observed with the serine-derived *N*-(2-oxo-3-oxetanyl)amides, a linear lipophilic chain attached to the carbamic acid was important to achieve high potency. An unsubstituted carbamate nitrogen turned out to be mandatory for enzyme inhibition, whereas monosubstitution at the carbon atom in α-position to the carbamate oxygen with a small substituent, such as a methyl group, could be beneficial for potency, depending on the substituent configuration. Finally, the replacement of the phenylpentyl chain of compound **4** with a *p*-biphenylmethyl moiety led to the discovery of compound **14q**, the first single-digit nanomolar inhibitor of both human and rat NAAA. This molecule represents a promising probe that may help characterize the functional roles of NAAA and assess the therapeutic potential of NAAA inhibitors as novel anti-inflammatory and analgesic agents.

■ EXPERIMENTAL SECTION

a. Chemistry. *Chemicals, Materials, and Methods.* All of the commercially available reagents and solvents were used as purchased from vendors without further purification. Dry solvents (THF, Et_2O, CH_2Cl_2, DMF, DMSO, MeOH) were purchased from Sigma-Aldrich. Optical rotations were measured on a Rudolf Research Analytical Autopol II Automatic polarimeter using a sodium lamp (589 nm) as the light source; concentrations are expressed in g/100 mL using $CHCl_3$ as a solvent and a 1 dm cell. Automated column chromatography purifications were done using a Teledyne ISCO apparatus (Combi*Flash* R_f) with prepacked silica gel columns of different sizes (from 4 to 120 g). Mixtures of increasing polarity of cyclohexane and ethyl acetate (EtOAc) or cyclohexane and methyl *tert*-butyl ether (MTBE) were used as eluents. Hydrogenation reactions were performed using H-Cube continuous hydrogenation equipment (SS-reaction line version), employing disposable catalyst cartridges (CatCart) preloaded with the required heterogeneous catalyst. Microwave heating was performed using an Explorer-48 positions instrument (CEM). NMR experiments were run on a Bruker Avance III 400 system (400.13 MHz for ^{1}H and 100.62 MHz for ^{13}C), equipped with a BBI probe and *Z*-gradients. Spectra were acquired at 300 K, using deuterated dimethyl sulfoxide (DMSO-d_6) or deuterated chloroform ($CDCl_3$) as solvents. UPLC-MS analyses were run on a Waters ACQUITY UPLC-MS system consisting of a SQD (single-quadrupole detector) mass spectrometer equipped with an electrospray ionization interface and a photodiode array detector. The PDA range was 210−400 nm. Analyses were performed on an ACQUITY UPLC HSS T3 C_{18} column (50 × 2.1 mm i.d., particle size = 1.8 μm) with a VanGuard HSS T3 C_{18} precolumn (5 × 2.1 mm i.d., particle size = 1.8 μm). Mobile phase was either 10 mM NH_4OAc in H_2O at pH 5 adjusted with AcOH (A) or 10 mM NH_4OAc in MeCN/H_2O (95:5) at pH 5. 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. The 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 an XBridge Prep C_{18} OBD column (100 × 19 mm i.d., particle size = 5 μm) with an XBridge Prep C_{18} (10 × 19 mm i.d., particle size = 5 μm) guard cartridge. The mobile phase was 10 mM NH_4OAc in MeCN/H_2O (95:5) at pH 5. Electrospray ionization in positive and negative mode was used. Analyses by chiral HPLC were run on a Waters Alliance HPLC instrument consisting of

an e2695 separation module and a 2998 photodiode array detector. PDA range was 210−400 nm. Analyses were performed isocratically on a Daicel ChiralPak AD column (250 × 4.6 mm i.d., particle size = 10 μm). Mobile phase was 0.1% TFA heptane/2-propanol (75:25). Separations by preparative chiral HPLC were run on a Waters Alliance HPLC instrument consisting of a 1525 binary HPLC pump, Waters Fraction Collector III, and a 2998 photodiode array detector. UV detection was at 240 nm. Purifications were performed isocratically on a Daicel ChiralPak AD column (250 × 10 mm i.d., particle size = 10 μm). Mobile phase was 0.1% TFA heptane/2-propanol (75:25). All tested compounds (**4**, **14a−u**, **19−22**, and **29**) showed ≥95% purity by NMR and UPLC-MS analysis.

Synthesis of 5-Phenylpentyl-[(2*S*,3*R*)-2-methyl-4-oxooxetan-3-yl]carbamate (4). **4** was obtained as an off-white solid. Experimental procedure and NMR are according to the literature.[28,37]

General Procedure I for the Synthesis of Carbamates 14b,e,h (Scheme 4). *Preparation of β-Hydroxycarboxylic Acids 15b,e,h (Step 1).* To a suspension of $NaHCO_3$ (2.5 equiv) in THF and H_2O was added D-threonine (1.0 equiv). The suitable chloroformate **6b,e,h** (1.1 equiv) was slowly added, followed by a catalytic amount of $(n\text{-Bu})_4NBr$. After stirring at room temperature for 18 h, the mixture was diluted with H_2O and washed with Et_2O. The aqueous layer was acidified with 2 M HCl solution and extracted with EtOAc. The combined organic layers were dried over Na_2SO_4, filtered, and concentrated to give **15b,e,h**, which were used in the next step without further purification.

Preparation of Carbamates 14b,e,h (Step 2). To a solution of β-hydroxycarboxylic acid was added **15b,e,h** (1.0 equiv) in dry CH_2Cl_2, Et_3N (3.0 equiv) under argon. After cooling at 0 °C, HBTU (1.5 equiv) or PyBOP (1.3 equiv) was added, and the mixture was stirred at 0 °C for 3 h and then at room temperature for 16 h. The obtained solid was filtered off and the solvent removed under vacuum. The crude was purified by typical silica gel column chromatography, eluting with cyclohexane/EtOAc (from 80:20 to 30:70) to give compounds **14b,e,h**.

3-Phenylpropyl-[(2*S*,3*R*)-2-methyl-4-oxo-oxetan-3-yl]-carbamate (14b). The reaction was carried out following the general procedure I (step 2) employing Et_3N (1.4 mL, 10.1 mmol), (2*R*,3*S*)-3-hydroxy-2-(3-phenylpropoxycarbonylamino)butanoic acid (**15b**) (0.95 g, 3.38 mmol), dry CH_2Cl_2 (40 mL), and HBTU (1.92 g, 5.07 mmol) to give **14b**, obtained as a white solid: yield, 31% (0.240 g); $[\alpha]^{25}_D$ −16.6 (*c* 0.1, $CHCl_3$); MS (ESI), *m/z* 262 [M − H]$^-$; FTIR (cm^{-1}), 3328, 3064, 3030, 2972, 2929, 2859, 1851, 1691, 1542, 1333, 1270, 1130, 1083, 1024, 843, 822, 697; ^{1}H NMR (DMSO-d_6), δ 1.36 (d, *J* = 6.4 Hz, 3H), 1.83−1.92 (m, 2H), 2.64 (t, *J* = 7.6 Hz, 2H), 4.00 (m, 2H), 4.86 (dq, *J* = 6.1, 6.4 Hz), 5.42 (dd, *J* = 6.1, 9.5 Hz, 1H), 7.10−7.40 (m, 5H), 8.19 (d, *J* = 9.5 Hz, 1H); ^{13}C NMR (DMSO-d_6), δ 14.52, 30.13, 31.27, 59.91, 64.05, 74.65, 125.89, 128.23, 128.33, 141.15, 155.87, 169.87.

Pentyl-[(2*S*,3*R*)-2-methyl-4-oxooxetan-3-yl]carbamate (14e). The reaction was carried out following the general procedure I (step 2) employing Et_3N (3.75 mL, 26.9 mmol), (2*R*,3*S*)-3-hydroxy-2-(pentyloxycarbonylamino)butanoic acid (**15e**) (2.0 g, 8.96 mmol), dry CH_2Cl_2 (100 mL), and PyBOP (6.1 g, 11.6 mmol) to give **14e** as a white solid: yield, 22.5% (0.539 g); $[\alpha]^{25}_D$ −25.2 (*c* 0.1, $CHCl_3$); MS (ESI), *m/z* 214 [M − H]$^-$; FTIR (cm^{-1}), 3323, 3074, 2958, 2931, 2871, 1854, 1692, 1545, 1471, 1391, 1334, 1270, 1150, 1126, 1087, 1025, 982, 844,823; ^{1}H NMR (DMSO-d_6), δ 0.87 (t, *J* = 7.0 Hz, 3H), 1.23−1.36 (m, 4H), 1.34 (d, *J* = 6.4 Hz, 3H), 1.56 (m, 2H), 4.00 (m, 2H), 4.84 (dq, *J* = 6.2, 6.4 Hz, 1H), 5.40 (dd, *J* = 6.2, 9.5 Hz, 1H), 8.19 (d, *J* = 9.5 Hz, 1H); ^{13}C NMR (DMSO-d_6), δ 13.87, 14.50, 21.77, 27.5, 28.2, 59.89, 64.67, 74.63, 155.82, 169.90.

Octyl-[(2*S*,3*R*)-2-methyl-4-oxooxetan-3-yl]carbamate (14h). The reaction was carried out following the general procedure I (step 2) employing Et_3N (1.5 mL, 10.9 mmol), (2*R*,3*S*)-3-hydroxy-2-(octyloxycarbonylamino)butanoic acid (**15h**) (1.0 g, 3.63 mmol), dry CH_2Cl_2 (40 mL), and HBTU (2.07 g, 5.45 mmol) to give **14h** as a white solid: yield, 18% (0.165 g); $[\alpha]^{25}_D$ −19.3 (*c* 0.1, $CHCl_3$); MS (ESI), *m/z* 256 [M − H]$^-$; FTIR (cm^{-1}), 3326, 2958, 2924, 2856, 1857, 1692, 1547, 1333, 1270, 1087, 1025, 845; ^{1}H NMR (DMSO-d_6), δ 0.85 (t, *J* = 7.0 Hz, 3H), 1.21−1.34 (m, 10 H), 1.34 (d, *J* = 6.4 Hz, 3H), 1.51−2.01 (m, 2H), 4.95−5.05 (m, 2H), 4.84 (dq, *J* = 6.1, 6.4 Hz, 1H), 5.40 (dd, *J* = 6.1, 9.3 Hz, 1H), 8.18 (d, *J* = 9.3 Hz, 1H); ^{13}C NMR (DMSO-d_6), δ 13.94, 14.43, 22.02, 25.27, 28.46, 28.59, 28.59, 31.18, 59.87, 64.66, 74.63, 155.83, 169.88.

Synthesis of Carbamate 14c (Scheme 4). (2*R*,3*S*)-3-Hydroxy-2-(4-phenylbutoxycarbonylamino)butanoic acid (15c). To a stirred mixture of 4-phenylbutan-1-ol (**5c**) (5.0 mL, 32.8 mmol, 1.0 equiv) in dry DMF (70 mL) under argon atmosphere was added CDI (10.6 g, 65.5 mmol, 2.0 equiv). After the mixture had stirred at room temperature for 2 h, D-threonine (3.90 g, 32.8 mmol, 1.0 equiv) dissolved in H_2O (70 mL) and Et_3N (6.8 mL, 49.1 mmol, 1.5 equiv) were added. The mixture was heated at 50 °C for 16 h and then allowed to cool. Water was added and the mixture washed with Et_2O (three times). The aqueous phase was acidified with 2 M HCl solution and then extracted with EtOAc (three times). The collected organic phases were dried over Na_2SO_4 and filtered and the solvent removed under vacuum. The crude was purified through silica gel column chromatography eluting with cyclohexane/EtOAc (20:80) + 1% CH_3COOH to afford **15c** as a pale yellow oil: yield, 54% (5.2 g); MS (ESI), *m/z* 294 [M − H]$^-$, 296 [M − H]$^+$; FTIR (cm^{-1}), 3332 (br), 3027, 2978, 2938, 2863, 1719 (br), 1525, 1253, 1069, 779, 748, 699; ^{1}H NMR ($CDCl_3$), δ 1.26 (d, *J* = 6.4 Hz, 3H), 1.39 (m, 2H), 1.63−1.67 (m, 2H), 2.62 (t, *J* = 7.7 Hz, 2H), 4.05−4.12 (m, 2H), 4.30−4.38 (m, 1H), 4.39−4.47 (m, 1H), 5.68 (m, 1H), 7.16−7.78 (m, 3H), 7.26−7.28 (m, 2H).

4-Phenylbutyl-[(2*S*,3*R*)-2-methyl-4-oxooxetan-3-yl]-carbamate (14c). To a solution of (2*R*,3*S*)-3-hydroxy-2-(4-phenylbutoxycarbonylamino)butanoic acid (**15c**) (3.9 g, 13.1 mmol, 1.0 equiv) in dry CH_2Cl_2 (170 mL) was added Et_3N (5.5 mL, 39.4 mmol, 3.0 equiv) under argon. After cooling at 0 °C, HBTU (7.5 g, 19.7 mmol, 1.5 equiv) was added and the mixture stirred at 0 °C for 3 h and then at room temperature for 3.5 h. The crude was purified by silica gel column chromatography, eluting with cyclohexane/EtOAc (from 95:5 to 60:40). The resulting pale yellow oil was further crystallized from cyclohexane to give **14c** as a white solid: yield, 6% (0.23 g); $[\alpha]^{25}_D$ −17.8 (*c* 0.1, $CHCl_3$); MS (ESI), *m/z* 276 [M − H]$^-$; FTIR (cm^{-1}), 3312, 3060, 3026, 2942, 2861, 1826, 1694, 1545, 1337, 1269, 1126, 1023; ^{1}H NMR ($CDCl_3$), δ 1.45 (d, 3H), 1.66−1.70 (m, 4H), 2.63−2.66 (m, 2H), 4.09−4.18 (m, 2H), 4.83−4.89 (m, 1H), 5.36 (br d, 1H), 5.45 (m, 1H), 7.16−7.31 (m, 5H).

General Procedure II for the Synthesis of Carbamates 14i,l,m,p,q,s (Scheme 3). Synthesis of (3*R*,4*S*)-4-Methyl-2-oxo-1-oxetan-3-ylammonium Toluene-4-sulfonate (13). **13** was obtained as an off-white solid. Experimental procedure and NMR data are according to the literature.[24]

Preparation of Activated Alcohols as Alkyl-2-pyridyl Carbonates 8i,l,m,p,q,s and Alkyl-2-oxopyridine-1-carboxylates 9i,l,m,p,q,s (Step 1). To a stirred mixture of the suitable alcohol **5i,l,m,p,q,s** (1.0 equiv) in dry CH_2Cl_2 and under nitrogen atmosphere was added Et_3N (1.5 equiv) or DMAP (0.1 equiv) and DPC (1.1 equiv). The mixture was reacted at room temperature for 15 h and then diluted with CH_2Cl_2, washed with a saturated NH_4Cl solution and, subsequently, with a saturated $NaHCO_3$ solution (three times). The combined organic layers were dried over Na_2SO_4, filtered, and concentrated to dryness to give an oil as a mixture (ca. 3:1 ratio if Et_3N was used and ca. 1.6:1 ratio if DMAP was used) of alkyl-2-pyridyl carbonate **8i,l,m,p,q,s** and alkyl-2-oxopyridine 1-carboxylate **9i,l,m,p,q,s**. The mixture of isomers was not separated and used in the next step without any further purification.

Preparation of Carbamates 14i,l,m,p,q,s (Step 2). To a stirred mixture of **13** (1.0 equiv) in dry CH_2Cl_2 (1.0 mL) under nitrogen atmosphere was added dropwise DIPEA (1.0 equiv). Subsequently, the isomeric mixture of **8i,l,m,p,q,s** and **9i,l,m,p,q,s** (3.0 equiv) dissolved in dry CH_2Cl_2 (2.0 mL) was added. The mixture was stirred at room temperature for 15 h, concentrated to dryness, and purified by column chromatography eluting with cyclohexane/MTBE (from 100:0 to 70:30). Compounds **14i,l,m,p,q,s** were further purified by preparative HPLC-MS.

3-Benzyloxypropyl-*N*-[(2*S*,3*R*)-2-methyl-4-oxo-oxetan-3-yl]-carbamate (14i). The reaction was carried out following the general procedure II (step 2) employing **13** (0.10 g, 0.36 mmol), DIPEA (0.06 mL, 0.36 mmol), and the isomeric mixture of **8i** and **9i** (0.294 g, 1.02 mmol). The crude product was further purified by preparative HPLC-MS to give **14i** as a white solid: yield, 41% (0.045 g); $[\alpha]^{25}_{D}$ −9.8 (*c* 0.1, $CHCl_3$); MS (ESI), *m/z* 292 [M − H]$^-$, 294 [M − H]$^+$; ^{1}H NMR (DMSO-d_6), δ 1.34 (d, *J* = 6.3 Hz, 3H), 1.81−1.91 (m, 2H), 3.50 (t, *J* = 6.3 Hz, 2H), 4.04−4.16 (m, 2H), 4.47 (s, 2H), 4.85 (dq, *J* = 6.2, 6.3 Hz, 1H), 5.42 (dd, *J* = 6.2, 9.4 Hz, 1H), 7.25−7.40 (m, 5H), 8.23 (d, *J* = 9.4 Hz, 1H); ^{13}C NMR (DMSO-d_6), δ 14.47, 28.89, 59.88, 62.08, 66.13, 71.87, 74.62, 127.36, 128.22, 138.47, 155.72, 169.83.

(1,1-Dimethyl-5-phenylpentyl)-*N*-[(2*S*,3*R*)-2-methyl-4-oxo-oxetan-3-yl]carbamate (14l). The reaction was carried out following the general procedure II (step 2) employing **13** (0.07 g, 0.25 mmol), DIPEA (0.044 mL, 0.25 mmol), and the isomeric mixture of **8l** and **9l** (0.321 g, 1.02 mmol). The crude product was further purified by preparative HPLC-MS to give **14l** as a white solid: yield, 31% (0.025 g); $[\alpha]^{25}_{D}$ −17.5 (*c* 0.1, $CHCl_3$); MS (ESI), *m/z* 318 [M − H]$^-$, 342 [M − Na]$^+$; ^{1}H NMR (DMSO-d_6), δ 1.32 (d, *J* = 6.4 Hz, 3H), 1.30−1.39 (m, 2H), 1.36 (s, 3H), 1.37 (s, 3H), 1.50−1.62 (m, 2H), 1.69−1.83 (m, 2H), 2.54−2.62 (m, 2H), 4.82 (dq, *J* = 6.1, 6.4 Hz, 1H), 5.34 (dd, *J* = 6.1, 9.3 Hz, 1H), 7.94 (d, *J* = 9.3 Hz, 1H), 7.10−7.30 (m, 5H); ^{13}C NMR (DMSO-d_6), δ 14.38, 22.94, 26.03, 31.32, 35.10, 59.61, 74.57, 81.15, 99.48, 125.57, 128.19, 128.21, 142.10, 154.71, 170.00.

[(1*R*) and (1*S*)-1-Methyl-5-phenylpentyl]-*N*-[(2*S*,3*R*)-2-methyl-4-oxo-oxetan-3-yl]carbamate (14m). The reaction was carried out following the general procedure II (step 2) employing **13** (0.100 g, 0.36 mmol), DIPEA (0.060 mL, 0.36 mmol), and the isomeric mixture of **8m** and **9m** (0.31 g, 1.02 mmol). The crude product was further purified by preparative HPLC-MS to give **14m** as a 1:1 diastereoisomeric mixture as a white solid: yield, 32% (0.035 g); MS (ESI), *m/z* 304 [M − H]$^-$, 306 [M − H]$^+$; ^{1}H NMR (DMSO-d_6), δ 1.12−1.21 (m, 6H), 1.26−1.38 (m, 10H), 1.46−1.64 (m, 8H), 2.52−2.61 (m, 4H), 4.64−4.75 (m, 2H), 4.84 (dq, *J* = 6.2, 6.4 Hz, 2H), 5.39 (dd, *J* = 6.2, 9.3 Hz, 2H), 7.12−7.31 (m, 10H), 8.12 (d, *J* = 9.3 Hz, 2H); ^{13}C NMR (DMSO-d_6), δ 14.90, 20.52, 24.94, 31.21, 31.25, 35.52, 35.81, 60.36, 71.68, 75.12, 126.08, 128.69, 142.57, 155.92, 170.26.

(3-Phenylphenyl)methyl-*N*-[(2*S*,3*R*)-2-methyl-4-oxo-oxetan-3-yl]carbamate (14p). The reaction was carried out following the general procedure II (step 2) employing **13** (0.120 g, 0.44 mmol), DIPEA (0.072 mL, 0.44 mmol), and the isomeric mixture of **8p** and **9p** (0.402 g, 1.32 mmol). The crude product was further purified by preparative HPLC-MS to give **14p** as a white solid: yield, 32% (0.045 g); $[\alpha]^{25}_{D}$ −11.4 (*c* 0.1, $CHCl_3$); MS (ESI), *m/z* 334 [M − Na]$^+$; ^{1}H NMR (DMSO-d_6), δ 1.36 (d, *J* = 6.4, 3H), 4.88 (dq, *J* = 6.2, 6.4, 1H), 5.08−5.29 (m, 2H), 5.47 (dd, *J* = 6.2, 9.3, 1H), 7.31−7.79 (m, 9H), 8.40 (d, *J* = 9.3, 1H); ^{13}C NMR (DMSO-d_6) δ 14.50, 59.95, 66.14, 74.62, 126.18, 126.31, 126.66, 126.85, 127.58, 128.94, 129.07, 137.21, 139.82, 140.33, 155.60, 169.73.

(4-Phenylphenyl)methyl-*N*-[(2*S*,3*R*)-2-methyl-4-oxo-oxetan-3-yl]carbamate (14q). The reaction was carried out following the general procedure II (step 2) employing **13** (0.12 g, 0.44 mmol), DIPEA (0.072 mL, 0.44 mmol), and the isomeric mixture **8q** and **9q** (0.40 g, 1.31 mmol). The crude product was further purified by preparative HPLC-MS to give **14q** as a white solid: yield, 30% (0.04 g); $[\alpha]^{25}_{D}$ −12.1 (*c* 0.1, $CHCl_3$); MS (ESI), *m/z* 310 [M − H]$^-$, 329 [M − NH_4]$^+$, 350 [M − K]$^+$; ^{1}H NMR (DMSO-d_6), δ 1.37 (d, *J* = 6.4 Hz, 3H), 4.88 (dq, *J* = 6.1, 6.4 Hz, 1H), 5.12 (d, *J* = 12.5 Hz, 1H), 5.16 (d, *J* = 12.5 Hz, 1H), 5.47 (dd, *J* = 6.1, 9.4 Hz, 1H), 7.35−7.41 (m, 1H), 7.45−7.51 (m, 4H), 7.66−7.71 (m, 4H), 8.40 (d, *J* = 9.4 Hz, 1H); ^{13}C NMR (DMSO-d_6), δ 14.51, 59.94, 65.89, 74.61, 126.65, 126.70, 127.52, 128.53, 128.91, 135.65, 139.68, 139.88, 155.59, 169.75.

(4-Benzyloxyphenyl)-*N*-[(2*S*,3*R*)-2-methyl-4-oxo-oxetan-3-yl]carbamate (14s). The reaction was carried out following the general procedure II (step 2) employing **13** (0.120 g, 0.44 mmol), DIPEA (0.072 mL, 0.44 mmol), and the isomeric mixture **8s** and **9s** (0.423 g, 1.31 mmol). The crude product was further purified by preparative HPLC-MS to give **14s** as a white solid: yield, 17% (0.025 g); $[\alpha]^{25}_{D}$ −21.6 (*c* 0.1, $CHCl_3$); MS (ESI), *m/z* 326 [M − H]$^-$, 328 [M − H]$^+$; ^{1}H NMR (DMSO-d_6), δ 1.43 (d, *J* = 6.4 Hz, 3H) 4.92 (dq, *J* = 6.1, 6.4 Hz, 1H), 5.10 (s, 2H), 5.51 (dd, *J* = 6.1, 9.4, Hz, 1H), 6.94−7.53 (m, 9H), 8.78 (d, *J* = 9.4 Hz, 1H); ^{13}C NMR (DMSO-d_6), δ 14.57, 59.99, 69.54, 74.61, 99.49, 115.23, 122.55, 127.63, 128.40, 136.98, 144.16, 154.33, 155.71, 169.44.

General Procedure III for the Synthesis of Carbamates 14a,d,f,g,j,k,n,o,r and 19−22 (Schemes 4 and 5). *Preparation of Activated Alcohols as Alkyl-2-pyridyl Carbonates **8a,d,f,g,j,k,n,o,r,t** and Alkyl-2-oxopyridine-1-carboxylates **9a,d,f,g,j,k,n,o,r,t** (Step 1).* To a stirred mixture of the suitable alcohol **5a,d,f,g,j,k,n,o,r,t** (1.0 equiv) in dry CH_2Cl_2, and under nitrogen atmosphere were added DMAP (0.1 equiv) and DPC (1.2 equiv). The reaction mixture was left to react at room temperature for 15 h, then diluted with CH_2Cl_2 and washed with a saturated NH_4Cl solution and, subsequently, with a saturated $NaHCO_3$ solution (three times). The combined organic layers were dried over Na_2SO_4, filtered, and concentrated to give a mixture (ratio 1.6:1) of alkyl-2-pyridyl carbonate **8a,d,f,g,j,k,n,o,r,t** and alkyl-2-oxopyridine 1-carboxylate **9a,d,f,g,j,k,n,o,r,t**. The mixture of isomers was not separated and used in the next step without any further purification.

*Preparation of β-Hydroxycarboxylic Acids **15a,d,f,g,j,k,n,o,r** and **16−18** (Step 2).* To a stirred mixture of D- or L- or D-*allo*- or L-*allo*-threonine (1.0 equiv) and $NaHCO_3$ (1.5 equiv) in H_2O (3.5 mL) was added the isomeric mixture containing the pyridyl carbonate **8a,d,f,g,j,k,n,o,r,t** and the 2-oxopyridine-1-carboxylate **9a,d,f,g,j,k,n,o,r,t** (1.5 equiv) in THF (3.5 mL). After 15 h at room temperature, the crude was evaporated and subsequently extracted with Et_2O (3 × 5 mL). The aqueous layer was acidified with 2 M HCl solution to pH 2−3 and subsequently extracted with EtOAc (3 × 10 mL). The combined organic layers were dried over Na_2SO_4, filtered, and concentrated to give the threonine derivatives **15a,d,f,g,j,k,n,o,r** and **16−18**, which were used in the next step without further purification.

*Preparation of Carbamates **14a,d,f,g,j,k,n,o,r** and **19−22** (Step 3).* To a stirred mixture of threonine derivatives **15a,d,f,g,j,k,n,o,r** and **16−18** (1.0 equiv) in dry CH_2Cl_2, at 0 °C, and under nitrogen atmosphere, were added Et_3N (3.0 equiv) and, subsequently, TBTU (1.2 equiv). The mixture was stirred at 0 °C for 1 h and at room temperature for 15 h, then concentrated, and the crude was purified by column chromatography, eluting with cyclohexane/EtOAc (from 100:0 to 0:100) to afford **14a,d,f,g,j,k,n,o,r** and **19−22**.

Phenethyl-*N*-[(2*S*,3*R*)-2-methyl-4-oxo-oxetan-3-yl]-carbamate (14a). The reaction was carried out following the general procedure III (step 3) employing (2*R*,3*S*)-3-hydroxy-2-(2-phenethyloxycarbonylamino)butanoic acid (**15a**) (0.35 g, 1.30 mmol), dry CH_2Cl_2 (35 mL), Et_3N (0.54 mL, 3.90 mmol), and TBTU (0.50 g, 1.56 mmol) to give **14a** as a white solid: yield, 57% (0.185 g); $[\alpha]^{25}_{D}$ −18.2 (*c* 0.1, $CHCl_3$); MS (ESI), *m/z* 249 [M − H]$^-$, 250 [M − H]$^+$; ^{1}H NMR (DMSO-d_6), δ 1.33 (d, *J* = 6.3 Hz, 3H), 2.89 (t, *J* = 6.8 Hz, 2H), 4.15−4.29 (m, 2H), 4.84 (dq, *J* = 6.1, 6.3 Hz, 1H), 5.40 (dd, *J* = 6.1, 9.4 Hz, 1H), 7.19−7.36 (m, 5H), 8.23 (d, *J* = 9.4 Hz, 1H); ^{13}C NMR (DMSO-d_6), δ 14.46, 34.65, 59.86, 65.15, 74.62, 126.31, 128.31, 128.81, 137.95, 155.61, 169.77.

6-Phenylhexyl-*N*-[(2*S*,3*R*)-2-methyl-4-oxo-oxetan-3-yl]-carbamate (14d). The reaction was carried out following the general procedure III (step 3) employing (2*R*,3*S*)-3-hydroxy-2-(6-phenylhexoxycarbonylamino)butanoic acid (**15d**) (0.25 g, 0.78 mmol), dry CH_2Cl_2 (25 mL), Et_3N (0.33 mL, 2.35 mmol), and TBTU (0.30 g, 1.2 mmol) to give **14d** as a white solid: yield, 50% (0.12 g); $[\alpha]^{25}_{D}$ −12.3 (*c* 0.1, $CHCl_3$); MS (ESI), *m/z* 304 [M − H]$^-$, 306 [M − H]$^+$; ^{1}H NMR (DMSO-d_6), δ 1.26−1.39 (m, 4H), 1.34 (d, *J* = 6.4 Hz, 3H), 1.48−1.65 (m, 4H), 2.54−2.61 (m, 2H), 3.91−4.06 (m, 2H), 4.84 (dq, *J* = 6.1, 6.4 Hz, 1H), 5.41 (dd, *J* = 6.1, 9.4 Hz, 1H), 7.11−7.31 (m, 5H), 8.19 (d, *J* = 9.4 Hz, 1H); ^{13}C NMR (DMSO-d_6), δ 14.47, 25.07, 28.23, 28.40, 30.88, 35.02, 59.88, 64.64, 74.63, 125.57, 128.19, 128.21, 142.20, 155.80, 169.85.

Hexyl-*N*-[(2*S*,3*R*)-2-methyl-4-oxooxetan-3-yl]carbamate (14f). The reaction was carried out following the general procedure III (step 3) employing (2*R*,3*S*)-2-(hexyloxycarbonylamino)-3-hydroxybutanoic acid (**15f**) (0.27g, 1.10 mmol), dry CH_2Cl_2 (27 mL), Et_3N

(0.46 mL, 3.31 mmol), and TBTU (0.42 g, 1.32 mmol) to give **14f** as a white solid: yield, 73% (0.185 g); $[\alpha]^{25}_{D}$ −25.1 (*c* 0.1, $CHCl_3$); MS (ESI), *m/z* 228 [M − H]$^-$, 230 [M − H]$^+$; ^{1}H NMR (DMSO-d_6), δ 0.86 (t, *J* = 6.9 Hz, 3H), 1.21−1.38 (m, 6H), 1.34 (d, *J* = 6.4 Hz, 3H), 1.50−1.62 (m, 2H), 3.93—4.07 (m, 2H), 4.84 (dq, *J* = 6.1, 6.4 Hz, 1H), 5.40 (dd, *J* = 6.1, 9.3 Hz, 1H), 8.19 (d, *J* = 9.3 Hz, 1H); ^{13}C NMR (DMSO-d_6), δ 13.83, 14.46, 21.98, 24.92, 28.42, 30.84, 59.88, 64.67, 74.62, 155.80, 169.85.

Heptyl-*N*-[(2*S*,3*R*)-2-methyl-4-oxooxetan-3-yl]carbamate (14g). The reaction was carried out following the general procedure III (step 3) employing (2*R*,3*S*)-2-(heptyloxycarbonylamino)-3-hydroxybutanoic acid (**15g**) (0.3 g, 1.14 mmol), dry CH_2Cl_2 (30 mL), Et_3N (0.48 mL, 3.44 mmol), and TBTU (0.44 g, 1.37 mmol) to give **14g** as a white solid: yield, 37% (0.103 g); $[\alpha]^{25}_{D}$ −23.1 (*c* 0.1, $CHCl_3$); MS (ESI), *m/z* 242 [M − H]$^-$, 244 [M − H]$^+$; ^{1}H NMR (DMSO-d_6), δ 0.86 (t, *J* = 6.9 Hz, 3H), 1.20−1.32 (m, 8H), 1.34 (d, *J* = 6.4 Hz, 3H), 1.49−1.60 (m, 2H), 3.93—4.07 (m, 2H), 4.84 (dq, *J* = 6.1, 6.4 Hz, 1H), 5.40 (dd, *J* = 6.1, 9.4 Hz, 1H), 8.19 (d, *J* = 9.4 Hz, 1H); ^{13}C NMR (DMSO-d_6), δ 13.90, 14.46, 21.98, 25.22, 28.28, 28.47, 31.16, 59.88, 64.67, 74.63, 155.81, 169.86.

2-Phenethyloxyethyl-*N*-[(2*S*,3*R*)-2-methyl-4-oxo-oxetan-3-yl]carbamate (14j). The reaction was carried out following the general procedure III (step 3) employing (2*R*,3*S*)-3-hydroxy-2-(2-phenethyloxyethoxycarbonylamino)butanoic acid (**15j**) (0.46 g, 1.18 mmol), dry CH_2Cl_2 (35 mL), Et_3N (0.49 mL, 3.55 mmol), and TBTU (0.30 g, 1.42 mmol) to give **14j** as a white solid: yield, 60% (0.18 g); $[\alpha]^{25}_{D}$ −12.7 (*c* 0.1, $CHCl_3$); MS (ESI), *m/z* 292 [M − H]$^-$, 294 [M − H]$^+$; ^{1}H NMR (DMSO-d_6), δ 1.35 (d, *J* = 6.4 Hz, 3H), 2.81 (t, *J* = 7.1 Hz, 2H), 3.57−3.65 (m, 4H), 4.05−4.21 (m, 2H), 4.85 (dq, *J* = 6.1, 6.4 Hz, 1H), 5.43 (dd, *J* = 6.1, 9.4 Hz, 1H), 7.15−7.33 (m, 5H), 8.33 (d, *J* = 9.4 Hz, 1H); ^{13}C NMR (DMSO-d_6), δ 14.51, 35.45, 59.89, 64.03, 68.24, 71.16, 74.60, 126.01, 128.19, 128.77, 138.78, 155.65, 169.80.

5-Cyclohexylpentyl-*N*-[(2*S*,3*R*)-2-methyl-4-oxo-oxetan-3-yl]carbamate (14k). The reaction was carried out following the general procedure III (step 3) employing (2*R*,3*S*)-2-(5-cyclohexylpentyloxycarbonylamino)-3-hydroxybutanoic acid (**15k**) (0.32 g, 1.02 mmol), dry CH_2Cl_2 (30 mL), Et_3N (0.43 mL, 3.08 mmol), and TBTU (0.39 g, 1.2 mmol) to give **14k** as a white solid: yield, 39% (0.12 g); $[\alpha]^{25}_{D}$ −8.9 (*c* 0.1, $CHCl_3$); MS (ESI), *m/z* 296 [M − H]$^-$, 298 [M − H]$^+$; ^{1}H NMR (DMSO-d_6), δ 0.78−0.93 (m, 2H), 1.05−1.33 (m, 10H), 1.36 (d, *J* = 6.4 Hz, 3H), 1.51−1.72 (m, 7H), 3.92−4.09 (m, 2H), 4.86 (dq, *J* = 6.1, 6.4 Hz, 1H), 5.42 (dd, *J* = 6.1, 9.4 Hz, 1H), 8.20 (d, *J* = 9.4 Hz, 1H); ^{13}C NMR (DMSO-d_6), 14.45, 25.58, 25.83, 25.87, 26.19, 28.49, 32.85, 36.83, 36.92, 59.88, 64.65, 74.61, 155.79, 169.84.

(1*R*) and (1*S*)-1-Methyloctyl-*N*-[(2*S*,3*R*)-2-methyl-4-oxo-oxetan-3-yl]carbamate (14n). The reaction was carried out following the general procedure III (step 3) employing (2*R*,3*S*)-3-hydroxy-2-(1-methyloctyloxycarbonylamino)butanoic acid (**15n**) (0.24 g, 0.83 mmol), dry CH_2Cl_2 (24 mL), Et_3N (0.35 mL, 2.49 mmol), and TBTU (0.32 g, 0.99 mmol) to give **14n** as a 1:1 diastereoisomeric mixture white solid: yield, 51%; MS (ESI), *m/z* 270 [M − H]$^-$, 272 [M − H]$^+$; ^{1}H NMR (DMSO-d_6), δ 0.82−0.90 (m, 6H), 1.13−1.19 (m, 6H), 1.21−1.30 (m, 20H), 1.31−1.37 (m, 6H), 1.40−1.59 (m, 4H), 4.63−4.74 (m, 2H), 4.79−4.88 (m, 2H), 5.35−5.43 (m, 2H), 8.08−8.16 (m, 2H); ^{13}C NMR (DMSO-d_6), δ 13.91, 14.40, 14.45, 20.07, 22.04, 24.78, 24.81, 28.60, 28.75, 28.77, 31.16, 35.51, 35.56, 59.87, 71.22, 74.59, 74.63, 155.54, 169.84, 169.91.

(1*R*)- and (1*S*)-(1-Isopropyl-5-phenylpentyl)-*N*-[(2*S*,3*R*)-2-methyl-4-oxo-oxetan-3-yl]carbamate (14o). The reaction was carried out following the general procedure III (step 3) employing (2*R*,3*S*)-3-hydroxy-2-[(1-isopropyl-5-phenylpentyloxy)-carbonylamino]butanoic acid (**15o**) (0.16 g, 0.45 mmol), dry CH_2Cl_2 (16 mL), Et_3N (0.19 mL, 1.36 mmol), and TBTU (0.17 g, 0.55 mmol) to give **14o** as a 1:1 diastereoisomeric mixture white solid: yield, 37% (0.05 g); MS (ESI), *m/z* 332 [M − H]$^-$, 334 [M − H]$^+$; ^{1}H NMR (DMSO-d_6), δ 0.84 (d, *J* = 6.7 Hz, 6H), 0.86 (d, *J* = 6.7 Hz, 6H), 1.22−1.35 (m, 4H), 1.30 (d, *J* = 6.2 Hz, 3H), 1.32 (d, *J* = 6.2 Hz, 3H), 1.42−1.66 (m, 8H), 1.69−1.80 (m, 2H), 2.52−2.61 (m, 4H), 4.46−4.56 (m, 2H), 4.78−4.90 (dq, *J* = 6.2 Hz, 2H), 5.33−5.43 (m, 2H), 7.13−7.21 (m, 6H), 7.23−7.30 (m, 4H), 8.13 (d, *J* = 7.0 Hz, 1H), 8.15 (d, *J* = 7.0 Hz, 1H); ^{13}C NMR (DMSO-d_6), δ 14.38, 17.40, 18.48, 24.68, 30.70, 30.76, 31.31, 35.04, 59.96, 74.64, 78.51, 125.59, 128.18, 128.20, 142.09, 156.06, 156.10, 169.72.

(1*S*,4*S*)- and (1*r*,4*R*)-(4-Benzylcyclohexyl)-*N*-[(2*S*,3*R*)-2-methyl-4-oxo-oxetan-3-yl]carbamate (14r). The reaction was carried out following the general procedure III (step 3) employing (2*R*,3*S*)-2-[(1*r*,4*R*)-4-benzylcyclohexyloxycarbonylamino]-3-hydroxybutanoic acid and (2*R*,3*S*)-2-[(1*s*,4*S*)-4-benzylcyclohexyloxycarbonylamino]-3-hydroxybutanoic acid (**15r**) (0.4 g, 1.20 mmol), dry CH_2Cl_2 (40 mL), Et_3N (0.5 mL, 3.6 mmol), and TBTU (0.46 g, 1.44 mmol) to give **14r**, as a 7.3 isomeric mixture white solid: yield, 43% (0.17 g); MS (ESI), *m/z* 316 [M − H]$^-$, 318 [M − H]$^+$; ^{1}H NMR (DMSO-d_6), δ 1.34 (d, *J* = 6.4 Hz, 3H, minor isomer), 1.37 (d, *J* = 6.4 Hz, 3H, major isomer), 1.00−1.99 (m, 20H), 2.48 (d, *J* = 7.2 Hz, 2H), 4.40−4.52 (m, 1H, minor isomer), 4.73−4.80 (m, 1H, major isomer), 4.81−4.92 (m, 2H), 5.36−5.47 (m, 2H), 7.13−7.23 (m, 6H), 7.24−7.34 (m, 4H), 8.12−8.23 (m, 2H); ^{13}C NMR (DMSO-d_6), δ 14.49, 26.79, 28.94, 30.11, 31.31, 31.35, 37.62, 38.04, 42.22, 59.85, 70.27, 73.74, 74.58, 74.64, 125.69, 128.11, 128.89, 140.51, 140.60, 155.23, 155.30, 169.96.

5-Phenylpentyl-*N*-[(2*R*,3*S*)-2-methyl-4-oxo-oxetan-3-yl]-carbamate (19). The reaction was carried out following the general procedure III (step 3) employing (2*S*,3*R*)-3-hydroxy-2-(5-phenylpentyloxycarbonylamino)butanoic acid (**16**) (0.3 g, 0.89 mmol), dry CH_2Cl_2 (30 mL), Et_3N (0.37 mL, 2.67 mmol), and TBTU (0.34 g, 1.06 mmol) to give **19** as a white solid: yield, 70% (0.18 g); $[\alpha]^{25}_{D}$ +21.3 (*c* 0.1, $CHCl_3$); MS (ESI), *m/z* 290 [M − H]$^-$, 292 [M − H]$^+$; ^{1}H NMR (DMSO-d_6), δ 1.28−1.37 (m, 2H), 1.33 (d, *J* = 6.4 Hz, 3H), 1.53−1.64 (m, 4H), 2.57 (t, *J* = 7.7 Hz, 2H), 3.93−4.06 (m, 2H), 4.84 (dq, *J* = 6.1, 6.4 Hz, 1H), 5.40 (dd, *J* = 6.1, 9.4 Hz, 1H), 7.13−7.30 (m, 5H), 8.19 (d, *J* = 9.4 Hz, 1H); ^{13}C NMR (DMSO-d_6), δ 14.46, 24.96, 28.33, 30.58, 35.03, 59.87, 64.63, 74.62, 125.60, 128.20, 128.23, 142.07, 155.78, 169.84.

5-Phenylpentyl-*N*-[(2*S*,3*S*)-2-methyl-4-oxo-oxetan-3-yl]-carbamate (20). The reaction was carried out following the general procedure III (step 3) employing (2*S*,3*S*)-3-hydroxy-2-(5-phenylpentyloxycarbonylamino)butanoic acid (**17**) (0.35 g, 1.13 mmol), dry CH_2Cl_2 (35 mL), Et_3N (0.47 mL, 3.39 mmol), and TBTU (0.43 g, 1.35 mmol) to give **20** as a white solid: yield, 76% (0.22 g); $[\alpha]^{25}_{D}$ −29.2 (*c* 0.1, $CHCl_3$); MS (ESI), *m/z* 290 [M − H]$^-$, 292 [M − H]$^+$; ^{1}H NMR (DMSO-d_6), δ 1.27−1.37 (m, 2H), 1.47 (d, *J* = 6.1 Hz, 3H), 1.53−1.64 (m, 4H), 2.57 (t, *J* = 7.6 Hz, 2H), 3.98 (t, *J* = 6.6 Hz, 2H), 4.63−4.77 (m, 2H), 7.12−7.31 (m, 5H), 8.04 (d, *J* = 8.0 Hz, 1H); ^{13}C NMR (DMSO-d_6) δ 18.62, 25.47, 28.80, 31.07, 35.52, 63.82, 65.08, 75.80, 126.09, 128.69, 128.72, 142.57, 156.02, 169.53.

5-Phenylpentyl-*N*-[(2*R*,3*R*)-2-methyl-4-oxo-oxetan-3-yl]-carbamate (21). The reaction was carried out following the general procedure III (step 3) employing (2*R*,3*R*)-3-hydroxy-2-(5-phenylpentyloxycarbonylamino)butanoic acid (**18**) (0.23 g, 0.74 mmol), dry CH_2Cl_2 (23 mL), Et_3N (0.31 mL, 2.22 mmol), and TBTU (0.28 g, 0.88 mmol) to give **21** as a white solid: yield, 69% (0.15 g); $[\alpha]^{25}_{D}$ +28.23 (*c* 0.1, $CHCl_3$); MS (ESI), *m/z* 290 [M − H]$^-$, 292 [M − H]$^+$; ^{1}H NMR (DMSO-d_6), δ 1.27−1.38 (m, 2H), 1.47 (d, *J* = 6.1 Hz, 3H), 1.53−1.64 (m, 4H), 2.54−2.59 (m, 2H), 3.98 (t, *J* = 6.6 Hz, 2H), 4.64−4.77 (m, 2H), 7.13−7.21 (m, 3H), 7.24−7.31 (m, 2H), 8.04 (d, *J* = 7.9 Hz, 1H); ^{13}C NMR (DMSO-d_6), δ 18.62, 25.47, 28.80, 31.07, 35.52, 63.82, 65.08, 75.80, 126.09, 128.69, 128.73, 142.57, 156.02, 169.53.

[(*S*)-1-Methyloctyl]-*N*-[(2*S*,3*R*)-2-methyl-4-oxo-oxetan-3-yl]-carbamate (22). Compound **22** was synthesized in the same manner as compound **14n** starting from (*S*)-nonan-2-ol: yield, 53% (0.1 g); white solid; $[\alpha]^{25}_{D}$ −4.39 (*c* 0.1, $CHCl_3$); MS (ESI), *m/z* 270 [M − H]$^-$, 272 [M − H]$^+$; ^{1}H NMR (DMSO-d_6), δ 0.85 (t, *J* = 6.8 Hz, 3H), 1.15 (d, *J* = 6.3 Hz, 3H), 1.20−1.31 (m, 10H), 1.34 (d, *J* = 6.4 Hz, 3H), 1.41−1.56 (m, 2H), 4.63−4.74 (m, 1H), 4.83 (dq, *J* = 6.3, 6.4 Hz, 1H), 5.39 (dd, *J* = 6.3, 9.3 Hz, 1H), 8.11 (d, *J* = 9.3 Hz, 1H); ^{13}C NMR (DMSO-d_6), δ 13.91, 14.44, 20.05, 22.03, 24.80, 28.59, 28.76, 31.16, 35.55, 59.86, 71.23, 74.63, 155.55, 169.92.

[(*R*)-1-Methyloctyl]-*N*-[(2*S*,3*R*)-2-methyl-4-oxo-oxetan-3-yl]-carbamate (23). Compound **23** was obtained by chiral HPLC purification of the diastereomeric mixture **14n**: white solid; $[\alpha]^{25}_{D}$ −28.39 (*c* 0.1, $CHCl_3$); ee >99% [chiral HPLC, AD column; flow, 1.0 mL/min; t_s, 26.28 min]; MS (ESI), *m/z* 270 $[M - H]^-$, 272 $[M - H]^+$; ^1H NMR (DMSO-d_6), δ 0.85 (t, *J* = 6.8 Hz, 3H), 1.17 (d, *J* = 6.3 Hz, 3H), 1.20−1.31 (m, 10H), 1.34 (d, *J* = 6.3 Hz, 3H), 1.42−1.56 (m, 2H), 4.63−4.74 (m, 1H), 4.84 (dq, *J* = 6.1, 6.3 Hz, 1H), 5.39 (dd, *J* = 9.4, 6.1 Hz, 1H), 8.13 (d, *J* = 9.3 Hz, 1H); ^{13}C NMR (DMSO-d_6), δ 14.55, 14.91, 20.58, 22.49, 25.27, 29.20, 31.66, 36.08, 60.40, 71.76, 75.06, 155.54, 169.84.

Synthesis of Carbamate 29 (Scheme 6). (2*R*,3*S*)-3-Benzyloxy-2-[*tert*-butoxycarbonyl(methyl)amino]butanoic Acid (25). To a solution of (2*R*,3*S*)-3-benzyloxy-2-(*tert*-butoxycarbonylamino)-butanoic acid (**24**) in dry THF (15 mL) at 0 °C, and under argon atmosphere, was added in one portion a 60% NaH dispersion in mineral oil (0.21 g, 5.33 mmol). The reaction mixture was stirred at 0 °C for 10 min, and then MeI (0.95 mL, 15.22 mmol) followed by DMF (0.75 mL) were sequentially added. The reaction was stirred at 0 °C for 2 h and at room temperature for 15 h, then quenched with H_2O (15 mL), diluted with EtOAc (30 mL), and acidified to pH 2 by dropwise addition of 2 N HCl aqueous solution. The organic layer was separated, and the aqueous layer was extracted with EtOAc (2 × 30 mL). The combined organic layers were dried over Na_2SO_4, filtered, and concentrated. The crude mixture was purified by column chromatography using an ISCO apparatus and cyclohexane/MTBE (60:40) as eluent. A mixture of two rotamers in a 2:1 ratio was obtained as a colorless oil: yield, 67% (1.1 g); ^1H NMR (DMSO-d_6), δ 1.13−1.20 (m, 6H), 1.36 (s, 9H, minor rotamer), 1.41 (s, 9H, major rotamer), 2.84 (s, 3H, minor rotamer), 2.87 (s, 3H, major rotamer), 4.13−4.24 (m, 2H), 4.34−4.40 (m, 2H), 4.49 (d, *J* = 5.5 Hz, 1H, minor rotamer), 4.57−4.62 (m, 2H), 4.71 (d, *J* = 5.2 Hz, 1H, major rotamer), 7.23−7.35 (m, 10H).

(2*R*,3*S*)-2-[*tert*-Butoxycarbonyl(methyl)amino]-3-hydroxybutanoic Acid (26). Compound **25** (0.5 g, 1.55 mmol) was dissolved in absolute EtOH (100 mL). The solution was passed through the H-Cube hydrogenator flow reactor provided with a 10% Pd/C cartridge. The system was set to full hydrogen mode at 60 °C and 1 bar (1.0 mL/min flow rate). The hydrogenated solution was concentrated to afford **26** as a 1:1.3 rotameric mixture colorless oil: yield, 96% (0.35 g); ^1H NMR (DMSO-d_6), δ 1.06−1.12 (m, 6H), 1.36 (s, 9H, minor rotamer), 1.40 (s, 9H, major rotamer), 2.86 (s, 3H, minor rotamer), 2.89 (s, 3H, major rotamer), 4.15−4.27 (m, 2H), 4.33 (m, 1H, minor rotamer), 4.47 (d, *J* = 5.3 Hz, 1H, major rotamer).

[(1*R*,2*S*)-1-Carboxy-2-hydroxypropyl]methylammonium Toluene-4-sulfonate (27). In a heart-shaped flask, **26** (0.086 g, 0.37 mmol) was mixed with *p*-TsOH (0.073, 0.39 mmol), and the mixture was cooled to 0 °C. Subsequently, TFA (2.0 mL) was added over 10 min, and the mixture was reacted at 0 °C for 15 min and then concentrated, maintaining the bath below 30 °C. The obtained oil was left under vacuum for 1 h, then dissolved in dry Et_2O to form a white precipitate. The solution was decanted and the solid washed several times with Et_2O, providing a white sticky solid: yield, 97% (0.11 g); ^1H NMR (DMSO-d_6), δ 1.25 (d, *J* = 6.5 Hz, 3H), 2.29 (s, 3H), 2.60 (s, 3H), 3.73−3.82 (m, 1H), 4.02−4.11 (m, 1H), 7.11 (d, *J* = 8.1 Hz, 2H), 7.48 (d, *J* = 8.1 Hz, 2H); ^{13}C NMR (DMSO-d_6), δ 21.25, 33.24, 40.01, 65.56, 66.57, 125.96, 128.50, 138.04, 146.24, 169.28.

(2*R*,3*S*)-3-Hydroxy-2-[methyl(5-phenylpentoxycarbonyl)-amino]butanoic Acid (28). To a stirred mixture of **27** (0.1 g, 0.33 mmol) and $NaHCO_3$ (0.05 g, 0.65 mmol) in H_2O (1.0 mL) was added the isomeric mixture containing the pyridyl-carbonate **8t** and the 2-oxopyridine-1-carboxylate **9t** (0.14 g, 0.49 mmol) in THF (1.0 mL). After 15 h at room temperature, the mixture was concentrated and extracted with Et_2O (3 × 5 mL). The aqueous layer was acidified with 2 M HCl solution to pH 2−3 and extracted with EtOAc (3 × 10 mL). The combined organic layers were dried over Na_2SO_4, filtered, and concentrated to give **28** as a 1.5:1 rotameric mixture colorless sticky oil: yield, 45% (0.05 g); ^1H NMR (DMSO-d_6), δ 1.07 (d, *J* = 6.3 Hz, 6H), 1.27−1.40 (m, 4H), 1.51−1.66 (m, 8H), 2.53−2.60 (m, 4H), 2.90 (s, 3H, minor rotamer), 2.91 (s, 3H, major rotamer), 3.92−4.02 (m, 4H), 4.16−4.27 (m, 2H), 4.32 (d, *J* = 5.8 Hz, 1H, minor rotamer), 4.47 (d, *J* = 5.4 Hz, 1H, major rotamer), 7.13−7.31 (m, 10H).

5-Phenylpentyl-*N*-methyl-*N*-[(2*S*,3*R*)-2-methyl-4-oxo-oxetan-3-yl]carbamate (29). To a stirred mixture of **28** (0.04 g, 0.14 mmol) in dry CH_2Cl_2 (5.0 mL), at 0 °C, and under nitrogen atmosphere, were added Et_3N (0.06 mL, 0.41 mmol) and subsequently TBTU (0.05 g, 0.17 mmol). The mixture was stirred at 0 °C for 1 h and at room temperature for 15 h and concentrated. The crude was absorbed over silica gel and purified by column chromatography, eluting with cyclohexane/EtOAc (from 100:0 to 0:100) to afford **29** as a 1:1.5 rotameric mixture colorless oil: yield, 23% (0.01 g); $[\alpha]^{25}_{D}$ −16.66 (*c* 0.1, $CHCl_3$); ^1H NMR (DMSO-d_6), δ 1.29−1.41 (m, 10H), 1.53−1.69 (m, 8H), 2.58 (t, *J* = 7.6 Hz, 4H), 2.93 (s, 6H), 3.98−4.11 (m, 4H), 4.77−4.89 (m, 2H), 5.24−5.33 (m, 1H, major rotamer), 5.35−5.43 (m, 1H, minor rotamer), 7.13−7.32 (m, 10H); ^{13}C NMR (DMSO-d_6), δ 14.25, 24.84, 28.07, 30.45, 34.95, 65.60, 66.95, 74.70, 125.59, 128.18, 128.23, 142.06, 167.32.

b. Pharmacology. *NAAA in Vitro Assay.* Rat native NAAA was obtained from lungs of male Sprague−Dawley rats (275−300 g, Charles River Italia, Calco, Italy). Rats were sacrificed by decapitation and lungs rapidly dissected out, finely chopped on ice, and transferred into ice-cold homogenizing buffer (20 mM Tris-HCl buffer, pH 7.4, 0.32 M sucrose). Samples were then homogenized with an IKA T-18 Ultraturrax for 1 min at 14000 rpm. Recombinant HEK−h-NAAA pellets were resuspended in homogenizing buffer and sonicated. Samples were then spun at 800*g* for 15 min at 4 °C, and the resultant supernatants were then ultracentrifuged at 12000*g* for 30 min at 4 °C. The pellets were resuspended in PBS, pH 7.4, on ice and subjected to a freeze/thaw cycle at −80 °C. The suspension was finally centrifuged at 105000*g* for 1 h at 4 °C. Protein concentration was measured and samples aliquoted and stored at −80 °C until use. NAAA protein preparation (10 μg) was preincubated with various concentrations of test compound or vehicle control in 100 mM NaH_2PO_4, 100 mM trisodium citrate dehydrate, 0.1% Triton-X 100, and 3 mM DTT, pH 4.5, for 30 min at 37 °C. Duplicate samples were then incubated with 50 μM C17:1 10-*cis*-heptadecenoylethanolamide (Avanti Polar Lipids, Alabaster, AL, USA) at 37 °C for 30 min. The reaction was terminated by the addition of 0.2 mL of cold methanol containing 1 nmol of heptadecanoic acid (NuChek Prep, Elysian, MN, USA) as internal standard. Samples were then analyzed by UPLC-MS. Heptadecenoic and heptadecanoic acids were eluted on an ACQUITY UPLC BEH C18 column (50 mm length, 2.1 mm i.d., 1.7 μm pore size, Waters) isocratically at 0.5 mL/min for 1.5 min with a solvent mixture of 95% methanol and 5% water, both containing 0.25% acetic acid and 5 mM ammonium acetate. The column temperature was 40 °C. Electrospray ionization was in the negative mode, capillary voltage was 2.7 kV, cone voltage was 45 V, and extractor voltage was 3 V. The source temperature was 150 °C with a desolvation temperature of 400 °C. N_2 was used as drying gas at a cone flow of 100 L/h and a desolvation flow of 800 °C. The $[M - H]^-$ ion was monitored in the selected ion monitoring mode (*m/z* values: heptadecenoic acid, 267.37; heptadecanoic acid, 269.37). Calibration curves were generated using commercial heptadecenoic acid (NuCheck Prep). Inhibition of NAAA activity was calculated as reduction of heptadecenoic acid in the samples compared to vehicle controls. IC_{50} values were calculated by nonlinear regression analysis of log[concentration]/inhibition curves using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA) applying a fixed slope curve fitting.

Human Recombinant NAAA. The coding sequence of human NAAA (NM_014435.3) was amplified by PCR (HotStar HiFidelity kit, Qiagen) from a human spleen cDNA library (catalog no. 639124, Clontech, Mountain View, CA, USA). To generate the construct h-NAAA(6× His)-pCDNA3.1 a sequence of h-NAAA fused with a C-terminal 6× histidine tag was amplified [forward primer, 5′-AAGCTTGAGCCCGAGCCATGCGGACCG−*Hin*dIII; reverse primer, 5′-CTCGAGTTAGTGGTGGTGGTGGTGGTGCTTTCT-ACTCGGGTTTC−*Xho*I] and cloned into *Hin*dIII/*Xho*I digested pCDNA 3.1 vector (Invitrogen, Carlsbad, CA, USA). HEK293 wild-type cells (American Type Culture Collection, Manassas, VA, USA) were cultured at 37 °C in a humidified incubator (5% CO_2) using

Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 1% penicillin−streptomycin and transfected with h-NAAA(6× His)-pCDNA3.1 construct using JetPEI transfection reagent (Polyplus, Illkirch, France) following the manufacturer's instructions. Stably transfected cells were selected by addition of G418 1 mg/mL to the cell culture media, and cell clones were then generated by limiting dilution plating. Growing clones were analyzed by Western blot (mAb anti-hASAHL, R&D Systems) for their capacity to produce h-NAAA and further characterized by the activity assay described below.

Rat Fatty Acid Amide Hydrolase (r-FAAH). Compounds were preincubated for 10 min with 50 μg of proteins from rat brain homogenates, followed by 30 min of incubation at 37 °C with [^{3}H]anandamide (1 μM cold AEA/0.6 nM [^{3}H]AEA (arachidonyl-[$^{1-3}$H]ethanolamine, 60 Ci/mmol, 1 mCi/mL; American Radiolabel Chemicals, Inc., St. Louis, MO, USA) in assay buffer (50 mM TRIS, pH 7.4, 0.05% fatty acid free BSA). The reaction was stopped with cold $CHCl_3/CH_3OH$ 1:1, and the aqueous phase was counted by liquid scintillation (Microbeta2 Lumijet, Perkin-Elmer Inc., Boston, MA, USA).[49]

Rat Acid Ceramidase (r-AC). Compounds were preincubated for 30 min at 37 °C with 25 μg of enzyme derived from rat AC-overexpressing HEK293 cells in assay buffer (100 mM NaH_2PO_4, 100 mM trisodium citrate dehydrate, 0.1% Triton-X 100, 3 mM DTT, pH 4.5).[24] *N*-Lauroyl ceramide 100 μM (Nu-Chek Prep) was added as substrate and analyzed by LC-MS in the negative-ion mode using heptadecanoic acid (HDA, NuChek Prep) as internal standard (m/z 199 for lauric acid, m/z 269 for HDA).

c. Computational Methods. Quantum chemical calculations were performed by means of the Gaussian 09 (G09) program suite.[50] For all stationary points, both geometry and analytical frequency calculations were carried out at the DFT level, using the B3LYP functional.[51] The employed basis set was Pople's 6-311G++(d,p). All calculations were performed in implicit water using the C-PCM solvent model.[52] To mimic the nucleophilic attack of the reactive cysteine, we built four model systems composed of a cysteamine molecule, in its zwitterionic form, and each of the compounds **4** and **19**−**21**. The molecules were truncated at the first carbon atom linked to the carbamate moiety and therefore modeled as *O*-methyl carbamate. Each Michaelis complex was built as reported by Lodola et al.[53] Then, we performed a scan on the potential energy surface using as reaction coordinate the distance between the sulfur atom of the cysteamine molecule and the carbon atom of the carbonyl group of the β-lactone ring. These calculations allowed us to identify the TS structures, which were further characterized by analyzing the Hessian matrix. In particular, at the TS, the diagonalized mass-weighted Hessian matrix showed only one negative eigenvalue, revealing in all of the reactions a first-order saddle point. Moreover, for each identified saddle point, the corresponding normal mode (related to the negative eigenvalue) involved nuclear displacements along the investigated reaction coordinate. Finally, to confirm the reaction path, we performed intrinsic reaction coordinate (IRC) calculations.

d. Proteomics. *h-NAAA Expression and Purification.* The best producing h-NAAA−HEK clone was adapted to serum-free growing condition (free style medium, Invitrogen) and cultivated in spinning culture at 37 °C in a humidified incubator (8% CO_2). The cell medium was harvested every 48 h and stored at −20 °C until use. For purification, 400 mL of clarified cell supernatant was incubated overnight with 3 mL of NiNTA (Qiagen) at 4 °C under stirring. The collected affinity resin was washed with a buffer of 20 mM Hepes, pH 7.0, 300 mM NaCl, and 20 mM imidazole. Proteins were eluted with the same buffer containing 500 mM imidazole and then dialyzed overnight with a buffer without imidazole.

Sample Preparation. A 5.0 μM solution of purified h-NAAA was incubated in 100 mM sodium phosphate buffer, 100 mM sodium citrate, 3 mM TCEP, and 0.1% Triton X-100, pH 4.5, for 90 min at 37 °C. **14q** was then added to a final 5.0 μM concentration, and the solution was incubated for a further 90 min (0.5% final DMSO). A control sample with 0.5% DMSO only was also prepared. At the end of the incubation, samples were precipitated with 10 volumes of ice-cold acetone, vortexed, and centrifuged at 14000 rpm for 10 min. The supernatant was removed, and pelletted h-NAAA was resuspended with Rapigest 0.5% (Waters, Milford, MA, USA) and digested with trypsin (DigestTip Trypsin, ProteoGen Bio, Italy). Prior to digestion, a small aliquot was run on SDS-PAGE to verify h-NAAA activation.

High-Resolution NanoLC-MS/MS Analysis. Tryptic peptides were loaded in a NanoAcquity LC system coupled with a Synapt G2 qTOF mass spectrometer (Waters) equipped with a nanospray ion source. The analytical column was a Waters BEH C18 75 μm × 10 cm working at 300 nL/min. Eluents were A, H_2O + 0.1% HCOOH, and B, CH_3CN + 0.1% HCOOH. A linear gradient from 3 to 55% of B in 30 min was applied to the column followed by a ramp to 90% in 10 min, an isocratic step at 90% for 10 min, and a reconditioning to 3% of B. Mass spectrometry parameters were as follows: spray, 1.8 kV; cone, 25 V. Data-dependent acquisition of tandem mass spectra was activated for doubly charged ions in the m/z 300−800 range. A linear ramp of the collision energy from 15 to 35 eV was applied to the precursor ion to collect tandem mass spectra. Glucofibrinopeptide (500 nM) infused at 500 nL/min was used as lockspray mass. MS/MS data were analyzed using the Biolynx software embedded in the MassLynx software suite. MassLynx and Protein Lynx softwares (Waters) were used for the interpretation of LC-MS data.

■ ASSOCIATED CONTENT

Supporting Information

Detailed experimental procedures, analytical and spectroscopical data of intermediate and final compounds, and ^{1}H and ^{13}C NMR spectra of compounds **4, 14i,k,q**, and **22**; additional computational details on the reaction mechanism and proteomic data of *S*-acylated *h*-NAAA tryptic peptide with **14q**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): Piomelli, D.; Bandiera, T.; Mor, M.; Tarzia, G.; Bertozzi, F.; Ponzano, S. are inventors in the patent application WO 2013078430 protecting the class of compounds disclosed in this paper.

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■ ABBREVIATIONS USED

FAEs, fatty acid ethanolamides; OEA, oleoylethanolamide; PEA, palmitoylethanolamide; NAAA, *N*-acylethanolamine acid amidase; FAAH, fatty acid amide hydrolase; AC, acid ceramidase; PPAR-α, peroxisome proliferator-activated receptor-α; HMGS, 3-hydroxy-3-methylglutaryl-CoA synthase; (*S*)-OOPP and (*R*)-OOPP, (*S*)- and (*R*)-*N*-(2-oxo-3-oxetanyl)-3-phenylpropionamide; CDI, 1,1′-carbonyldiimidazole; DPC, di-2-pyridyl carbonate; DMAP, 4-dimethylaminopyridine; TS, transition state; E_a, activation energy; DFT, density functional theory; LHMDS, lithium bis(trimethylsilyl)amide; PyBOP, benzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluoro-

phosphate; HBTU, [2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate; TBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate.

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