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Synthesis, Biological Evaluation, and 3D QSAR Study of 2-Methyl-4-oxo-3-oxetanylcarbamic Acid Esters as N-Acylethanolamine Acid Amidase (NAAA) Inhibitors

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Supporting Information

ABSTRACT: N-(2-Oxo-3-oxetanyl)carboxylic acid esters have recently been reported to be noncompetitive inhibitors of the N-acylethanolamine acid amidase (NAAA) potentially useful for the treatment of pain and inflammation. In the present study, we further explored the structure–activity relationships of the carboxylic acid ester side chain of 2-methyl-4-oxo-3-oxetanylcarbamic acid ester derivatives. Additional favorable features in the design of potent NAAA inhibitors have been found together with the identification of a single digit nanomolar inhibitor. In addition, we devised a 3D QSAR using the atomic property field method. The model turned out to be able to account for the structural variability and was prospectively validated by designing, synthesizing, and testing novel inhibitors. The fairly good agreement between predictions and experimental potency values points to this 3D QSAR model as the first example of quantitative structure–activity relationships in the field of NAAA inhibitors.

INTRODUCTION

The fatty acid ethanamides (FAEs) are a class of multifunctional lipid mediators that have attracted significant attention because of their potential role in physiological and pathological processes including pain,1–3 innate immunity,4 reward regulation,5 and feeding.6,7 In particular, palmitoylethanolamide (PEA), the endogenous amide of palmitic acid and ethanolamine, has been shown to inhibit peripheral inflammation.1,2,7 Only a restricted number of NAAA inhibitors, belonging to a narrow range of chemical classes, has been reported so far.21,22 Generally most of the first discovered compounds have been shown to block the enzyme hydrolytic activity with micromolar potencies.23–27 Rational drug discovery efforts aimed at either identifying novel scaffolds or improving already known ones have been severely hampered by the limited amount of detailed structural information on this target.

NAAA is a cysteine hydrolase that belongs to the N-terminal nucleophile (Ntn) family of enzymes.20,28,29 Like other Ntn enzymes, NAAA is produced as an inactive proenzyme and is activated at acidic pH by autocatalytic cleavage at a specific site of the peptide chain.30 Recently, a comparative model of NAAA active site was generated starting from the coordinates of conjugated bile acid hydrolase (CBAH).31 The insights gained from this model eventually led to the discovery of the serine-derived 2-oxo-3-oxetanamid, (S)-OOPP, as a first submicromolar NAAA inhibitor. The model turned out to be consistent with both mutagenesis data, explaining the role of several residues in the catalytic process, as well as with the SAR exploration performed around the newly identified potent NAAA inhibitors, featuring an α-amino-β-lactone ring as a reactive electrophile warhead.32 Structure–activity relationship (SAR) studies of α-amino-β-lactone derivatives, as carboxylic acid esters, investigated the...
effects on NAAA inhibition of side chain modifications and the stereochemical requirements of the introduction of a β-substitution.33–35 These works led to the identification of compounds that were highly potent at inhibiting both rat and human NAAA, such as β-lactones 1 (ARN077)33,34,36 and 234 (Figure 1). The β-lactone ring of these compounds was proved to be responsible for the inhibition mechanism, interacting covalently with the N-terminal catalytic cysteine (Cys126), through the formation of a thioester bond.36

In the present study, we further extended the exploration of the SAR for this class of inhibitors, focusing on the carbamic acid ester side chain. Besides generating new compounds, a more extensive investigation of the structural determinants for NAAA activity could also shed light on some three-dimensional features of NAAA binding pocket, thus facilitating future development of new molecular scaffolds.

To this aim, combining previously reported results34 together with data on 17 new compounds purposely synthesized to enhance the structural diversity of the set, we devised a three-dimensional quantitative structure–activity relationship (3D QSAR) model. Our model was built adopting the recently reported atomic property field (APF) method.37 APF can be described as a grid-based model in which the discrete features of a classic pharmacophore are replaced by continuous, regularly spaced three-dimensional lattices. Each molecule from our training set contributed to this composite set of grids proportionally to its reported activity. With respect to more traditional approaches, the main advantage of this technique is that the strength of the overlap between a new putative compound and the APF signature of the template molecules can be directly translated into a prediction of activity.

Finally, our model was prospectively validated. On the basis of the predictions generated on a list of virtual compounds, we selected five high-scoring derivatives to be actually synthesized and tested. Notably, the activities of the prospectively tested compounds turned out to be in line with the predictions.

## CHEMISTRY

Novel 2-methyl-4-oxo-3-oxetanylcarnamic acid esters 9a–v (Table 1 and Table 4) were synthesized using previously reported synthetic protocols (Scheme 1).34,35 First, alcohols 3 were activated either as imidazole 1-carboxylates 4 or as a mixture of 2-pyridyl carbonates 5 and 2-oxopyridine-1-carboxylates 6. The desired final 2-methyl-4-oxo-3-oxetanycarbamic acid esters 9a–v were obtained either by reaction of (2S,3R)-2-methyl-4-oxo-3-oxetanlammonium toluene-4-sulfonate (7) with compounds 4–6 or by cyclization of intermediate α-substituted-β-hydroxycarboxylic acids 8, synthesized from commercially available D-threonine and the corresponding activated alcohols 4–6.

Most of the alcohols used in the above-mentioned methodologies were commercially available or obtained via reduction of the corresponding commercially available carboxylic acids. Alcohols 3c–e,p were prepared as reported

![Figure 1. Potent α-amino β-lactone NAAA inhibitors.](image)

**Table 1. Inhibitory Potencies (IC50) of Compounds 9a–q on Rat NAAA Activity**

| Compounds | Structure | IC50 (μM) ± SD |
|-----------|-----------|---------------|
| 9a        | ![Structure](image) | 0.24 ± 0.07   |
| 9b        | ![Structure](image) | 0.296 ± 0.017 |
| 9c        | ![Structure](image) | 3.76 ± 1.72   |
| 9d        | ![Structure](image) | 0.52 ± 0.19   |
| 9e        | ![Structure](image) | 3.43 ± 0.52   |
| 9f        | ![Structure](image) | 0.02 ± 0.01   |
| 9g        | ![Structure](image) | 5.42 ± 0.56   |
| 9h        | ![Structure](image) | 0.039 ± 0.011 |
| 9i        | ![Structure](image) | 1.22 ± 0.30   |
| 9j        | ![Structure](image) | 0.02 ± 0.003  |
| 9k        | ![Structure](image) | 0.022 ± 0.01  |
| 9l        | ![Structure](image) | 0.007 ± 0.002 |
| 9m        | ![Structure](image) | 0.011 ± 0.006 |
| 9n        | ![Structure](image) | 0.029 ± 0.007 |
| 9o        | ![Structure](image) | 0.036 ± 0.021 |
| 9p        | ![Structure](image) | 0.023 ± 0.008 |
| 9q        | ![Structure](image) | 0.176 ± 0.06  |

*IC50 values are reported as mean values of three or more determinations.*
in Scheme 2. While the oxetane derivative 3c was synthesized in a straightforward manner from oxetan-3-one (10) by reaction with butyllithium, compound 3d was obtained in a one pot procedure starting from 4-bromobutylbenzene (11), which was first converted into its lithium analogue and subsequently reacted with 10. The syntheses of alcohols 3e and 3p were accomplished according to literature procedures. The commercially available 2-methylpropanoic acid (12) was reacted with (2-bromoethyl)benzene using sodium hydride and in situ prepared lithium disopropylamine to afford the carboxylic acid 13. Lewis acid addition (boron trifluoride diethyl etherate) to a mixture of ethyl 4-hydroxybenzoate (14) and cyclohexene afforded the ester 15. Both intermediates 13 and 15 were submitted to lithium aluminum hydride reduction to furnish the desired alcohols 3e and 3p.

RESULT AND DISCUSSION

The main objectives of the present study were to (i) perform an extended structural exploration specifically tailored on the side chain of 2-methyl-4-oxo-3-oxetanylcarbamic acid ester derivatives, (ii) exploit this structural variability to devise a 3D QSAR model, and (iii) prospectively validate the model by synthesizing novel active compounds in line with the predictions.

SAR Study. Starting from previously identified compound 1, extensive SAR studies led to the discovery of the first single-digit nanomolar inhibitor of intracellular rat (and
human) NAAA activity. Here, a new series of 2-methyl-4-oxo-3-oxetanylcarbamoylacetic acid esters 9a–q (Table 1) were synthesized to verify additional features, which could help confirm or better define the structural determinants for NAAA activity. We therefore explored the effect on NAAA inhibition of different side chains, taking into account structural modifications, such as the introduction of heteroatoms, the length of the side-chain, and the disubstitution in α and β position with respect to the carbamic acid function. Finally, to further define the contribution of conformationally constrained moieties, more rigid structures based on the tridimensional shape of 2 were taken into consideration.

The potency of the new synthesized 2-methyl-4-oxo-3-oxetanylcarbamoylacetic acid esters 9a–q was evaluated by their ability to inhibit the hydrolysis of 10-cis-heptadecenoylthanolamide (an unnatural FAE) by native NAAA prepared from rat lungs. Median inhibitory concentration (IC$_{50}$) values obtained using the rat enzyme (r-NAAA) are reported in Table 1 and Table 4.

Previously described analogues (1, 2, and 16–29) and their corresponding r-NAAA inhibitory activity are reported in Table S1 (see Supporting Information).

The analysis of the IC$_{50}$ values reported in Table 1 together with those of the previously reported analogues (Table S1) allows some general considerations to be made. The introduction of a fluorine atom in the para position of the phenyl ring (9a, IC$_{50}$ = 0.24 μM) was detrimental for the activity, leading to a ~5-fold drop in potency compared to 1 (Table S1). Taking into consideration possible modifications on compound 22 (IC$_{50}$ = 0.76 μM, Table S1), a substitution of a methyl group with a more lipophilic chlorine atom (9b) resulted in an increased inhibitory potency (IC$_{50}$ = 0.28 μM), while the introduction of an oxetane moiety in α-position with respect to the carbamate (9c) turned out to be detrimental for activity (IC$_{50}$ = 3.76 μM). The same negative effect of a substitution in the β-position of the carbamate was observed in analogue 9d (IC$_{50}$ = 0.52 μM) compared to 1, therefore indicating a limited space in the region of the enzyme proximal to the carbamic function. A similar loss in activity was observed with the insertion of a gem-dimethyl substitution in the β-position as for compound 9e (IC$_{50}$ = 3.43 μM), which was 9-fold less active than the corresponding unsubstituted analogue 20 (IC$_{50}$ = 0.39 μM, Table S1).

Whereas the presence of a terminal cyclohexyl moiety in a four-carbon aliphatic linker (9f) retained approximately the same potency as for the previously reported five-carbon aliphatic linker analogue 28 (IC$_{50}$ = 0.013 μM, Table S1), a ~300-fold loss in activity was observed when the cyclohexyl moiety was directly connected to the carbamic acid function (9g, IC$_{50}$ = 5.42 μM).

The addition of a methylene unit to the aliphatic linker of 1, leading to a six-carbon atom alkyl chain, was reported to be detrimental for activity (21, IC$_{50}$ = 1.17 μM, Table S1), whereas the insertion of an additional carbon atom (9h) surprisingly restored the original activity (IC$_{50}$ = 0.039 μM). A short alkyl chain bearing a terminal p-methylphenyl group (9i, IC$_{50}$ = 1.22 μM) confirmed low micromolar activity as
previously observed for the phenethyl derivative (18, IC$_{50}$ = 1.22 μM, Table S1).

To further investigate and possibly confirm the importance of the rigidity of the side chain for potent enzyme inhibition, new β-lactones structurally related to 2 were designed and tested against r-NAAA. The substitution of the proximal phenyl ring with an acetylene functionality (9j), which retains the overall shape of the side chain of 2, led to a 3-fold drop in potency. The same variation in activity was observed when substituting the terminal phenyl ring with a thiophene moiety (9k, IC$_{50}$ = 0.022 μM), whereas the substitution with a cyclohexyl moiety (9l, IC$_{50}$ = 0.007 μM) resulted in a compound equally active to 2 (Table S1). To the best of our knowledge, compounds 2 and 9l are the most active, single-digit nanomolar NAAA inhibitors so far reported in literature.

We then evaluated the effect of small substituents at the para position of the biphenyl moiety. No significant variation in potency was observed for the p-fluro derivative 9m (IC$_{50}$ = 0.011 μM), while a methyl or trifluoromethyl group in the same position led to slightly less potent compounds (9n and 9o, IC$_{50}$ = 0.029 μM and IC$_{50}$ = 0.036 μM, respectively). Interestingly, increasing the flexibility of the side chain of compound 9l by insertion of an oxygen atom between the phenyl and cyclohexyl ring (9p) led to only a 3-fold decrease in activity (IC$_{50}$ = 0.023 μM). Finally, replacing the p-piperonyl ring with a more polar, fused bicyclic piperonyl moiety (9q) led to a 25-fold drop in potency.

**3D QSAR Model.** We assembled a training set encompassing 33 compounds: 17 new, purposely synthesized 2-methyl-4-oxo-3-oxetanylcarbamic acid esters derivatives (Table 1) and 16 analogues taken from a previous SAR study (see also the covalent docking studies and Figure S2 in Supporting Information). The 3D QSAR model was built using the APF approach, namely, a continuous 3D pharmacophoric potential implemented on a set of regularly spaced grids. This multicomponent 3D potential was generated solely by the training set compounds. Each individual contribution reflected preferences for various atomic properties at each point in space, and it was rescaled according to the compound’s experimental potency. Multiple ligands consistently displaying atoms with similar properties in the same location generated a strong pharmacophoric signal for these common features. In line with the original APF 3D QSAR implementation proposed by Totrov, we selected seven properties to be analyzed and assigned: charge, hydrogen bond donor propensity, hydrogen bond acceptor propensity, sp$^2$ carbon atom hybridization, and lipophilicity to encompass the classic pharmacophoric features, plus two more unconventional properties, namely, size and electropositivity/negativity, to take into account more subtle differences among substituents that would have been very difficult to capture otherwise.

First, the training set compounds were superimposed using, as template, the transition state model of compound 2, as previously obtained by quantum chemical calculations (see Experimental Section for details). In Figure 2A, the structural alignment of the training set compounds is reported. As expected, the β-lactone ring perfectly matched. Conversely, the conformational arrangement of the side chains captured the diversity of the training set and represented the truly informative core of our model. For each molecule, a seven-component property vector was evaluated and used to generate the 3D QSAR model. The optimal number of latent vectors to be used in the partial least squares (PLS) analysis was selected in order to optimize the overall squared correlation coefficient between the predicted and the observed activities ($R^2$) as well as the leave-one-out cross-validation results ($Q^2$) on the training set.

In Table 2, $R^2$ and $Q^2$ values as a function of the number of latent vectors are reported. Eventually, we decided to use six latent vectors meeting the generally accepted threshold of one latent vector for every five compounds employed in the training set. In this way, our model showed a good $R^2$ coefficient between the experimental pIC$_{50}$ (pIC$_{50exp}$) and the predicted pIC$_{50}$ (pIC$_{50pred}$) ($R^2$ = 0.98, see Table S1) and a good predictivity with a $Q^2$ = 0.76 (see Figure S1).

In Table 3, the experimental, predicted, and leave-one-out cross-validated pIC$_{50}$ values for the training set are reported. The structural implication of the generated 3D QSAR model can be visualized in terms of isopotential contour maps of the various APF components. For example, the lipophilicity field of the model was useful to pinpoint regions that react to the presence of a lipophilic moiety, enhancing or reducing the inhibitory potency. As already reported, the size and the shape of the lipophilic tail are key determinants for the inhibitory potency of the β-lactones compounds. In Figure 2C, only the regions in which a lipophilic moiety improved NAAA inhibition are reported. Interestingly, the structural implications of the proposed model are in agreement with those proposed by Solorzano and colleagues by means of mutagenesis and homology modeling studies (see also the covalent docking studies and Figure S2 in Supporting Information).

The shape of this lipophilic region suggests a preference for linear moieties in the carbamic acid esters for optimal enzyme recognition. The sp$^2$ hybridization atomic property field generated by the training set is reported in Figure 2D. In particular, the regions in which the presence of sp$^2$ hybridized atoms was detrimental for the inhibitory potency are shown. For example, a terminal cyclohexyl moiety in a four units aliphatic linker was preferred to a terminal phenyl ring, as confirmed by the drop in potency of 20 with respect to compound 9l. In Figure 2E, the regions in which large substituents led to a drop in the inhibitory potency are reported. This seems to suggest that the access to the lipophilic subpocket is quite tight and, as such, ill-suited to lodge bulky substituents like the tert-butyl group of compound 16.

**Model Validation.** The 3D QSAR model was then challenged by designing, synthesizing, and testing novel compounds. An in silico library of 200 β-lactones derivatives was assembled starting from a list of commercially available alcohols. Their activity data were predicted through the 3D

![Table 2. Squared Correlation Coefficients ($R^2$) and Leave-One-Out Squared Correlation Coefficients ($Q^2$) According to the Number of Latent Vectors](image-url)
Table 3. Experimental, Predicted, and Leave-One-Out Cross-Validated pIC₅₀ Values for the Training Set Compounds

| Compounds | pIC₅₀ (exp) | pIC₅₀ (pred) | pIC₅₀ (LOOO) |
|-----------|------------|-------------|--------------|
| 9l        | 8.18       | 8.28        | 8.37         |
| 2         | 8.14       | 7.63        | 7.50         |
| 9m        | 7.96       | 7.74        | 7.65         |
| 9j        | 7.81       | 7.43        | 6.39         |
| 28        | 7.79       | 7.94        | 7.99         |
| 9k        | 7.68       | 7.58        | 7.50         |
| 9f        | 7.67       | 7.25        | 7.05         |
| 9p        | 7.64       | 7.69        | 8.00         |
| 9n        | 7.62       | 7.73        | 7.77         |
| 9o        | 7.56       | 7.68        | 7.71         |
| 25        | 7.52       | 7.38        | 7.19         |
| 9h        | 7.41       | 7.74        | 7.50         |
| 24        | 7.35       | 7.09        | 6.94         |
| 26        | 7.29       | 7.24        | 6.65         |
| 1         | 7.28       | 7.04        | 6.97         |
| 19        | 7.07       | 7.34        | 7.15         |
| 27        | 6.83       | 7.13        | 7.02         |
| 9q        | 6.75       | 6.97        | 6.93         |
| 9a        | 6.62       | 7.16        | 7.30         |
| 23        | 6.57       | 6.61        | 6.58         |
| 9b        | 6.54       | 6.43        | 6.23         |
| 29        | 6.50       | 6.27        | 6.24         |
| 20        | 6.41       | 6.30        | 6.37         |
| 9d        | 6.28       | 6.21        | 6.30         |
| 22        | 6.17       | 6.19        | 6.16         |
| 21        | 5.93       | 6.08        | 7.01         |
| 9i        | 5.91       | 6.06        | 6.49         |
| 17        | 5.68       | 5.60        | 5.12         |
| 18        | 5.60       | 5.77        | 6.06         |
| 9e        | 5.46       | 5.80        | 6.75         |
| 9c        | 5.42       | 5.39        | 5.55         |
| 9g        | 5.27       | 5.00        | 4.92         |
| 16        | 5.20       | 5.35        | 5.48         |

The resulting 23 compounds along with pIC₅₀pred values equal to 7.45 were selected for visual inspection. The structures of these compounds not only retrospectively explained the SAR in the lipophilic region of the NAAA binding pocket but could also be used prospectively to design novel NAAA inhibitors. In this regard, it should be pointed out that a hopping exercise aimed at identifying completely novel chemical scaffolds was outside the scope of the present 3D QSAR study. Furthermore, given the robustness of the present model, the compounds could be used not only retrospectively to explain the SAR in the lipophilic region of the NAAA binding pocket but could also be used prospectively to design novel NAAA inhibitors.

In Table S2 (see Supporting Information). Eventually, we selected five new derivatives for chemical synthesis and biological evaluation. The selection was based on the following criteria: (i) good predicted activity (rank), (ii) chemical feasibility, (iii) actual availability of the alcohol substituent, (iv) introduction of new chemical features with respect to already known β-lactones NAAA inhibitors. The compounds (9r–v) were synthesized according to Scheme 1, and their rank and activity data are reported in Table 4. For comparison, in Table 3 (see Supporting Information) we also report the structures of the five least active compounds as predicted by the model.

It clearly emerges the quite good agreement between predicted and observed pIC₅₀ values, further highlighting the robustness of the present 3D QSAR model. Indeed, our model not only retrospectively explained the SAR in the lipophilic region of the NAAA binding pocket but could also be used prospectively to design novel NAAA inhibitors.

In Figure 3, the novel compounds are reported. The new molecules showed a good superimposition with respect to the biphenyl moiety of 2. In particular, the APF lipophilic contribution of these molecules matched well with the ideal lipophilic shape and size of the side chain obtained by our model. These compounds were not profoundly different from those used in the training set to devise the model. In this regard, it should be pointed out that a hopping exercise aimed at identifying completely novel chemical scaffolds was outside the scope of the present 3D QSAR study. Furthermore, given the robustness of the present model, the compounds could be used not only retrospectively to explain the SAR in the lipophilic region of the NAAA binding pocket but could also be used prospectively to design novel NAAA inhibitors.

Conclusions

In this study, we investigated a series of NAAA inhibitors in terms of three-dimensional quantitative structure–activity relationship (3D QSAR), and to the best of our knowledge,
this represents the first 3D QSAR model in this field. To this objective, we first expanded the SAR around the 2-methyl-4-oxo-3-oxetanylcarbamic acid esters as NAAA inhibitors, hence investigating the role of previously unexplored structural features on the inhibition of this enzyme. In particular, we further characterized the size and shape of the side chain linked to the carbamic acid function, features that were extensively investigated here for the first time. This eventually led to the discovery of a quite potent NAAA inhibitor, compound 9l, endowed with single-digit nanomolar activity against this enzyme. In addition, this allowed us to generate a consistent series of analogues that was then utilized to build the 3D QSAR model. The statistical model was obtained using the innovative APF methodology, where discrete features of classic pharmacophores are replaced by continuous, regularly spaced three-dimensional lattices. The 3D QSAR model showed quite robust statistics and good consistency with docking experiments carried out using a homology built model of NAAA. These promising results prompted us to generate a small set of novel NAAA inhibitors, which were purposely designed and predicted using the 3D QSAR model. The fairly good agreement between theoretical and experimental IC50 values for the new 2-methyl-4-oxo-3-oxetanylcarbamic acid esters highlights the good descriptive and predictive power of the

Table 4. Inhibitory Potencies (IC50) of QSAR Model Predicted Compounds 9r–v on Rat NAAA Activitya

| Compounds | Structure | pIC50pred | Rank | IC50 (μM) ± SD | pIC50exp |
|-----------|-----------|-----------|------|---------------|---------|
| 9r        | ![Structure](image1.png) | 7.61 | 8th | 0.015 ± 0.005 | 7.82 |
| 9s        | ![Structure](image2.png) | 7.60 | 9th | 0.022 ± 0.005 | 7.66 |
| 9t        | ![Structure](image3.png) | 7.55 | 11th | 0.028 ± 0.003 | 7.55 |
| 9u        | ![Structure](image4.png) | 7.46 | 21th | 0.013 ± 0.004 | 7.89 |
| 9v        | ![Structure](image5.png) | 7.45 | 23th | 0.077 ± 0.029 | 7.11 |

*aIC50 values are reported as mean values of three or more determinations.

Figure 3. Molecules selected for the validation of the 3D QSAR model are shown in yellow, as licorice, superimposed to 2 in pink: (A) molecule 9r, (B) molecule 9s, (C) molecule 9t, (D) molecule 9v, (E) molecule 9u.
model. From a more general standpoint in the field of NAA inhibitors, the present study shows that an extended and lipophilic moiety could be the ideal pharmacophoric function for substituting the side chain of covalent inhibitors of this enzyme. In conclusion, the present SAR exploration and the 3D QSAR model can help the future design of novel NAA inhibitors, which can find therapeutic applications in the treatment of pain and inflammation.

**EXPERIMENTAL SECTION**

**a. Chemicals, Materials, and Methods.** All the commercially available reagents and solvents were used as purchased from vendors without further purification. Dry solvents (THF, EtO, 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 (Gumbshall II) 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. NMR experiments were run on a Bruker Avance III 400 system (400.13 MHz for 1H and 100.62 MHz for 13C), equipped with a BBI probe and Z-gradients. Spectra were acquired at 300 K, using deuterated dimethylsulfoxide (DMSO-d₆) or deuterated chloroform (CDCl₃) as solvents. UPLC was run on a Waters AutoPurifcation System consisting of a SQD cation system consisting of a 3100 electrospray ionization interface and a photodiode array detector. PDA (single quadrupole detector) mass spectrometer equipped with an 

**Preparation of Carbamates 9r–v (Step 3).** To a stirred mixture of threonine derivatives 8r–v (1.0 equiv) in dry CH₂Cl₂ at 0 °C and under nitrogen atmosphere, EtN (5.0 equiv) and subsequently TBTr (1.2 equiv) were added. 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 100:0) to afford pure 9r–v.

**Dec-2-ylinyl(25,3Rr)-2-methyl-4-o xo oxetan-3-yl)carbamate (9v).** The reaction was carried out following the general procedure (step 3) employing (2S,3R)-2-(4-bromo-2-thienyl)phenyl)methyl-carbamate (9r). The reaction was carried out following the general procedure (step 3) employing (2S,3R)-2-(4-bromo-2-thienyl)phenyl)methyl-carbamate (9s). The reaction was carried out following the general procedure (step 3) employing (2S,3R)-2-(4-bromo-2-thienyl)phenyl)methyl-carbamate (9t).

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procedure (step 3) employing (2R,3S)-2-[(2R)-2-(5-nitro-2-fur-2-yl)-5-(3-pyridyl)pentanamido]-3-hydroxybutanoic acid (8v) (0.35 g, 1.18 mmol), dry CH2Cl2 (35 mL), Et3N (0.49 mL, 3.56 mmol), and TBDU (0.46 g, 1.42 mmol) to give 9v. White solid; yield, 26% (0.090 g).13 [13C]D-15.83 (c 0.1, CHCl3), MS (ESI) m/z: 280 [M − H]+, 282 [M − H]−.1H NMR (400 MHz, DMSO-d6); δ 0.83–0.88 (m, 3H), 1.20–1.37 (m, 12H), 1.38–1.48 (m, 2H), 2.17–2.24 (m, 1H), 4.65 (d, J = 2.02 Hz, 1H), 4.67 (d, J = 2.57 Hz, 1H), 4.86 (dq, J = 6.30 Hz, 1H), 5.43 (dd, J = 6.07, 9.41 Hz, 1H), 8.38 (dd, J = 9.41 Hz, 1H).13C NMR (101 MHz, DMSO-d6); δ C 13.92, 14.48, 17.87, 22.03, 27.88, 28.13, 31.14, 52.98, 59.92, 74.57, 75.11, 87.12, 99.50, 154.98, 169.62.

b. Pharmacology. Rat NAAA in Vitro Assay. For a detailed description of in vitro rat-NAAA assay, see Supporting Information.
c. Computational Methods. Compounds Preparation. For each compound, we generated a two-dimensional representation in which bond orders, tautomeric forms, stereochemistry, hydrogen atoms, and protonation states were manually assigned. Conversion from a two- to a three-dimensional representation was automatically performed by the ICM converting procedure (ICM 3.7, Molsoft LLC, San Diego, CA).41 Cartesian coordinates were translated into internal coordinates, and each molecule was assigned the MMFF force field atom types and charges.

Three-Dimensional Alignment of the Training Set Molecules. We generated our initial structural alignment starting from a rigid template, namely, the structure of the transition state model of α-amino-β-lactamase. Compound 2 was converted to a transition state as modeled as follows: the 2-methyl-4-oxo-3-oxetanylcarbamic acid ester region was assigned the optimized coordinates that we previously identified: the 2-methyl-4-oxo-3-oxetanylcarbamic acid ester region was validated to provide a simulation of suitable length. In order to identify a plausible starting conformation of the molecule, we performed an energy optimization on the three torsional angles describing the orientation of the biphenyl moiety by means of the biased probability Monte Carlo (BPMC) stochastic optimizer as implemented in ICM. The vicinity threshold, namely, the root-mean-square deviation threshold for the rotatable torsions, was set equal to 15 degrees. Having used our lower threshold for activity equal to 7.45, corresponding to the potency of the less active biphenyl derivative (9o), we then evaluated all the compounds with a predicted IC50 greater than a prespecified threshold. Having used 2 as rigid template for the alignment, we set our lower threshold for activity equal to 9.45.

3D QSAR Model Validation. We generated a virtual library of β-lactones molecules through the Markush Combinatorial Library tool of ICM using a library of commercially available alcohols reacting with the β-lactone amino group. In this way we automatically created a list of 200 new compounds: their activity data were then derived through the 3D QSAR model. The molecules were then ranked according to their predicted pIC50. We then selected all the compounds with a predicted IC50 greater than a prespecified threshold. Having used 2 as rigid template for the alignment, we set our lower threshold for activity equal to 7.45, corresponding to the potency of the less active biphenyl-bearing derivative (9o). Within the selected 23 molecules, only five compounds were then chosen to be synthesized for the subsequent model validation. The choice of these compounds was based on the following criteria: chemical feasibility, good predicted activity data, interesting new chemical features that are not kept by the available known β-lactones molecules.

ASSOCIATED CONTENT

Supporting Information
Detailed experimental procedures, analytical and spectroscopic data of intermediate and final compounds, 1H and 13C NMR spectra of compounds 9r−v, in vitro pharmacology procedures, and additional computational details on the 3D QSAR predicted activity. 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): D. Piomelli, T. Bandiera, F. Bertozzi, and S. Ponzano are inventors in the patent application WO2013078430 protecting the class of compounds disclosed in this paper.

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ABBREVIATIONS USED
FAE, fatty acid ethanolamide; OEA, oleoylethanolamide; PEA, palmitoylethanolamide; NAAA, N-acylethanolamide acid amide; FAAH, fatty acid amide hydrolase; CBAH, conjugated bile acid hydrolase; PPAR-α, peroxisome proliferator-activated receptor α; (S)-OOPP, (S)-N-(3-oxo-3-exetanyl)-3-phenylpropionamide; DPC, di-2-pyridyl carbonate; DMAP, 4-dimethylaminopyridine; APF, atomic property field; PLS, partial least squares.
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