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Permalink
https://escholarship.org/uc/item/28k244m2

Journal
Journal of Medicinal Chemistry, 56(17)

ISSN
0022-2623

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Publication Date
2013-09-12

DOI
10.1021/jm400739u

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Synthesis and Structure—Activity Relationship (SAR) of 2-Methyl-4-oxo-3-oxetanlycarbamic 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 oleoylthanolamide (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-(2S,3R)-2-methyl-4-oxo-oxetan-3-yl)carbamate (14q) as the first single-digit nanomolar inhibitor of intracellular NAAA activity (IC50 = 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 arachidonoylthanolamide (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 oleoylthanolamide (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 degranulation8,9 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-acylthanolamine 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 nuclease (Ntn) family of enzymes18,19 and bears a significant degree of sequence homology with the cholinephycyan hydro-
lases, which are characterized by the ability to cleave nonpeptide amide bonds.\textsuperscript{29} Like other Ntn enzymes, NAAA is activated by autoproteolysis, which occurs at acidic pH and generates a catalytically competent form of the enzyme.\textsuperscript{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.\textsuperscript{23,31}–\textsuperscript{33}

Compounds containing chemical groups susceptible to nucleophilic attack, such as a $\beta$-lactone moiety, are known to inhibit enzymes that contain a catalytic cysteine.\textsuperscript{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,\textsuperscript{35} was found to inhibit NAAA activity with micromolar potency.\textsuperscript{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 $\mu$M).\textsuperscript{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.\textsuperscript{24} In addition, compound 2 does not inhibit FAAH or other lipid hydrolases, such as monoacylglycerol lipase and diacylglycerol lipase type-$\alpha$, 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 $\beta$-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 $\mu$M), which prevented carrageenan-induced decrease of FAE levels in vivo with a potency that paralleled its ability to inhibit NAAA in vitro.\textsuperscript{25} A first investigation of threonine-derived $\beta$-lactone derivatives, which have enhanced chemical stability compared to their serine-derived analogues,\textsuperscript{28,35,36} led to the discovery of ARN077 (4, Figure 1), a potent NAAA inhibitor\textsuperscript{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 $\beta$-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$S$)-2-methyl-4-oxo-3-oxetanylaminium 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 $\alpha$-substituted-$\beta$-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. Chlorormate 6b was prepared by...
D-Threonine-Derived \( \beta \)-Lactones 14i,l,m,p,q,s via Tosylate Salt 13a

```
\[
\begin{align*}
\text{R-OH} & \quad \text{a} \quad \text{b} \quad \text{c} \\
\text{7c} & \quad \text{8a,d,f,g,i-t} \quad + \text{9a,d,f,g,i-t} \\
\text{6b} & \quad \text{1-carboxylates} 7c \quad \text{chloroformates 6} \\
\end{align*}
\]
```

Reagents and conditions: (a) 5b, (Cl\textsubscript{3}CO)\textsubscript{2}CO, pyridine, toluene, room temperature, 16 h; (b) 5c, CDI, DMF, room temperature, 2 h; (c) 5a,d,f,g,i-t, Et\textsubscript{3}N or DMAP, DPC, CH\textsubscript{2}Cl\textsubscript{2}, room temperature, 15 h.

More 2-methyl-4-oxo-3-oxetanylcarbamic acid esters were obtained in moderate yields (17–41%) by coupling the tosylate salt 13 with a slight excess (1.5 equiv) of di-2-pyridyl carbonate (DPC), to be reacted with 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 carbones 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).31,42 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-oxetanylcarboxylic 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).

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-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethylammonium hexafluorophosphate (HBTU), benzotriazol-1-yl-oxytris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP), or 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethylmethylammonium tetrafluoroborate (TBTU)] to afford the

Reagents and conditions: (a) Boc\textsubscript{2}O, NaHCO\textsubscript{3}, H\textsubscript{2}O, MeOH, room temperature, 36 h; (b) PyBOP, Et\textsubscript{3}N, CH\textsubscript{2}Cl\textsubscript{2}, room temperature, 15 h; (c) CF\textsubscript{3}COOH, p-TsOH, 0 °C, 15 min; (d) DIPEA, mixture of 8i,l,m,p,q,s and 9i,l,m,p,q,s, CH\textsubscript{2}Cl\textsubscript{2}, room temperature, 15 h.

dx.doi.org/10.1021/jm400739u J. Med. Chem. 2013, 56, 6917–6934
desired 2-methyl-4-oxo-3-oxetanycarbamic 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).
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,l,m,o 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 BF₃- 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$^a$

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Structure</th>
<th>IC$_{50}$ (µM) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td><img src="image1.png" alt="Structure" /></td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>14a</td>
<td><img src="image2.png" alt="Structure" /></td>
<td>2.50 ± 0.85</td>
</tr>
<tr>
<td>14b</td>
<td><img src="image3.png" alt="Structure" /></td>
<td>0.09 ± 0.07</td>
</tr>
<tr>
<td>14c</td>
<td><img src="image4.png" alt="Structure" /></td>
<td>0.39 ± 0.17</td>
</tr>
<tr>
<td>14d</td>
<td><img src="image5.png" alt="Structure" /></td>
<td>1.17 ± 0.40</td>
</tr>
<tr>
<td>14e</td>
<td><img src="image6.png" alt="Structure" /></td>
<td>0.76 ± 0.35</td>
</tr>
<tr>
<td>14f</td>
<td><img src="image7.png" alt="Structure" /></td>
<td>0.27 ± 0.14</td>
</tr>
<tr>
<td>14g</td>
<td><img src="image8.png" alt="Structure" /></td>
<td>0.04 ± 0.02</td>
</tr>
<tr>
<td>14h</td>
<td><img src="image9.png" alt="Structure" /></td>
<td>0.03 ± 0.03</td>
</tr>
<tr>
<td>14i</td>
<td><img src="image10.png" alt="Structure" /></td>
<td>0.05 ± 0.02</td>
</tr>
<tr>
<td>14j</td>
<td><img src="image11.png" alt="Structure" /></td>
<td>0.14 ± 0.05</td>
</tr>
<tr>
<td>14k</td>
<td><img src="image12.png" alt="Structure" /></td>
<td>0.013 ± 0.004</td>
</tr>
</tbody>
</table>

$^a$IC$_{50}$ values are reported as mean values of three or more determinations.

Nium bromide to afford compound 37. 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.
RESULTS AND DISCUSSION

Previous pharmacological studies by our group have identified the 2-methyl-4-oxo-3-oxetanycarbamic acid ester derivative 4 (ARN077) as a potent NAAA inhibitor. In vitro experiments have shown that compound 4 inhibits rat NAAA through a mechanism that is rapid, noncompetitive, and fully reversible after overnight dialysis. 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.

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\textsubscript{50}) of 0.42 μM.24

Table 2. Inhibitory Potencies (IC\textsubscript{50}) of Compounds 14l−s, 22, 23, and 29 on Rat NAAA Activity\textsuperscript{a}

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Structure</th>
<th>IC\textsubscript{50} (μM) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>14l</td>
<td><img src="image" alt="Structure" /></td>
<td>0.31 ± 0.14</td>
</tr>
<tr>
<td>14m</td>
<td><img src="image" alt="Structure" /></td>
<td>0.029 ± 0.001</td>
</tr>
<tr>
<td>14n</td>
<td><img src="image" alt="Structure" /></td>
<td>0.06 ± 0.03</td>
</tr>
<tr>
<td>14o</td>
<td><img src="image" alt="Structure" /></td>
<td>0.10 ± 0.05</td>
</tr>
<tr>
<td>22</td>
<td><img src="image" alt="Structure" /></td>
<td>0.016 ± 0.003</td>
</tr>
<tr>
<td>23</td>
<td><img src="image" alt="Structure" /></td>
<td>0.27 ± 0.12</td>
</tr>
<tr>
<td>14p</td>
<td><img src="image" alt="Structure" /></td>
<td>23.0 ± 6.6</td>
</tr>
<tr>
<td>14q</td>
<td><img src="image" alt="Structure" /></td>
<td>0.007 ± 0.001</td>
</tr>
<tr>
<td>14r</td>
<td><img src="image" alt="Structure" /></td>
<td>0.31 ± 0.08</td>
</tr>
<tr>
<td>14s</td>
<td><img src="image" alt="Structure" /></td>
<td>0.29 ± 0.22</td>
</tr>
<tr>
<td>29</td>
<td><img src="image" alt="Structure" /></td>
<td>No inhibition</td>
</tr>
</tbody>
</table>

\textsuperscript{a}IC\textsubscript{50} values are reported as mean values of three or more determinations.
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 (IC₅₀ = 6.0 µM).²⁴ The benzyl carbamate 1 was less potent than 2 at inhibiting NAAA (IC₅₀ = 2.96 µM),²⁸ whereas its enantiomer, (R)-2-oxo-3-oxetanylbenzyl carbamate, showed potency comparable to that of 2 (IC₅₀ = 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 (IC₅₀ = 1.0 µM),²⁸ showed an increased chemical

Table 3. Inhibitory Potencies (IC₅₀) of Compounds 4 and 19–21 on Rat NAAA Activity

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Structure</th>
<th>IC₅₀ (µM) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td><img src="image" alt="Structure" /></td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>19</td>
<td><img src="image" alt="Structure" /></td>
<td>3.53 ± 2.22</td>
</tr>
<tr>
<td>20</td>
<td><img src="image" alt="Structure" /></td>
<td>0.48 ± 0.26</td>
</tr>
<tr>
<td>21</td>
<td><img src="image" alt="Structure" /></td>
<td>0.02 ± 0.01</td>
</tr>
</tbody>
</table>

*IC₅₀ values are reported as mean values of three or more determinations.

Table 4. Inhibitory Potencies (IC₅₀) on Rat and Human NAAA and Percent Inhibitory Activity on Rat FAAH and Rat AC of Compounds 4, 14i,k,q and 22

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Structure</th>
<th>r-NAAA IC₅₀ (µM) ± SD</th>
<th>h-NAAA IC₅₀ (µM) ± SD</th>
<th>r-FAAH (%Inhib.)</th>
<th>r-AC (%Inhib.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td><img src="image" alt="Structure" /></td>
<td>0.05 ± 0.01</td>
<td>0.007 ±0.001</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>14i</td>
<td><img src="image" alt="Structure" /></td>
<td>0.05 ± 0.02</td>
<td>0.02 ± 0.01</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>14k</td>
<td><img src="image" alt="Structure" /></td>
<td>0.013 ±0.004</td>
<td>0.005 ± 0.002</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>14q</td>
<td><img src="image" alt="Structure" /></td>
<td>0.007 ±0.001</td>
<td>0.007 ± 0.0002</td>
<td>n.a.</td>
<td>30%</td>
</tr>
<tr>
<td>22</td>
<td><img src="image" alt="Structure" /></td>
<td>0.016 ±0.003</td>
<td>0.005 ± 0.002</td>
<td>19%</td>
<td>45%</td>
</tr>
</tbody>
</table>

*IC₅₀ values are reported as mean values of three or more determinations. Inhibitory activities are reported as a % value of two determinations at 10 µM. n.a. = < 10% inhibition.
stability with respect to serine-derived \( \beta \text{-lactone} \) analogues.\(^{28,35,36}\) These results prompted us to focus our attention on carboxylic acid ester derivatives bearing a methyl substitution at the \( \beta \)-position of the \( \beta \text{-lactone} \) ring.

2-Methyl-4-oxo-3-oxetanycarbamic acid esters were tested for their ability to inhibit the hydrolysis of 10-cis-heptadecenoylthanolamide (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-oxetanycarbamic 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 \( \mu \text{M} \)) 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 \( \mu \text{M} \)). However, potency was recovered by increasing the length of the aliphatic chain, with compounds bearing an n-heptyl (14g, IC\(_{50}\) = 0.04 \( \mu \text{M} \)) or an n-octyl (14h, IC\(_{50}\) = 0.03 \( \mu \text{M} \)) 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 \( \mu \text{M} \)). The replacement of a carbon atom with an oxygen in the aliphatic chain of 4 (14i, Table 1) was tolerated only at a specific position, as shown by the different potencies of compounds 14i (IC\(_{50}\) = 0.05 \( \mu \text{M} \)) and 14j (IC\(_{50}\) = 0.14 \( \mu \text{M} \)). To evaluate the influence of the shape of the phenylalkyl chain, we synthesized compounds 14i–n (Table 2), which derive from secondary and tertiary alcohols bearing branched aliphatic chains. Introduction of a gem-dimethyl (14l) moiety close to the carboxylic function was detrimental for potency, indicating a limited space in the region of the enzyme occupied by the carboxylic 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 \( \mu \text{M} \)), 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 \( \alpha \)-position to the carboxylic function, we synthesized compounds 14n, as a diastereomeric mixture, and compound 22, from optically pure (S)-1-methyloctanol. Compound 14n (IC\(_{50}\) = 0.06 \( \mu \text{M} \)) displayed a slightly decreased potency compared to the unsubstituted derivative 14h (IC\(_{50}\) = 0.03 \( \mu \text{M} \)). The optically pure (S)-1-methyloctyl diastereoisomer 22 inhibited NAAA with IC\(_{50}\) = 0.016 \( \mu \text{M} \), a value comparable to that of the unsubstituted compound 14h, whereas the (R)-diastereoisomer 23 (IC\(_{50}\) = 0.27 \( \mu \text{M} \)), obtained by chiral HPLC purification of 14n, was 10 times less potent than 14n. These results indicate a stereorecognition among the isomers and, specifically, a preference for the (S)-configuration at the carbon atom in position \( \alpha \) 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 \( \mu \text{M} \)) or a p-benzoxylphenyl (14s, IC\(_{50}\) = 0.29 \( \mu \text{M} \)) 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 \( \mu \text{M} \). On the contrary, the m-biphenylmethyl analogue 14p did not effectively inhibit NAAA (IC\(_{50}\) = 23 \( \mu \text{M} \)). These results show a clear preference for a linear moiety in the carboxylic acid ester for optimal enzyme recognition.

The role of the carboxylic function was also investigated by preparing compound 29 (Table 2), a derivative in which the carboxyte 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 \( \beta \)-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 (2S,3S) epimer 20 (IC\(_{50}\) = 0.48 \( \mu \text{M} \)) showed a 10-fold decrease in potency compared to 4. By contrast, the (2R,3R) epimer 21 turned out to be a potent NAAA inhibitor (IC\(_{50}\) = 0.02 \( \mu \text{M} \)). 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 \( \beta \)-lactone ring, the first step of the covalent inhibition mechanism hypothesized for the present series of compounds.\(^{45,46}\) In our simulations, the four compounds were truncated and modeled as methyl carbamate, and the sulfur atom was provided by...
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 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-oxetanycarbamic acid esters were tested in a functional assay using h-NAAA (Table 4). A common trend between relative potencies at rat and human 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 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, and rat lung acid ceramidase (r-AC), a cysteine amidase that exhibits 33% amino acid identity with r-NAAA (Table 4). 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. 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 trypsin ion (167.08 m/z) and a side-chain loss of the biphenyl group and CO₂ (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-oxetanycarbamic acid esters as NAAA inhibitors. Those studies had led to the discovery of S-phenylpentyl-N-[2S,3R]-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 activity. 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, Et₂O, CH₂Cl₂, 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 CHCl₃ as a solvent and a 1 dm cell. Automated column chromatography purifications were done using a Teledyne ISCO apparatus (CombiFlash Rf) 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 1H and 100.62 MHz for 13C), with a BBI probe and a 2.1 mm i.d., particle size = 1.8 μm). Mobile phase was either 10 mN H₂O, DMSO, MeOH:CHCl₃ = 10:1 (210–400 nm. Analyses were performed on an ACQUITY UPLC HSS T3 C₁₈ column (50 × 2.1 mm i.d., particle size = 1.8 μm) with a VanGuard HSS T3 C₁₈ precolumn (5 × 2.1 mm i.d., particle size = 1.8 μm). Mobile phase was either 10 mN NH₄OAc in H₂O at pH 5, 5 adjusted with AcOH (A) or 10 mN NH₄OAc in MeCN/H₂O (95:5) at pH 5. Electrospray ionization in positive and negative mode was used. Purifications by preparative HPLC-MS 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 C₁₈ column (50 × 2.1 mm i.d., particle size = 1.8 μm) with a VanGuard HSS T3 C₁₈ precolumn (5 × 2.1 mm i.d., particle size = 1.8 μm). Mobile phase was either 10 mN NH₄OAc in H₂O at pH 5, 5 adjusted with AcOH (A) or 10 mN NH₄OAc in MeCN/H₂O (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 C₁₈ OBD column (100 × 19 mm i.d., particle size = 5 μm) with an XBridge Prep C₁₈ (10 × 19 mm i.d., particle size = 5 μm) guard cartridge. The mobile phase was 10 mN NH₄OAc in MeCN/H₂O (95:5) at pH 5. Electrospray ionization in positive and negative mode was used. Purifications by chiral HPLC were run on a Waters Alliance HPLC instrument consisting of...
Experimental procedure and NMR are according to the literature.28,37 4 by NMR and UPLC-MS analysis.

**Synthesis of 5-Phenylpentyl-[(2S,3R)-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.8,37

**General Procedure I for the Synthesis of Carbamates 14b,e,h (Scheme 4).** Preparation of β-Hydroxyalkylcarboxylic Acids 15b,e,h (Step 1). To a suspension of NaHCO₃ (2.5 equiv) in THF and H₂O was added t-threonine (1.0 equiv). The suitable chloroformate (1.0 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 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 15b,e,h.

**Preparation of Carbamates 14b,e,h (Step 2).** A solution of β-hydroxyalkylcarboxylic acid was added 15b,e,h (1.0 equiv) in dry CH₂Cl₂, and PyBOP (6.1 g, 11.6 mmol) to give 14b,e,h, which were used in the next step without further purification.

**Preparation of Carbamates 14b,e,h (Scheme 4).** A solution of (2S,3S)-3-hydroxy-2-(4-phenoxybutylcarboxy)butanoylcarboxylic acid (15c) (5.9 g, 13.1 mmol, 1.0 equiv) in dry CH₂Cl₂ (170 mL) was added Et₃N (5.5 mL, 39.4 mmol, 3.0 equiv) under argon. After cooling at 0 °C, HBTU (7.5 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); [α]D=−17.8 (c 0.1, CHCl₃); MS (ESI), m/z 276 [M−H]−; FTIR (cm⁻¹), 3312, 3060, 3026, 2964, 2861, 1826, 1694, 1545, 1337, 1269, 1126, 1023; 1H NMR (CDCl₃), δ 1.34, 1.91, 2.54 (2H, 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, SH), 8.19 (d, J = 9.5 Hz, 1H); 13C NMR (DMSO-d₆), 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.

**General Procedure II for the Synthesis of Carbamates 14i,l,m,p,q,s (Scheme 4).** Synthesis of (3S,4S)-3-methyl-4-oxooxetan-3-yl]carbamate (14d). To a solution of (2S,3S)-3-hydroxy-2-(4-phenoxybutylcarboxy)butanoylcarboxylic acid (15c) (5.9 g, 13.1 mmol, 1.0 equiv) in dry CH₂Cl₂ (100 mL) was added Et₃N (3.0 equiv) under argon. After cooling at 0 °C, HBTU (7.5 mmol, 1.5 equiv) was added and the mixture stirred at 0 °C for 18 h, the mixture was diluted with H₂O and washed with Et₂O. The aqueous layer was acidified with 2 M HCl solution and extracted with EtOAc. The combined organic layers were dried over Na₂SO₄, filtered, and the solvent removed under vacuum. The crude was purified by typical silica gel column chromatography, eluting with cyclohexane/CH₂Cl₂ (100:0 to 30:70) to give compounds 14i,l,m,p,q,s.

**Preparation of Carbamates 14i,l,m,p,q,s (Scheme 3).** Preparation of 3-methyl-4-oxooxetan-3-yl]lammonium 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,lm,p,q,s and Alkyl-2-oxopyridin-1-oxocarboxylates 9i,lm,p,q,s (Step 1).** To a stirred mixture of the suitable alcohol 8i,lm,p,q,s (1.0 equiv) in dry CH₂Cl₂, and under nitrogen atmosphere was added Et₃N (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 CH₂Cl₂, washed with a saturated NH₄Cl solution and, subsequently, with a saturated NaHCO₃ solution (three times). The combined organic layers were dried over Na₂SO₄, filtered, and concentrated to dryness without any further purification.

**Preparation of Carbamates 14i,lm,p,q,s (Step 2).** To a stirred mixture of 13 (1.0 equiv) in dry CH₂Cl₂ (1.0 mL) under nitrogen atmosphere was added dropwise DIPEA (1.0 equiv). Subsequently, the isomeric mixture of 8i,lm,p,q,s and 9i,lm,p,q,s (3.0 equiv) dissolved in dry CH₂Cl₂ (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,lm,p,q,s were further purified by preparative HPLC-MS.
3-Benzylxopropyl-N-(25,3R)-2-methyl-4-oxo-oxetan-3-ylcarbamate (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 8f 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); [α]D, 15° = −9.8° (c 0.3, CHCl3); MS (EI), m/z 292 [M − H]+; 1H NMR (DMSO-d6), δ 1.34 (d, J = 6.3 Hz, 3H), 1.81–1.91 (m, 2H), 3.50 (J = 6.3 Hz, 2H), 4.04–4.16 (m, 2H), 4.47 (s, 2H), 4.85 (dq, J = 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). 13C NMR (DMSO-d6), 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.

General Procedure III for the Synthesis of Carbamates 14a,d,f,g,k,n,o,r and 19–22 (Schemes 4 and 5). Preparation of Activated Alcohols as Alkyl-2-pyridyl Carbonates 8a,d,f,g,k,n,o,r, and Alkyl-2-oxopiperidine-1-carboxylates 9a,d,f,g,k,n,o,r (Step 1). To a stirred mixture of the suitable alcohol 8a,d,f,g,k,n,o,r (1.0 equiv) in dry CH3Cl2 and under nitrogen atmosphere was added DMAP (0.1 equiv) and DIPC (1.2 equiv). The reaction mixture was left to react at room temperature for 15 h, then diluted with CH3Cl2 and washed with a saturated NH4Cl solution and, subsequently, with a saturated NaHCO3 solution (3 times). The combined organic layers were dried over Na2SO4, filtered, and concentrated to give a mixture (ratio 1.6:1) of alkyl-2-pyridyl carbonate 8a,d,f,g,k,n,o,r, and alkyl-2-oxopiperidine-1-carboxylate 9a,d,f,g,k,n,o,r. The mixture of isomers was not separated and used in the next step without any further purification.

Preparation of 2-Hydroxycarboxylic Acids 15a,d,f,g,k,n,o,r and 16–18 (Step 2). To a stirred mixture of n- or t-n-butyl-5-threonine (1.0 equiv) and NaHCO3 (1.5 equiv) in H2O (3.5 mL) was added the isomeric mixture containing the pyridyl carbonate 8a,d,f,g,k,n,o,r and the 2-oxopiperidine-1-carboxylate 9a,d,f,g,k,n,o,r (1.0 equiv in THF (3.5 mL)). After 15 h at room temperature, the crude was evaporated and subsequently extracted with EtO (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 Na2SO4, filtered, and concentrated to give the threonine derivatives 15a,d,f,g,k,n,o,r and 16–18, which were used in the next step without further purification.

Preparation of Carbamates 14a,d,f,g,k,n,o,r and 19–22 (Step 3). To a stirred mixture of threonine derivatives 15a,d,f,g,k,n,o,r and 16–18 (1.0 equiv) in dry CH3Cl2, at 0 °C, and under nitrogen atmosphere, were added Et3N (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 100:3) to afford 14a,d,f,g,k,n,o,r and 19–22.

Phenethyl-N-[125,3R]-2-methyl-4-oxo-oxetan-3-ylcarbamate (14p). 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 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); [α]D, 15° = −11.4° (c 0.1, CHCl3); MS (EI), m/z 334 [M − Na]+; 1H NMR (DMSO-d6), 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 (d, J = 6.2, 6.4 Hz, 2H), 5.39 (d, J = 6.2, 9.3 Hz, 2H), 7.12–7.31 (m, 10H), 8.12 (d, J = 9.3 Hz, 2H). 13C NMR (DMSO-d6), δ 14.90, 20.52, 24.94, 31.21, 31.25, 35.52, 35.81, 60.76, 71.68, 75.12, 126.08, 126.89, 147.52, 159.72, 170.26.

2-Benzylxopropyl-N-[25,3R]-2-methyl-4-oxo-oxetan-3-ylcarbamate (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 of 8q and 9q (0.405 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); [α]D, 15° = −12.1° (c 0.1, CHCl3); MS (EI), m/z 310 [M − H]+; 329 [M − NH3]−, 350 [M − K]+; 1H NMR (DMSO-d6), δ 1.37 (d, J = 6.4 Hz, 3H), 4.88 (d, J = 6.2, 6.4 Hz, 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). 13C NMR (DMSO-d6), 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, 155.61, 169.73.

6-Phenethyl-N-[25,3R]-2-methyl-4-oxo-oxetan-3-ylcarbamate (14d). The reaction was carried out following the general procedure III (step 3) employing (2R,3S)-3-hydroxy-2-(2-phenethylxoycarbonyl)butanoic acid (15a) (0.5 g, 1.50 mmol), dry CH3Cl2 (35 mL), Et3N (0.54 mL, 3.90 mmol), and TBTU (0.50 g, 1.56 mmol) to give 14d as a white solid yield; 57% (0.75 g, 85.7%); [α]D, 15° = −18.2° (c 0.1, CHCl3); MS (EI), m/z 329 [M − H]+, 250 [M − H]+; 1H NMR (DMSO-d6), δ 1.33 (d, J = 6.3 Hz, 3H), 2.89 (s, J = 6.8 Hz, 2H), 4.15–4.29 (m, 2H), 4.84 (d, 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). 13C NMR (DMSO-d6), δ 14.46, 34.65, 59.86, 65.15, 74.62, 126.31, 128.31, 128.81, 137.95, 155.61, 169.77.

Hexyl-(N-[25,3R]-2-methyl-4-oxo-oxetan-3-yl)carbamate (14f). The reaction was carried out following the general procedure III (step 3) employing (2R,3S)-2-(hexylxoycarbonyl)butanoic acid (15f) (0.27 g, 1.10 mmol), dry CH3Cl2 (27 mL), Et3N
The reaction was carried out following the general procedure III (step 3) employing (2R,3S)-3-hydroxy-2-(5-phenethyloxy-1-methyloctyl)-3-hydroxybutyric acid and (2R,3S)-3-(1-isopropyl-5-phenylpentyloxy)-2-methyl-4-oxo-oxetan-3-yl-carbamate (14r). The reaction was carried out following the general procedure III (step 3) employing (2R,3S)-3-hydroxy-2-(2-methyl-4-oxo-oxetan-3-yl)-carbamate (14f). The reaction was carried out following the general procedure III (step 3) employing (2R,3S)-3-hydroxy-2-(5-phenethyloxy-1-methyloctyl)-3-hydroxybutyric acid and (2R,3S)-3-(1-isopropyl-5-phenylpentyloxy)-2-methyl-4-oxo-oxetan-3-yl-carbamate (14n).
([R]-1-Methylcloyt)-N-[25,3R,2]-methyl-4-oxo-oxetan-3-yl]-carbamate (23). Compound 23 was obtained by chiral HPLC purification of the diastereomeric mixture 14a: white solid; $[\alpha]_D^{25}$ $-28.39$ ($c = 0.1, \text{CHCl}_3$); ee $> 99\%$ (chiral HPLC, AD column; flow, 1.0 mL/min; $t_0 = 28.26$ min); MS (ESI), m/z $270$ [M $- \text{H}^+]$; $272$ [M $- \text{H}^+ - \text{H}_2\text{O}]$; $^{1}H$ NMR (DMSO-$d_6$), $\delta$ 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-$d_6$), $\delta$ 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). (2R,3S)-3-Benzoyloxy-2-[tert-butoxycarbonyl[methyl]amino]butanoic Acid (25). To a solution of (2R,3S)-3-benzyloxy-2-[tert-butoxycarbonyl][methyl]-aniline (24) in dry THF (15 mL) at $0^\circ$C, and under argon atmosphere, were added Et$_3$N (0.06 mL, 0.41 mmol) and subsequently TBTU (0.05 g, 0.17 mmol). The mixture was stirred at $0^\circ$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). The resulting粗 products were then elutriated at 12000g for 30 min at $4^\circ$C. The pellets were resuspended in PBS, pH 7.4, and subjected to a freeze/thaw cycle at $-80^\circ$C. The suspension was finally centrifuged at 10500g for 1 h at $4^\circ$C. Protein concentration was measured and samples aliquoted and stored at $-80^\circ$C until use. NAA protein preparation (10 $\mu$g) was preincubated with various concentrations of test compound or vehicle control in 100 mM NaH$_2$PO$_4$, 100 mM trisodium citrate dehydrate, 0.1% Triton-X 100, and 3 mM DTT, pH 4.5, for 30 min at $37^\circ$C. Duplicate samples were then incubated with 50 $\mu$Ci M17:1 11-cis-heptadecenoylanethanolamide (Avanti Polar Lipids, Alabaster, AL, USA) at $37^\circ$C for 30 min. The reaction was terminated by the addition of 0.2 mL of cold methanol containing 1 nmol of heptadecanoyl acetic acid (NuChek Prep, Elysian, MN, USA) as internal standard. Samples were then analyzed by UPLC-MS. Heptadecanoyl and heptadecanoic acids were eluted on an ACQUITY UPLC BEH Amide column (50 mm length, 2.1 mm i.d., 1.7 $\mu$m 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 $40^\circ$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 $150^\circ$C with a desolvation temperature of $400^\circ$C. $N_2$ was used as drying gas at a cone flow of 100 L/h and a desolvation flow of $800^\circ$C. The $[M - H]^-$ ion was monitored in the selected ion monitoring mode ($m/z$ values: heptadecanoyl acid, 267.37; heptadecanoic acid, 269.37). Calibration curves were generated using commercial heptadecanoic acid (NuCheck Prep). Inhibition of NAA activity was calculated as reduction of heptadecanoyl 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 NAA.** The coding sequence of human NAA (NM_014435.3) was amplified by PCR (HotStar HiFidelity kit, Qiagen) from a human spleen cDNA library (catalog no. 693124, Clontech, Mountain View, CA, USA). To generate the construct h-NAAA (NM_014435.3) was amplified by PCR (HotStar HiFidelity kit, Qiagen) from a human spleen cDNA library (catalog no. 693124, Clontech, Mountain View, CA, USA). To generate the construct h-NAAA (6x His)-pcDNA3.1 a sequence of h-NAAA fused with a central 6x histidine tag was amplified [forward primer, 5'-AACAGTGGACGCGACATGCGGAGC-3'; reverse primer, 5'-CTCCAGTGTAGTGTTGAGCCGTTGTTCTACTCCGTTTCT-3'] and cloned into HindIII/XhoI digested pcDNA 3.1 vector (Invitrogen, Carlsbad, CA, USA). HEK293 wild-type cells (American Type Culture Collection, Manassas, VA, USA) were cultured at $37^\circ$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(6x His)-pCDNA3.1 construct using JetPEI transfection reagent (Polyplus, Illkirch, France) following the manufacturer’s instructions. Stably transected 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 protein from rat brain homogenates, followed by 30 min of incubation at 37 °C with [3H]anandamide (1 μM cold AEA/0.6 nM [3H]AEA (arachidonylethanolamine, 60 Ci/mmol), 1 mM DTT, 100 mM NaH2PO4, pH 7.4, 0.05% fatty acid free BSA). The reaction was stopped with cold CHCl3/CH3OH 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 NaH2PO4, 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 heptadecanonic 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

-f, cation, 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. 14C 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 pelleted 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, H2O + 0.1% HCOOH, and B, CH3CN + 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. Glucobifinopeptide (500 nM) infused at 500 nL/min was used as lockspray mass. MS/MS data were analyzed using the Biolyx 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 spectrophotometric data of intermediate and final compounds, and 1H and 13C 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.

**ACKNOWLEDGMENTS**

We thank Sine Mandrup Bertozzi for reverse phase and chiral HPLC purifications, Luca Goldoni for NMR technical support, Silvia Venzano for compounds handling, and Dr. Natalia Realian and Clara Albani for r-AC and r-FAAH screenings, respectively.

**ABBREVIATIONS USED**

FAEs, fatty acid ethanolamides; OEA, oleoyl ethanolamide; PEA, palmitoyl ethanolamide; NAAA, N-acylethanolamine acid amide; 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′-carbonyldimidazole; DPC, di-2-pryridyl carbonato; DMAP, 4-dimethylaminopyridine; TS, transition state; Eα activation energy; DFT, density functional theory; LHMDs, lithium bis(trimethylsilyl)amide; PyBOP, benzotriazol-1-yl-oxytris(pyrrolidino)phosphonium hexafluoro-
phosphate; HBTU, [2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethylthylammonium hexafluorophosphate; TBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethylthylammonium tetrafluoroborate.

■ REFERENCES


(47) Chiral HPLC purification of diastereomeric mixtures 14m and 14o afforded the corresponding pure diastereoisomers. No absolute configuration of the newly formed stereocenter was assigned. The activities of single diastereoisomers were the following, for 14m: isomer 1 IC50 = 0.018 ± 0.009 μm, isomer 2 IC50 = 5.1 ± 1.7 μm, and for 14o: isomer 1 IC50 = 0.08 ± 0.03 μm, isomer 2 IC50 = 12.2 ± 4.32 μm.

