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N-(2-Oxo-3-oxetanyl)carbamic Acid Esters as N-Acylethanolamine Acid Amidase Inhibitors: Synthesis and Structure—Activity and Structure—Property Relationships

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ABSTRACT: The β-lactone ring of N-(2-oxo-3-oxetanyl)amides, a class of N-acylethanolamine acid amidase (NAAA) inhibitors endowed with anti-inflammatory properties, is responsible for both NAAA inhibition and low compound stability. Here, we investigate the structure—activity and structure—property relationships for a set of known and new β-lactone derivatives, focusing on the new class of N-(2-oxo-3-oxetanyl)carbamates. Replacement of the amide group with a carbamate one led to different stereoselectivity for NAAA inhibition and higher intrinsic stability, because of the reduced level of intramolecular attack at the lactone ring. The introduction of a syn methyl at the β-position of the lactone further improved chemical stability. A tert-butyl substituent in the side chain reduced the reactivity with bovine serum albumin. (2S,3R)-2-Methyl-4-oxo-3-oxetanylcarbamic acid S-phenylpentyl ester (27, URB913/ARN077) inhibited NAAA with good in vitro potency (IC_{50} = 127 nM) and showed improved stability. It is rapidly cleaved in plasma, which supports its use for topical applications.

INTRODUCTION
Fatty acid ethanolamides (FAEs) make up a family of bioactive mediators that have stimulated pharmaceutical interest because compounds blocking their deactivating hydrolysis offer a new strategy for the treatment of pain and inflammation. An endogenous FAE that has attracted considerable attention is palmitoylethanolamide (PEA), which has been found to suppress pain behaviors induced by tissue injury, nerve damage, degranulation, and to exert antinociceptive effects in rodent models of acute and chronic pain. PEA has also been found to modulate pain and inflammation by engaging peroxisome proliferator-activated receptor type α. In particular, PEA has been shown to inhibit peripheral inflammation and mast cell degranulation and to exert antinociceptive effects in rodent models of acute and chronic pain. PEA has also been found to suppress pain behaviors induced by tissue injury, nerve damage, or inflammation in mice.

Along with PEA, other FAEs having neuromodulatory functions have been identified. These include the endogenous cannabinoid agonist N-arachidonoylethanolamine (anandamide, AEA) and the feeding regulator oleoylthanolamide. FAEs share similar anabolic and catabolic pathways, with their levels finely controlled by enzymes responsible for synthesis and degradation. FAEs are produced by the action of a selective phospholipase D, which catalyzes the cleavage of the membrane precursor N-acylphosphatidylethanolamine. The hydrolysis of anandamide is mostly attributed to fatty acid amide hydrolase (FAAH), an intracellular membrane-bound protein belonging to the amidase signature (AS) family, characterized by an optimal activity at pH 9.0. On the other hand, PEA is primarily hydrolyzed by N-acylethanolamine acid amidase (NAAA), which is not related to FAAH or other members of the AS family, is localized in the lysosomes, and shows an optimal activity at pH 5.0. NAAA is an N-terminal nucleophile hydrolyase (Ntn) and belongs to the chlorylglycine hydrolyase family of hydrolases, which are characterized by the ability to cleave nonpeptide amide bonds. Like other Ntn enzymes, NAAA is converted by self-catalyzed proteolysis into a shorter active form upon incubation at acidic pH. Processing of NAAA renders a cysteine residue (Cys131 in rat NAAA and Cys126 in human NAAA) the N-terminal amino acid. Site-directed mutagenesis and mass spectrometry studies demonstrated that this N-terminal cysteine plays a pivotal role in both catalytic activity and proteolytic processing. This is consistent with a catalytic mechanism involving the exchange of a proton between the sulphydryl and the amino groups of the terminal cysteine and a nucleophile attack on the amide carbonyl of the substrate, as supported by quantum
mechanics/molecular mechanics (QM/MM) simulations for cysteine Ntn hydrolases.26 Recently, the level of interest in NAAA as a pharmaceutically relevant target has increased, because of the observation that NAAA inhibitors locally administered to inflamed tissues, where the biosynthesis of PEA is downregulated,7 restore the physiological levels of PEA.24 While several classes of potent and selective FAAH inhibitors have been discovered and employed to study the central27,28 and peripheral29 role of FAAH, only few NAAA inhibitors have been reported.24,30−32 To further investigate the usefulness of NAAA inhibitors in acute and chronic inflammation, the discovery of new potent and selective compounds and the optimization of known chemical classes are both needed.

In this context, our research group has recently identified a class of β-lactone-based NAAA inhibitors, exemplified by (S)-N-2-oxo-3-oxetanyl-3-phenylpropanamide [(S)-OOPP, 1 (Table 1)], which weakens responses to inflammatory stimuli by elevating PEA levels in vitro and in vivo,24 and described the structure−activity relationships (SARs) of a series of N-(2-oxo-3-oxetanyl)amides.33 These compounds inhibit NAAA with a rapid, noncompetitive, and reversible mechanism, consistent with the acylation of the catalytic Cys131. Among the synthesized N-(2-oxo-3-oxetanyl)amides, compound 7 (Table 1) had an IC₅₀ of 115 nM and was effective at reducing the level of carrageenan-induced leukocyte infiltration in vivo.33 The α-amino-β-lactone moiety, although essential for the inhibitory activity of N-(2-oxo-3-oxetanyl)amides, is responsible for the low chemical stability of these compounds. Indeed, compounds incorporating an α-amino-β-lactone portion promptly react with bionucleophiles34 and are readily hydrolyzed in aqueous media.35,36 As a consequence, their use in pharmacological studies remains restricted to topical administration and requires caution in their handling and storage.

In this work, we investigated the mechanism of degradation of NAAA inhibitors with an α-amino-β-lactone scaffold, including the known N-(2-oxo-3-oxetanyl)amides, and expanded the series to new N-(2-oxo-3-oxetanyl)carbamic acid esters, which were designed and synthesized to explore the effect of the side chain on inhibitory activity and chemical stability. SARs and structure−property relationships (SPRs) demonstrated that the introduction of an α-carbamic acid ester side chain and a β-methyl group on the β-lactone ring resulted in potent NAAA inhibitors with enhanced chemical stability.

<table>
<thead>
<tr>
<th>Cpd.</th>
<th>Structure</th>
<th>pH 7.4 t₁/₂(min)</th>
<th>pH 5.0 t₁/₂(min)</th>
<th>pH 5.0 + DTT t₁/₂(min)</th>
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<td>1</td>
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<td>23.1 ± 0.8</td>
<td>19.0 ± 0.4</td>
</tr>
<tr>
<td>2</td>
<td><img src="image2" alt="Structure" /></td>
<td>9.8 ± 0.8</td>
<td>19.0 ± 1.7</td>
<td>15.9 ± 2.0</td>
</tr>
<tr>
<td>3</td>
<td><img src="image3" alt="Structure" /></td>
<td>12.1 ± 1.7</td>
<td>25.1 ± 2.3</td>
<td>20.9 ± 1.8</td>
</tr>
<tr>
<td>4</td>
<td><img src="image4" alt="Structure" /></td>
<td>8.2 ± 2.6</td>
<td>18.1 ± 1.9</td>
<td>19.1 ± 2.0</td>
</tr>
<tr>
<td>5</td>
<td><img src="image5" alt="Structure" /></td>
<td>9.8 ± 1.5</td>
<td>23.7 ± 2.0</td>
<td>19.5 ± 1.9</td>
</tr>
<tr>
<td>6</td>
<td><img src="image6" alt="Structure" /></td>
<td>13.4 ± 2.0</td>
<td>23.2 ± 1.7</td>
<td>18.8 ± 2.2</td>
</tr>
<tr>
<td>7</td>
<td><img src="image7" alt="Structure" /></td>
<td>12.6 ± 1.4</td>
<td>28.4 ± 1.7</td>
<td>20.9 ± 2.1</td>
</tr>
<tr>
<td>8</td>
<td><img src="image8" alt="Structure" /></td>
<td>73.8 ± 1.6</td>
<td>134.6 ± 2.4</td>
<td>100.2 ± 2.7</td>
</tr>
<tr>
<td>9</td>
<td><img src="image9" alt="Structure" /></td>
<td>93.5 ± 3.4</td>
<td>114.6 ± 3.9</td>
<td>113.8 ± 5.0</td>
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</table>

Table 1. Stabilities of Compounds 1−9 in Buffers and in the Presence of Thiols
Compounds 1–9 were obtained as reported elsewhere.\textsuperscript{33} \(10a\) was prepared by a literature procedure.\textsuperscript{36} N-Benzoylcarbonyl-\(L\)-serine (N-Cbz-\(L\)-serine, \(11a\)) was purchased from Lancaster.

The \(\beta\)-lactone \(10b\)\textsuperscript{37} and the cyclobutane derivative \(13\)\textsuperscript{38} were synthesized starting from benzyl chloroformate (12) (Scheme 1). 12 was treated with cyclobutylamine, in the case of 13, or \(D\)-serine, in aqueous sodium bicarbonate. In the latter case, the resulting intermediate N-Cbz-\(D\)-serine (11b) was cyclized to 10b by means of a modified Mitsunobu reaction, employing dimethyl azodicarboxylate (DMAD) and dry triphenylphosphine.\textsuperscript{36}

Cyclobutane derivative \(15\)\textsuperscript{36} was synthesized by a reaction between benzyl carbamate (14) and 1,2-bis(trimethylsilyloxy)-cyclobutene\textsuperscript{39} in ethereal hydrogen chloride (Scheme 1).\textsuperscript{36}

Ureas 19a and 19b were obtained by reacting benzyl isocyanate with (S)- and (R)-2-oxo-3-oxetanylammonium toluene-4-sulfonate (18a\textsuperscript{40} and 18b\textsuperscript{41}), respectively, in turn synthesized by deprotection of \(\beta\)-lactone derivatives 17a\textsuperscript{37} and 17b\textsuperscript{37}, respectively, with dry trifluoroacetic acid in the presence of dry p-toluene sulfonic acid (Scheme 2).\textsuperscript{40,41} Intermediates 17a and 17b were prepared starting from N-tert-butyloxycarbonyl-\(L\)-serine (N-Boc-\(L\)-serine, 16a) and N-Boc-\(D\)-serine (16b), respectively, employing the same method used for 10b (Scheme 2).\textsuperscript{37}

The synthesis of threonine \(\beta\)-lactones 23a,\textsuperscript{43} 23b,\textsuperscript{44} 24a,\textsuperscript{33} 24c,\textsuperscript{44} and 24d is illustrated in Scheme 2. The amino groups of L-, D-, \(L\text{-allo-}\)-, and \(D\text{-allo-}\)-threonine (20a–d, respectively) were protected with 12 or di-tert-butyldicarbonate (Boc-O) in aqueous sodium bicarbonate to give 21a,\textsuperscript{45} 21b,\textsuperscript{46} 22a,\textsuperscript{47} 22b,\textsuperscript{38} 22c,\textsuperscript{39} and 22d,\textsuperscript{38} respectively, employing a slightly modified literature procedure.\textsuperscript{51} Cyclization to \(\beta\)-lactones 23a,\textsuperscript{44}b and 24a–d was accomplished by treating the parent compounds 21a,\textsuperscript{44}b and 22a–d, respectively, with benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP reagent) by means of a literature procedure.\textsuperscript{52} The latter was treated with BOP and tetrabutylammonium bromide in aqueous sodium bicarbonate to afford 26, which was cyclized by means of the 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethylammonium hexafluorophosphate (HBTU) carboxylic acid activating agent to give 27.

### RESULTS AND DISCUSSION

**Chemical Stability of N-(2-Oxo-3-oxetanylamides).** The chemical stabilities of the previously published N-(2-oxo-3-oxetanylamides) 1–8 and compound 9 are summarized in Table 1. Those compounds revealed high susceptibility to chemical hydrolysis in buffered solution both at pH 7.4 (physiological pH) and at pH 5.0 (pH for the in vitro rat NAAA inhibitory assay). Both aliphatic (1) and aromatic (2–7) N-(2-oxo-3-oxetanylamides) have half-lives (\(t_{1/2}\)) of ≤18 min at pH 7.4 and ≤28 min at pH 5.0. The presence of dithiothreitol (DTT) (3 mM) in the pH 5.0 buffer did not significantly affect half-life values, indicating that the \(\beta\)-lactones do not preferentially react with thiols and that hydrolysis is the main factor for their instability under the assay conditions. This result is also relevant for the interpretation of the in vitro tests, as DTT was added to NAAA samples to maintain the catalytic activity of the enzyme over time.
The introduction of different electron-withdrawing (3 or 5) or electron-donating (4 or 6) substituents at conjugated positions did not significantly modify the stability of arylamides 2−7, which showed similar half-lives. Conversely, the introduction of a syn methyl group at position 4 (compound 8) led to a significant improvement in half-life, which was approximately 5-fold longer.

An investigation, performed by HPLC−ultraviolet (UV)−mass spectrometry (MS) analysis, of degradation products of amides 1−8 showed, at pH 7.4 and 5.0, the formation of N-acetylated L-serines, which resulted from the hydrolytic opening of the β-lactone ring (data not shown). An important difference in stability was observed between amides 1−7 and alkyl lactone 9, which was significantly more stable with half-lives passing from 8.2−18.0 to 93.5 min at pH 7.4 and from 18.1−28.4 to 114.6 min at pH 5.0. Thus, the amide group of the side chain plays a role in the chemical degradation of the lactone ring.

Taking compound 2 as the representative of compounds 1−8, we conducted HPLC−UV−MS analysis at pH 7.4 and 5.0 of its hydrolysis time course and that of formation of the resulting products (Figure 1).

Figure 2. Possible routes of formation of O- and N-(2-naphthoyl)-l-serine from compound 2.

N-(2-Naphthoyl)-l-serine was detected at pH 7.4, and its identity was confirmed by its m/z value ([M − H]− = 258.1) and superposition of its HPLC peak with that of a standard sample. The hydrolysis of the amide side chain was significantly slower, as measurable amounts of 2-naphthoic acid were detected after only 24 h. At pH 5.0, a degradation product not observed at pH 7.4 was detected. The compound showed a retention time on a C18 RP column shorter than that of N-(2-naphthoyl)-l-serine and had an m/z value corresponding to the addition of a water molecule to compound 2 ([M + H]+ = 260.1). The compound was identified as O-(2-naphthoyl)-l-serine on the basis of its retention time and that of one authentic sample.

Figure 1 shows the time course and degradation products of 2 at pH 5.0. At time ≤2 h, 2 was converted rapidly into N-(2-naphthoyl)-l-serine [product A (Figure 2)] and more slowly into O-(2-naphthoyl)-l-serine [product B (Figure 2)]. At 2 h, no starting compound or 2-naphthoic acid [product C (Figure 2)] was detected, and approximately equal amounts of A and B were present, accounting together for ∼60% of the total mass balance. At early time points, a peak with an m/z value corresponding to that of compound 2 ([M − H]− = 240.1) was also observed, corresponding to an additional degradation product [D (Figure 2)]. At longer time points, the slow decrease in the level of N-(2-naphthoyl)-l-serine (product A) was paralleled by a corresponding increase in the level of 2-naphthoic acid. After 3 days, the sum of molar concentrations for compounds A−C, measured through calibration curves with pure standards, was more than 90% of the starting concentration of compound 2, and the peak of the additional degradation product [D (Figure 2)] disappeared, probably because it converted into compound A or B. We hypothesized a degradation scheme for 2 involving an active role for the amide group, following what had been already proposed for the degradation of serine lactones in a different context (Figure 2).33 In particular, a nucleophilic attack from the amide carbonyl to the β-methylene group of N-(2-oxo-3-oxetanyl)-amides would give the intermediate 5-dihydro-2-(2-naphthalen-4-oxazolecarboxylic acid, having an m/z value consistent with product D, which would quickly be hydrolyzed to O-acylated amino acid B. At the same time, water could directly attack the lactone carbonyl of 2, giving N-acylated amino acid A. In fact, the time courses of starting lactone 2 and products A and B (Figure 1) suggest two simultaneous pathways: one involving hydrolysis of the lactone to give N-(2-naphthoyl)-l-serine (A) and the second yielding O-(2-naphthoyl)-l-serine (B) through the formation of oxazoline−carboxylic acid intermediate D, which could not be isolated or quantified but showed intense peaks in chromatograms at short time points (Figure 3). This interpretation is further supported by the experimental observation that N-(2-naphthoyl)-l-serine did not directly convert to O-(2-naphthoyl)-l-serine but was slowly hydrolyzed to 2-naphthoic acid when maintained at pH 5.0 for 3 days. O-(2-Naphthoyl)-l-serine was found to be stable at pH 5.0, whereas at pH 7.4, it was converted to N-(2-naphthoyl)-l-serine (t1/2 ~ 280 min).

As the only substitution that improved the chemical stability of the N-(2-oxo-3-oxetanyl)amides (i.e., the introduction of the methyl group at the β-position of the lactone) had shown a detrimental effect on rat NAAA inhibitory potency,35 our attempt to optimize both potency and stability focused on the
class of \(N\)-(2-oxo-3-oxetanyl)carbamic acid esters (Table 2), assuming that the lower nucleophilicity of the carbamate carbonyl would weaken the negative effect of the side chain on lactone stability. This hypothesis was based on quantum mechanical calculations for the internal nucleophilic attack on the \(\beta\)-carbon of the lactone ring by the side chain acyl oxygen, simulated for the two model compounds \(N\)-(2-oxo-3-oxetanyl)-acetamide and \(N\)-(2-oxo-3-oxetanyl)carbamic acid ester. Potential energy paths, calculated at the density functional theory (DFT) level (see Experimental Section for details), revealed that the carbamate has a higher potential energy barrier for oxazoline formation, probably because of the lower nucleophilicity of the carbamate carbonyl, compared to that of the carboxamide (Figure 4). The lower susceptibility of the carbamic acid ester to undergo internal cleavage was also confirmed by chemical stability data (e.g., carbamic acid esters 10a and 23b), which showed a significantly higher stability with respect to the corresponding amides 1 and 8, the half-life going at pH 7.4 from 18.0–73.8 to 26.7–107.7 min and at pH 5.0 from 23.1–134.6 to 39.2–185.1 min. To improve the potency, we started a SAR exploration of \(N\)-(2-oxo-3-oxetanyl)carbamic acid esters. SPR analysis was also conducted to investigate the stereoelectronic requirements of the substituents at the \(\alpha\)- and \(\beta\)-positions of the \(\alpha\)-amino-\(\beta\)-lactone and the role of the size and shape of the carbamic acid ester side chain.

SAR of \(N\)-(2-Oxo-3-oxetanyl)carbamic Acid Esters. In our previous SAR exploration of \(N\)-(2-oxo-3-oxetanyl)amides, enantiomers with the \((S)\) configuration at position 3 led to a 10-fold increase in rat NAAA inhibitory potency, compared to that of \((R)\) compounds. This trend is reversed in the present \(N\)-(2-oxo-3-oxetanyl)carbamic acid esters, as the \((R)\) analogue 10b became more potent than its \((S)\) enantiomer 10a, with IC\(_{50}\) values of 0.70 and 2.96 \(\mu\)M, respectively. As observed with \(N\)-(2-oxo-3-oxetanyl)amides, the essential role played in rat NAAA inhibitory potency by the intact \(\beta\)-lactone ring was confirmed by the lack of potency of open derivatives 11a and 11b, the cyclobutane, and cyclobutanone derivatives 13 and 15 (IC\(_{50}\) > 100 \(\mu\)M). Replacing the carbamate group with a urea, as in derivatives 19a and 19b, resulted in a marked decrease in potency (IC\(_{50}\) values of 15 and 49 \(\mu\)M, respectively). Two further structural modifications were attempted. First, we introduced a tert-butyl substituent onto the carbamate group. This substitution was tolerated for NAAA inhibition but caused a lack of stereoselectivity, with IC\(_{50}\) values of 0.58 and 0.50 \(\mu\)M for \((S)\) (17a) and \((R)\) (17b) enantiomers, respectively. The second modification was the introduction of a methyl group at the \(\beta\)-position of the ring. Within the class of \(N\)-(2-oxo-3-oxetanyl)amides, \(S\) methyl derivatives showed reduced inhibitory potency (IC\(_{50}\) values of >100 \(\mu\)M for 8 and 100 \(\mu\)M for its enantiomer), whereas the corresponding anti methyl derivatives were too unstable to be prepared or isolated (unpublished results); however, methyl substitution could be positively related to stability [e.g., compound 8 (Table 1 and related results)]. The first of the two benzyl derivatives 23a and 23b, formally related to \(L\)- and \(D\)-threonine, respectively, was slightly (10 times) more potent than the second. In the case of the tert-butyl carbamates (24a–d), it was possible to synthesize and test the four enantiomers corresponding to the relative configurations of the two substituents on the \(\beta\)-lactone ring. Among them, those having the \(C_\beta\) of the \(\alpha\)-amino-\(\beta\)-lactone ring in the \((S)\) configuration (24b and 24c) were endowed with good inhibitory potency (IC\(_{50}\) values of 0.22 and 0.19 \(\mu\)M, respectively), while their enantiomers were inactive (for 24a, IC\(_{50}\) > 80 \(\mu\)M) or significantly less potent (for 24d, IC\(_{50}\) = 4.9 \(\mu\)M). The role of the lipophilicity of the carbamic acid ester side chain was explored with the introduction of a 5-phenylpentyl substituent (27). This compound showed a very good potency with respect to rat NAAA inhibition, with an IC\(_{50}\) of 127 nM, which demonstrates the positive influence of lipophilicity on rat NAAA inhibition.

Chemical Stability of \(N\)-(2-Oxo-3-oxetanyl)carbamic Acid Esters. The decision to move from serine- to threonine-based carbamic acid esters was conducive to chemical stability. For example, half-lives of threonine lactone 23b were on
average >4-fold longer than that of its homologue serine-based 10a (Table 3). A similar lengthening of half-lives was also observed for all other threonine-based derivatives 24a–c and 27. The introduction of a bulky tert-butyl substituent onto the carbamate side chain had no additive effect on chemical stability; within this subset of derivatives, syn 24b was more stable than the anti derivative (24c). Other threonine lactones, characterized by the syn arrangement, showed half-lives of >100 min at pH 7.4 and 5.0.

**Biological Stability.** The N-(2-oxo-3-oxetanyl)amides and N-(2-oxo-3-oxetanyl)carbamic acid esters were also assayed for their biological stability in the presence of a prototypical off-target protein, bovine serum albumin (BSA), the most abundant protein in plasma, which is also endowed with esterase activity, and 80% (v/v) rat plasma; this is a well-established in vitro model for hydrolytic metabolism. Results are reported in Table 4.

In the presence of 20 mg/mL BSA, half-lives of all compounds significantly decreased with respect to chemical stability assays. Threonine-based derivatives were in general more stable than their serine-based analogues, which confirmed the important role of methyl substitution of the β-lactone system in chemical and BSA-catalyzed hydrolysis. For example, 1 had a half-life of 7.0 min in the presence of BSA, which doubled for threonine-based amide 8 (t_{1/2} = 16 min). In the set of carbamic acid esters, the very low stability of 10a (t_{1/2} ~ 1 min) was improved in analogues 23b (t_{1/2} ~ 5 min) and 27.
more pronounced in the case of threonine-based enzymatic inhibitors.

A representative of its class and subjected to a panel of stability 0.22

degradation of the molecules,

plasma hydrolases might be responsible for the rapid 1

inhibition of carboxylesterases and cholinesterases, while 63 phenylmethyl-

catalyzed hydrolysis.

Paraoxonase58 (Table 4. In Vitro Biological Stabilities for Compounds 1–9, 10a, 17a, 19a, 23b, 24a–c, and 27)

<table>
<thead>
<tr>
<th>compd</th>
<th>20 mg/mL BSA t_{1/2} (min)</th>
<th>80% (v/v) rat plasma t_{1/2} (s)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>7.0 ± 0.6</td>
<td>&lt;10</td>
</tr>
<tr>
<td>2</td>
<td>&lt;1</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>3.2 ± 0.7</td>
<td>75 ± 20</td>
</tr>
<tr>
<td>4</td>
<td>&lt;1</td>
<td>29 ± 8</td>
</tr>
<tr>
<td>5</td>
<td>2.7 ± 0.6</td>
<td>&lt;10</td>
</tr>
<tr>
<td>6</td>
<td>5.8 ± 1.0</td>
<td>65 ± 30</td>
</tr>
<tr>
<td>7</td>
<td>3.3 ± 0.8</td>
<td>32 ± 15</td>
</tr>
<tr>
<td>8</td>
<td>16.0 ± 1.2</td>
<td>&lt;10</td>
</tr>
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<td>9</td>
<td>15.1 ± 1.8</td>
<td>14 ± 9</td>
</tr>
<tr>
<td>10a</td>
<td>1.2 ± 0.7</td>
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</tr>
<tr>
<td>17a</td>
<td>19.6 ± 1.5</td>
<td>&lt;10</td>
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<td>4.6 ± 1.1</td>
<td>&lt;10</td>
</tr>
<tr>
<td>23b</td>
<td>5.1 ± 0.8</td>
<td>&lt;10</td>
</tr>
<tr>
<td>24a</td>
<td>120.7 ± 1.1</td>
<td>13 ± 6</td>
</tr>
<tr>
<td>24b</td>
<td>129.1 ± 7.0</td>
<td>11 ± 5</td>
</tr>
<tr>
<td>24c</td>
<td>87.7 ± 2.3</td>
<td>&lt;10</td>
</tr>
<tr>
<td>27</td>
<td>11.4 ± 2.4</td>
<td>&lt;10</td>
</tr>
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</table>

(t_{1/2} ∼ 11 min). Nevertheless, the most relevant stabilization over BSA-catalyzed cleavage was obtained by the introduction of the bulky tert-butyl substituent on the carbamic acid ester side chain. The substitution of the benzy! (as in 10a) with a tert-butyl group (as in 17a) had a marked effect on stability, with the half-life increasing from ∼1 to ∼20 min. The effect was more pronounced in the case of threonine-based tert-butyl carbamic acid esters 24a–c. In fact, they showed half-lives, in the presence of BSA, comparable to those observed in pH 7.4 buffered solution, indicating no significant BSA-catalyzed cleavage and chemical stability as the limiting factor for these compounds. With compound 24b, for example, it was possible to maintain a good inhibitory potency on rat NAAA (IC_{50} = 0.22 μM) and half-lives of ∼2 h in the case of both chemical and BSA-catalyzed hydrolysis.

The majority of the compounds were rapidly cleaved in the presence of 80% (v/v) rat plasma, with half-lives shorter than 1–2 min. To obtain preliminary indications of which types of plasma hydrolyses might be responsible for the rapid degradation of the molecules, 24b was chosen as a representative of its class and subjected to a panel of stability assays in rat and human plasma in the presence of different enzymatic inhibitors.

Rat and human plasma contain different types of esterases,57 including carboxylesterases, cholinesterases, and paraoxonase. Paraoxonase58–60 is a lipoprotein-associated esterase that hydrolyzes organophosphorous compounds but is not inhibited by them and is responsible for the cleavage of several arylesters and aromatic and aliphatic lactones; it requires calcium ions as cofactor for activity and stability and is consequently inactivated by ethylendiaminetetraacetic acid (EDTA).61,62 The esterase inhibitors bis-4-nitrophenyl phosphate (BNPP),63 phenylmethyl-

<table>
<thead>
<tr>
<th>inhibitor</th>
<th>50% (v/v) rat plasma</th>
<th>50% (v/v) human plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>0.53 ± 0.20</td>
<td>2.99 ± 0.54</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.34 ± 0.18</td>
<td>31.99 ± 2.60</td>
</tr>
<tr>
<td>BNPP</td>
<td>3.29 ± 1.70</td>
<td>4.70 ± 1.80</td>
</tr>
<tr>
<td>eserine</td>
<td>4.19 ± 0.90</td>
<td>13.75 ± 3.00</td>
</tr>
<tr>
<td>PMSF</td>
<td>7.13 ± 1.70</td>
<td>11.50 ± 2.80</td>
</tr>
</tbody>
</table>

Table 5. Plasma Stability of Compound 24b in the Presence of Enzymatic Inhibitors

plasma, the cleavage of 24b seems to be mainly dependent on the combined action of carboxylesterase and cholinesterase. In fact, while EDTA did not change the stability of the compound (t_{1/2} = 0.53 min vs 0.34 min in the presence of EDTA), BNPP, PMSF, and eserine markedly increased the half-life of 24b. In human plasma, which does not contain carboxylesterases,65 inhibition of calcium-dependent esterases by EDTA led to a 10-fold increase in the half-life, a value higher than that observed with other inhibitors. Human plasma paraoxonase had already been identified as the main factor responsible for the quick plasma hydrolysis of a set of anti-inflammatory glucocorticoid y-lactones.66 In that case, a potential application of these compounds as “soft drugs” for the treatment of asthma was postulated,67 based on the nonubiquitous distribution of paraoxonase, and in particular on its absence from the lung tissue.61 Soft drugs exert their therapeutic action in the target tissue and are then converted into inactive metabolites upon reaching the systemic circulation. In the literature, the soft drug strategy has been successfully exploited for topically applied drugs. With regard to the field of anti-inflammatory corticosteroids, attention was paid to carboxylic (thio)ester derivatives and the development of related plasma labile soft drugs68 or “antidrugs”69,70. In the dermatologic field, boron-containing type 4 phosphodiesterase inhibitors, developed for topical treatment of psoriasis and atopic dermatitis, have been successfully derivatized by incorporation of a cleavable ester group, thus weakening their systemic side effects.71 With regard to our set of N-(2-oxo-3-oxetanyl)carboxylic acid esters, low plasma stability may be turned to an advantage for topical applications. PEA is an endogenous component of the human epidermis and is generated from phospholipids in the stratum granulosum. Topical application of a PEA-containing product had been shown to significantly inhibit the development of UV light-induced erythema and thymine dimer formation in normal human skin.72 Topical application of plasma unstable NAAA inhibitors could therefore improve the anti-inflammatory effects of PEA, whereas the systemic instability of the compounds would restrict NAAA inhibition to the site of administration, thus weakening their potential systemic side effects.

### CONCLUSIONS

A series of N-(2-oxo-3-oxetanyl)carboxylic acid esters has been synthesized as rat NAAA inhibitors, and SARs and SPRs were studied to discover novel inhibitors endowed with an improved potency and stability profile. Figure 5 summarizes the SARs for the β-lactone carbamate NAAA inhibitors described here. They confirmed the importance of the β-lactone ring for rat NAAA inhibition and the positive role of the carbamate group in the side chain. The lipophilicity of the side chain was also positively correlated with NAAA inhibitory potency. The shift from serine- to threonine-based carbamic acid esters was tolerated in terms of NAAA inhibitory potency and led to an important

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improvement in the chemical stability profile, even if the preferred stereocchemical configuration of the two substituents showed a complex and not yet fully defined dependence on the alkyll substitution of the carbamate side chain. The introduction of a bulky tert-butyl substituent into the side chain made the compounds less reactive toward BSA.

In particular, structural modification led us to discover (2S,3R)-2-methyl-4-oxo-3-oxetanycarbamic acid 5-phenylpentyl ester (27), which (i) is a potent in vitro rat NAAA inhibitor (IC50 = 127 nM), (ii) has an improved chemical stability profile, (iii) has a reduced propensity to react with BSA, and (iv) is rapidly cleaved by plasma hydrolases. Compound 27, also termed URB913 and ARN077, is the first potent representative of a new class of NAAA inhibitors sharing a carbamic acid ester side chain, which has been expanded for further SAR investigation and potency improvement and reported elsewhere.73 27 may be a useful new tool for investigating the role of NAAA in inflammation and analgesia and a possible soft drug candidate for topical use.

**EXPERIMENTAL SECTION**

**Chemicals, Materials, and Methods.** All reagents were purchased from Sigma-Aldrich, Lancaster, Novabiochem, or Acros at the highest quality commercially available. Solvents were RP grade unless otherwise indicated. Dry tetrahydrofuran was distilled over sodium and benzophenone. Dry dimethylformamide, dichloromethane, and triethylamine were used as supplied. Petroleum ether refers to the highest quality commercially available. Solvents were RP grade.

**Synthesis of (R)-2-Oxo-3-oxetanycarbamic Acid Benzyl Ester (13).** To a stirred solution of NaHCO3 (0.504 g, 6.0 mmol) in H2O (7.0 mL) under a N2 atmosphere, were added Et3N (0.056 g, 0.085 mL, 0.55 mol) in dry THF (4 mL), at 0 °C, c-C4H7NH2 (0.213 g, 0.260 mL, 3.0 mmol). The mixture was stirred at room temperature for 3 h, diluted with H2O (10 mL), and extracted with EtOAc. The combined organic phases were washed with brine (Na2SO4), and concentrated. Purification of the residue by flash column chromatography (8:2 cyclohexane/PrOH; 1.96 (m, 4H), 2.26 – 2.41 (m, 2H), 4.08 – 4.24 (m, 2H), 4.15 (m, 2H), 5.15 (s, 1H), 5.51 (br s, 1H), 7.36 (m, 5H);13C NMR (CDCl3) δ 19.8, 41.6, 62.2, 66.5, 128.4, 128.6, 136.0, 155.5, 205.3; IR (neat) νmax 3317, 2977, 2945, 1682, 1539 cm⁻¹. Anal. (C12H15NO2) C, H, N.

**Synthesis of Cyclobutylcarbamic Acid Benzyl Ester (14).** To a stirred solution of NaHCO3 (0.504 g, 6.0 mmol) in H2O (7.0 mL) were added c-C4H7-NH2 (0.213 g, 0.260 mL, 3.0 mmol) and, dropwise, a solution of NaHCO3 (0.504 g, 6.0 mmol) in H2O (7.0 mL) under a N2 atmosphere, were added Et3N (0.056 g, 0.085 mL, 0.55 mol) in dry THF (4 mL), at 0 °C, c-C4H7NH2 (0.213 g, 0.260 mL, 3.0 mmol). The mixture was stirred at room temperature for 3 h, diluted with H2O (10 mL), and extracted with EtOAc. The combined organic phases were washed with brine (Na2SO4), and concentrated. Purification of the residue by flash column chromatography (8:2 cyclohexane/PrOH; 1.96 (m, 4H), 2.26 – 2.41 (m, 2H), 4.08 – 4.24 (m, 2H), 4.15 (m, 2H), 5.15 (s, 1H), 5.51 (br s, 1H), 7.36 (m, 5H);13C NMR (CDCl3) δ 14.7, 31.4, 46.2, 66.5, 128.1, 128.5, 136.6, 155.2; IR (neat) νmax 3092, 2973, 2870, 1701, 1522 cm⁻¹. Anal. (C12H13NO4) C, H, N.

**Synthesis of (S)-2-Oxocyclobutancarbamic Acid tert-Butyl Ester (17a).** For the white solid, the yield, mp, [α]D, MS (EI), and IR are given in ref 33. 1H NMR and IR data are given in ref 37. Anal. (C12H15NO2) C, H, N.

**Synthesis of (S)-1-Methyl-3-(2-oxo-3-oxetanyl)urea (19a).** To a stirred mixture of 18a (0.130 g, 0.5 mmol) in dry THF (4 mL), at 0 °C under a N2 atmosphere, were added Et3N (0.056 g, 0.805 mL, 0.55 mmol) and C2H5CH2NO (0.073 g, 0.808 mL, 0.55 mmol). The mixture was stirred at 0 °C for 0.5 h and at room temperature for 3 h and then concentrated. Purification of the residue by column chromatography (4:6 to 2:8 cyclohexane/PrOH) and recrystallization gave 19a as white crystals: 54% yield (0.060 g); mp 188 – 190 °C, 4831 dx.doi.org/10.1021/jm300349j J. Med. Chem. 2012, 55, 4824–4836
decomposition with color change starting from 130 °C, sealed capillary tube [(CH3)2CO(Ch3)/petroleum ether]; [α]20D = −12 (c 0.5, MeOH); MS (El) m/z 220 (M+), 91 (100); 1H NMR (DMSO-d6) δ 4.20 (d, 2H, J = 6 Hz), 4.31−4.36 (m, 2H), 5.07−5.16 (m, 1H), 6.80−6.92 (m, 2H), 7.21−7.32 (m, 5H); 13C NMR (DMSO-d6) δ 43.3, 59.2, 66.6, 127.1, 127.5, 128.7, 140.8, 157.4, 172.1; IR (Nujol) νmax 3431, 3295, 1845, 1815, 1561 cm−1. Anal. (C12H13NO4) C, H, N.

**Synthesis of (R)-1-Methyl-3-oxo-3-oxetanylfural (19b).** The same protocol applied to 18b, apart from the eluent for column chromatography (3:7 cyclohexane/EtOAc), gave 19b as white crystals: 42% yield (0.060 g); mp 183 °C; decomposition with color change starting from 125 °C, sealed capillary tube [(CH3)2CO(Ch3)/petroleum ether]; [α]20D = +10 (c 0.5, MeOH); MS (El) m/z 220 (M+), 91 (100); 1H NMR (DMSO-d6) δ 4.21 (d, 2H, J = 6 Hz), 4.29−4.37 (m, 2H), 5.08−5.17 (m, 1H), 6.78−6.87 (m, 2H), 7.17−7.32 (m, 5H); 13C NMR (DMSO-d6) δ 43.3, 59.2, 66.6, 127.1, 127.5, 128.7, 140.8, 157.4, 172.1; IR (Nujol) νmax 3431, 3295, 1845, 1815, 1561 cm−1. Anal. (C12H13NO4) C, H, N.

**Synthesis of (S)-2-Benzyloxycarbonylamino-3-hydroxybutyric Acid Benzyl Ester (23a).** White needles; 50% yield (0.429 g); mp 120−122 °C (CHCl3/n-hexane); [α]20D = +11.0 (c 0.29, (CH3)2CO(Ch3)); MS (El) m/z 235 (M+), 91 (100); 1H NMR (DMSO-d6) δ 1.33 (d, 3H, J = 6.3 Hz), 4.85 (m, 1H), 5.01−5.14 (Abg, 2H), 5.44 (dd, 1H, J1 = 4.9 Hz, J2 = 6.1 Hz), 7.27−7.43 (m, 5H), 8.36 (d, 1H, J = 9.4 Hz); 13C NMR (CDCl3) δ 15.1, 60.4, 67.9, 74.7, 128.3, 128.6, 128.7, 135.5, 155.3, 168.7; IR (Nujol) νmax 3284, 1813, 1695, 1553 cm−1. Anal. (C12H13NO4) C, H, N.

**Synthesis of (S)-2-Benzyloxycarbonylamino-3-hydroxybutyric Acid tert-Butyl Ester (23c).** White solid; 55% yield (0.472 g); mp 120−121 °C (CHCl3/n-hexane); [α]20D = −9.5 (c 0.2, (CH3)2CO(Ch3)); MS (El) m/z 235 (M+), 91 (100); 1H NMR (DMSO-d6) δ 1.33 (d, 3H, J = 6.4 Hz), 4.85 (m, 1H), 5.01−5.14 (Abg, 2H), 5.44 (dd, 1H, J1 = 4.9 Hz, J2 = 6.1 Hz), 7.27−7.43 (m, 5H), 8.36 (d, 1H, J = 9.4 Hz); 13C NMR (CDCl3) δ 15.1, 60.4, 67.9, 74.7, 128.3, 128.6, 128.7, 135.5, 155.3, 168.7; IR (Nujol) νmax 3284, 1813, 1695, 1553 cm−1. Anal. (C12H13NO4) C, H, N.

**Synthesis of (R)-2-Benzyloxycarbonylamino-3-hydroxybutyric Acid tert-Butyl Ester (24a).** For the off-white solid, the yield, mp, and 1H NMR are given in ref 40. (R)-2-Benzyloxycarbonylamino Thiochrome-4-sulfonate (18a). For the white solid, the yield, mp, and 1H NMR are given in ref 41.

**General Procedure for the Synthesis of Threonine Derivatives 21a,b and 22a,d.** To a stirred mixture of the opportune threonine (2S,3R)-2-Methyl-4-oxo-3-oxetanylcarbamic Acid 5-Cbzl- and (2R,3S)-2-Methyl-4-oxo-3-oxetanylcarbamic Acid tert-Butyl Ester (24a,d):43,44 white solid; 76% yield (0.566 g); mp 119−121 °C (0.353 g, 2.96 mmol). After gas evolution, 5-phenylpentyl chlorofomate (0.740 g, 3.26 mmol), previously prepared as reported in the literature,48 was added slowly followed by (n-Bu)4NB (0.035 g, 0.92 mmol). The mixture was stirred at room temperature for 18 h, diluted with H2O, and washed with EtOAc. The combined organic layers were washed with brine, dried (Na2SO4), and concentrated to give 21a,d and 22a,d−

For the white solid, the 1H NMR is given in ref 41.

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phosphate-buffered saline (PBS) (pH 7.4)] and acidic [0.01 M phosphate buffer (pH 5.0)] pH conditions, and in the presence of a 3 mM solution of the thiol nucleophile DTT in 0.01 M phosphate buffer (pH 5.0), as previously reported. Stock solutions of compounds were prepared in DMSO, and each sample was incubated at a final concentration of 1–100 µM in a prewarmed (37 °C) buffer solution; the final DMSO concentration in the samples was kept at 1%. The samples were maintained at 37 °C in a temperature-controlled shaking water bath (60 rpm). At various time points, 100 µL aliquots were removed and analyzed by HPLC. Apparent half-lives \( t_{1/2} \) for the disappearance of test compounds were calculated from the pseudo-first-order rate constants obtained by linear regression of plots of log[compound] versus time and are listed in Tables 1 and 3 as mean values of three experiments along with their standard deviations.

**In Vitro Rat Plasma Stability.** Rat plasma stability was investigated as previously reported. Briefly, plasma was quickly thawed and diluted to 80% (v/v) with PBS (pH 7.4) to buffer the solution pH, which was checked during the course of the experiments. Pooled rat plasma (400 µL) was incubated with PBS buffer (95 µL, pH 7.4) and a compound stock solution (5 µL) in DMSO (final DMSO concentration in samples of 1%; final compound concentrations of 5–100 µM). Samples were maintained at 37 °C in a temperature-controlled shaking water bath (60 rpm) throughout the experiments. At regular time points, aliquots (50 µL) were withdrawn, supplemented with 2 volumes of CH3CN centrifuged at 8000g for 5 min at 4 °C, and analyzed by RP-HPLC. Apparent half-lives \( t_{1/2} \) for the disappearance of test compounds were calculated from the pseudo-first-order rate constants obtained by linear regression of plots of log[compound] versus time; apparent half-life values listed in Table 4 as the mean values of three experiments along with standard deviations.

**In Vitro Stability in the Presence of BSA.** A 20 mg/mL solution of bovine serum albumin in PBS (pH 7.4) was employed for stability assays at 37 °C. Test compounds were incubated at final concentrations of 5–100 µM with a final DMSO percentage of 1% (v/v). Samples were processed as reported above for rat plasma stability experiments.

**In Vitro and Human Plasma Stability in the Presence of Selected Inhibitors.** Rat and human plasma stability experiments in the presence of selected inhibitors (BNPP, eserine, PMSF, and EDTA) took place at 37 °C in the presence of 50% (v/v) plasma, because of the very rapid hydrolysis of the test compounds under the previously reported experimental conditions [50% (v/v) plasma at 37 °C]. Briefly, rat and human plasma were preincubated in the presence of selected inhibitors or vehicle according to literature conditions. After preincubation, compound 24b was added, and at regular time points, aliquots of the sample (50 µL) were withdrawn, processed, and analyzed as reported for in vitro rat plasma stability assays.

**Conditions for HPLC–UV–MS Analysis.** The degradation of compounds 1–9, 10a, 19a, and 23b was monitored by RP-HPLC and UV by employing a Shimadzu gradient system (Shimadzu Corp., Kyoto, Japan) consisting of two Shimadzu LC-10Amp solvent delivery modules, a 20 µL Rheodyne sample injector (Rheodyne LLC, Rohnert Park, CA), and an SPD-10Avp UV–vis detector, equipped with a reversed-phase C18 column [LC-18-DB, 5 µm, 150 mm × 4.6 mm (inside diameter) (Supelco, Bellefonte, PA)]. The HPLC system was interfaced with PeakSimple version 2.83 for data acquisition. Mobile phases consisted of various percentages of CH3CN and 0.1% (v/v) formic acid delivered at a flow rate of 1 mL/min. Each compound was monitored at its relative absorbance maximum for UV detection. Compounds 17a, 24a–c, and 27 were monitored with an API1100EX single-quadrupole HPLC–MS system (AB/Sciex, Toronto, ON) equipped with a atmospheric pressure chemical ionization (APCI) ion source working in positive and negative ion mode and coupled to an Agilent 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany) that consisted of a G1312A binary pump, a G1379A degasser, and a 10 µL Rheodyne sample injector. Compound-dependent parameters were optimized by flow injection analysis (FIA) and ramping of the potentials. The final settings were as follows: declustering potential (DP), ±3.0 V; focusing potential (FP), ±100 V; entrance potential (EP), ±10 V. In all cases, higher voltages led to compound in-source fragmentation. The ion source temperature was set at 400 °C. A Supelcosil C18-DB column (150 mm × 4.6 mm, 5 µm) (Supelco) was employed; the flow rate was kept at 1 mL/min. Mobile phases consisted of various percentages of acetonitrile and 10 mM ammonium acetate (pH 7.0). Data were acquired by employing Analyst version 1.4 (AB/Sciex).
ACKNOWLEDGMENTS
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DEDICATION
This article is dedicated to the memory of Andrea Tontini, who sadly died after the manuscript had been submitted. Andrea, a valuable scientist and a great friend, joined the research group in Urbino in 1991 and was a pillar of our teamwork. His sudden death leaves a professional void in our research groups and tremendous grief in our hearts.

ABBREVIATIONS USED
AEEA, N-arachidonoylethanolamine; AS, amidase signature; BNPP, bis-4-nitrophenylphosphate; BOP, benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate; DFT, density functional theory; FAE, fatty acid ethanolamide; HBTA, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HPLC, high-performance liquid chromatography; NAAA, N-acyl ethanolamide acid amidase; Ntn, N-terminal nucleophile; PEA, palmitoylethanolamide; PMSF, phenylmethanesulfonyl fluoride; PBS, phosphate-buffered saline; QM/MM, quantum mechanics/molecular mechanics; RP, reverse phase; SPR, structure--property relationship.

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