Journal of Medicinal Chemistry

Article

pubs.acs.org/jmc

Discovery of a New Class of Highly Potent Inhibitors of Acid Ceramidase: Synthesis and Structure−Activity Relationship (SAR)

Daniela Pizzirani,[†,‡] Chiara Pagliuca,[†,‡] Natalia Realini,[†] Davide Branduardi,[⊥] Giovanni Bottegoni,[†] Marco Mor,[§] Fabio Bertozzi,[†] Rita Scarpelli,[†] Daniele Piomelli,*[,†,∥] and Tiziano Bandiera*[,†]

[†]Drug Discovery and Development, Fondazione Istituto Italiano di Tecnologia, Via Morego 30, I-16163 Genova, Italy

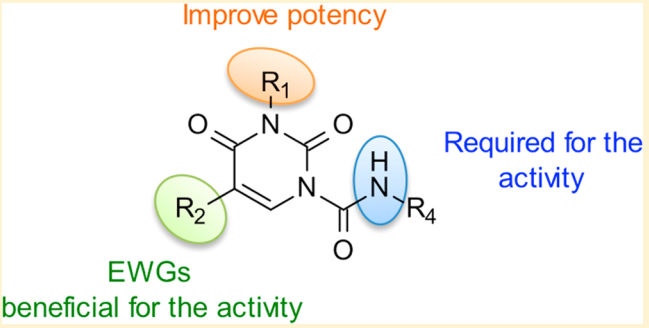
[⊥]Theoretical Molecular Biophysics Group, Max Planck Institute for Biophysics, Max-von-Laue Strasse 3, 60438, Frankfurt am Main, Germany

[§]Dipartimento di Farmacia, Università degli Studi di Parma, Viale delle Scienze 27/A, I-43124 Parma, Italy

[∥]Department of Anatomy and Neurobiology, University of California—Irvine, Irvine, California 92697-4625, United States

Supporting Information

ABSTRACT: Acid ceramidase (AC) is an intracellular cysteine amidase that catalyzes the hydrolysis of the lipid messenger ceramide. By regulating ceramide levels in cells, AC may contribute to the regulation of cancer cell proliferation and senescence and to the response to cancer therapy. We recently identified the antitumoral agent carmofur (**4a**) as the first nanomolar inhibitor of intracellular AC activity (rat AC, IC_{50} = 0.029 μM). In the present work, we expanded our initial structure−activity relationship (SAR) studies around **4a** by synthesizing and testing a series of 2,4-dioxopyrimidine-1-carboxamides. Our investigations provided a first elucidation of the structural features of uracil derivatives that are critical for AC inhibition and led us to identify the first single-digit nanomolar inhibitors of this enzyme. The present results confirm that substituted 2,4-dioxopyrimidine-1-carboxamides are a novel class of potent inhibitors of AC. Selected compounds of this class may represent useful probes to further characterize the functional roles of AC.



INTRODUCTION

Sphingosine-containing lipids are essential structural components of cell membranes that also serve important signaling functions in cell migration, cell recognition, and inflammation, as well as in the control of cell growth and differentiation.[1] Among the various groups of sphingolipids, the ceramides, amides of sphingosine with long-chain fatty acids, have attracted attention for their roles in the replication and differentiation of normal and neoplastic cells.[2] Stress-related signals such as tumor necrosis factor α (TNF-α) stimulate the production of ceramides in cells, which can cause in turn replicative senescence and apoptosis.[3] Thus, enzymes involved in the biological deactivation of ceramides have emerged as potential targets for therapeutic manipulation by small molecules.[4]

Acid ceramidase (AC) is a ubiquitous cysteine amidase that operates within the acidic milieu of the lysosome to catalyze the hydrolysis of ceramides into sphingosine and fatty acid. By regulating the intracellular levels of ceramides, AC may influence the survival, growth, and death of tumor cells.[5] Consistent with this view, cells that express abnormally high levels of AC are more resistant than normal cells to pharmacological induction of programmed cell death.[6] Indeed, several types of cancer (including prostate, head and neck, colon and melanoma) overexpress AC, which is suggestive of a role for this enzyme in the resistance to radiotherapy and chemotherapy.[7]

Although several AC inhibitors have been described in the literature, potent small-molecule compounds capable of inhibiting this enzyme in vivo are still needed.[4,7b,8] The majority of compounds reported so far, including oleoylethanolamide (**1**, OEA), B-13 (**2**), D-MAPP (**3**) (Figure 1),[7d,9] and their derivatives, are structural analogues of ceramide and therefore suffer from several limitations, including low potency and insufficient druglikeness. For example, pharmacological inhibition of AC with **1** has been related to sensitization of hepatoma cells to daunorubicin-induced cell death, but the low potency ($K_i \approx 500$ μM) and rapid metabolic degradation of this compound preclude its therapeutic use.[10] Compound **2** inhibits AC activity with a median inhibitory concentration (IC_{50}) of approximately 10 μM, causes apoptosis in several human cancer cell lines, and sensitizes prostate tumors to the effects of radiation.[11] Compound **3**, which is structurally related to **2**, inhibits both AC and neutral ceramidase activities and triggers apoptosis in squamous carcinoma cells.[12] These results suggest

Received: December 20, 2012

   dx.doi.org/10.1021/jm301879g | J. Med. Chem. XXXX, XXX, XXX−XXX

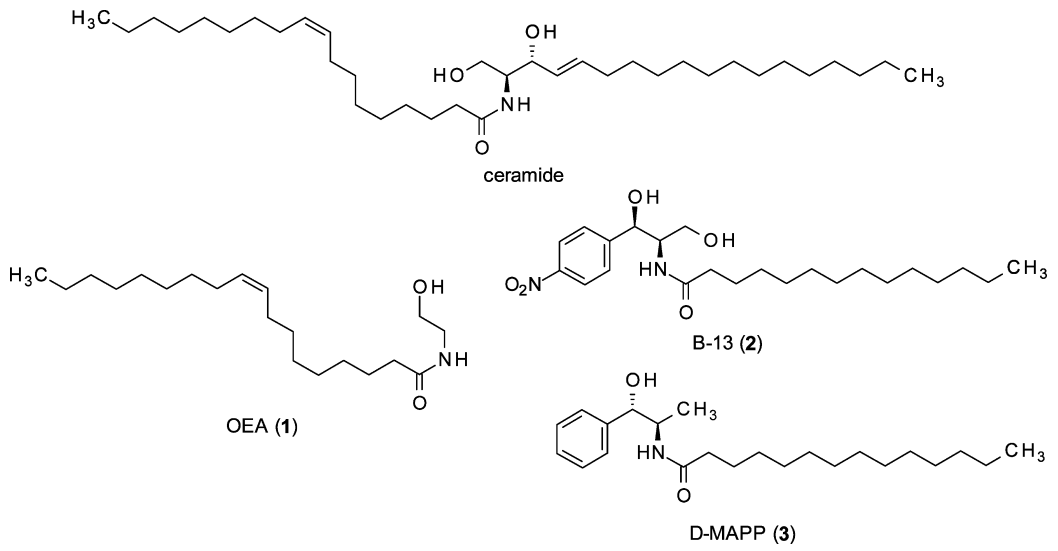


Figure 1. Structures of a representative ceramide and various AC inhibitors (**1**–**3**) reported in the literature.

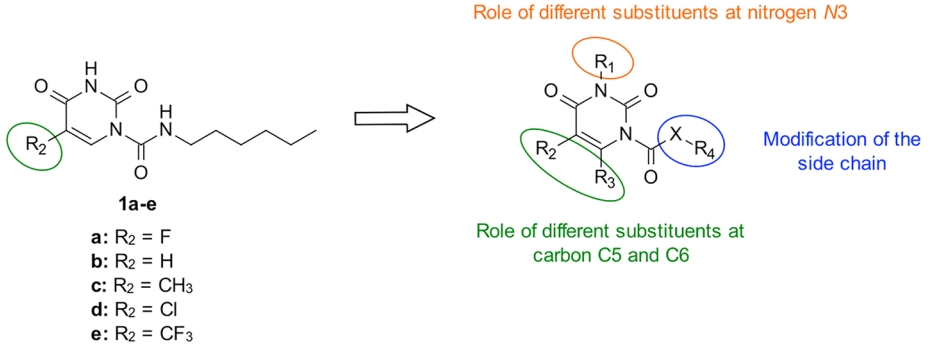
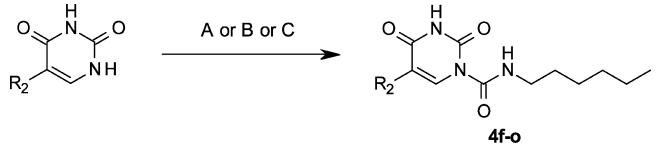


Figure 2. Illustration of the design strategy for structure–activity relationship studies.

Table 1. Reaction Conditions for the Preparation of Compounds 4f–o[a]



entry	R_2	method	product (yield, %)	entry	R_2	method	product (yield, %)
1	CH_2CH_3	B	**4f** (16)	6	*N*-methylpiperazine	A	**4k** (17)
2	CH_2OH	C	**4g** (27)	7	Ph	A	**4l** (49)
3	OCH_3	B	**4h** (56)	8	I	A	**4m** (19)
4	$N(CH_3)Bn$	A	**4i** (75)	9	Br	A	**4n** (62)
5	morpholine	A	**4j** (60)	10	CN	C	**4o** (13)

[a]Method A: isocyanate, DMAP, pyridine, room temperature, 12 h. Method B: isocyanate, DMSO, 50 °C, 4–12 h. Method C: isocyanate, pyridine, microwave, 100 °C, 10 min.

that AC blockade may promote or facilitate cancer cell death, especially when combined with the use of other chemotherapeutic agents. The findings also highlight the need to discover more potent and druglike compounds, which might be used to investigate further the usefulness of AC as a target for chemosensitizing drugs.

We recently reported that the 5-fluorouracil (5-FU) releasing drug carmofur (**4a**, Figure 2) inhibits AC activity with nanomolar potency (IC_{50} for rat AC of 0.029 μM) and increases intracellular ceramide levels both in vitro and in vivo.[13] These effects are independent of carmofur's ability to generate 5-FU and are accompanied by a marked enhancement in the antiproliferative actions of other antitumoral agents. Preliminary structure–activity relationship (SAR) studies revealed that replacement of the fluorine atom of carmofur with selected chemical groups with different stereoelectronic properties leads to molecules that retain the ability to inhibit AC with high potency (e.g., **4e**, IC_{50} = 0.012 μM).[13]

In the present report, we expanded our SAR studies around carmofur with the objective of identifying other potent small-molecule inhibitors for this enzyme. We synthesized a series of 2,4-dioxopyrimidine-1-carboxamides to explore the role of

dx.doi.org/10.1021/jm301879g | J. Med. Chem. XXXX, XXX, XXX–XXX

Scheme 1. Synthesis of 5-Substituted Uracils 5i–l

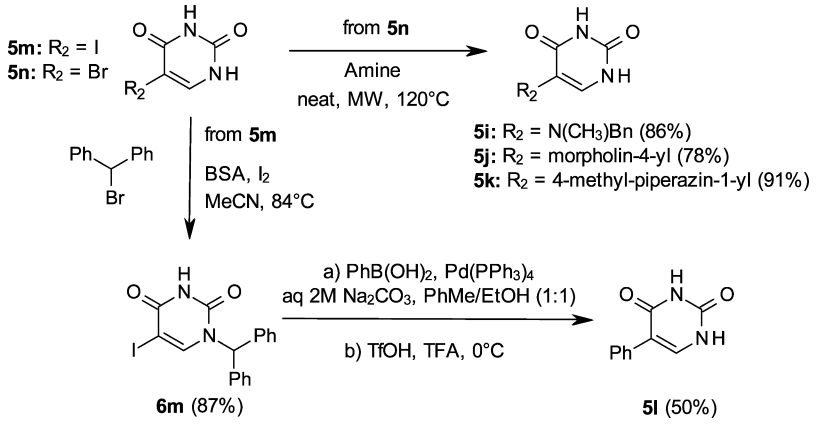
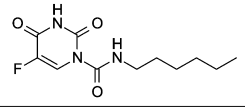
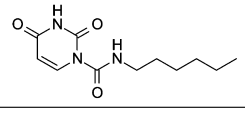
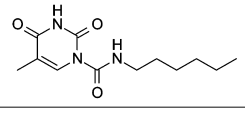
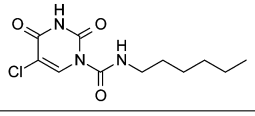
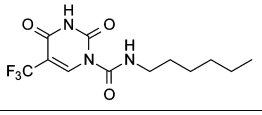
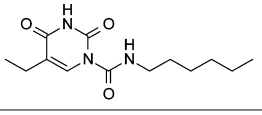
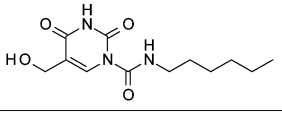
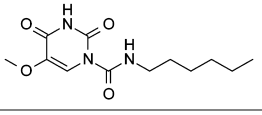
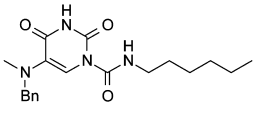
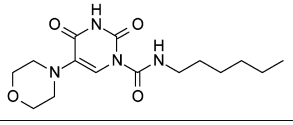
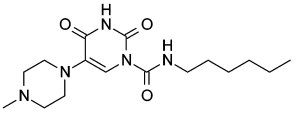
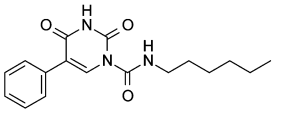
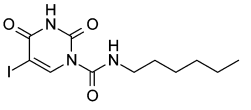
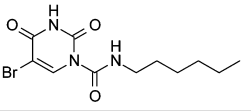
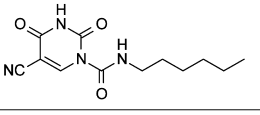
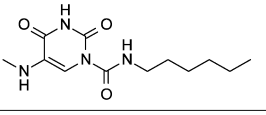
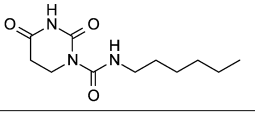
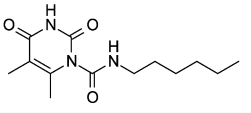
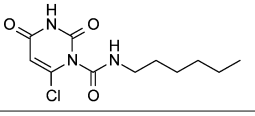


Table 2. Inhibitory Potencies (IC_{50}) of Compounds on Rat AC Activity[a]

Compounds	Structure	IC_{50} (μM) ± S.E.M
4a		0.029 ± 0.005[b]
4b		0.426 ± 0.104[b]
4c		1.46 ± 0.125[b]
4d		0.067 ± 0.005[b]
4e		0.012 ± 0.002[b]
4f		0.733 ± 0.054
4g		0.417 ± 0.024
4h		1.1 ± 0.03
4i		5.5 ± 0.45
4j		0.875 ± 0.073

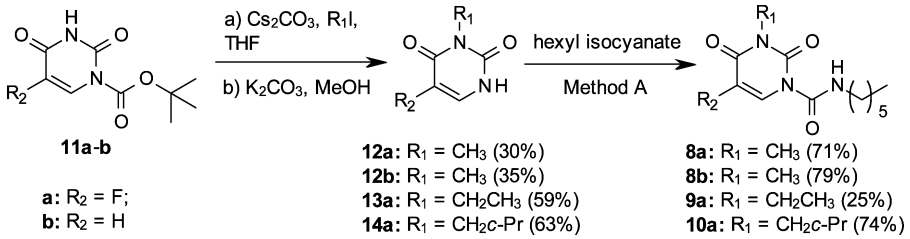
Compounds	Structure	IC_{50} (μM) ± S.E.M
4k		1.4 ± 0.013
4l		0.177 ± 0.01
4m		0.147 ± 0.02
4n		0.051 ± 0.008
4o		unstable
4p		3.4 ± 0.45
7		No inhibition
26		unstable
30		unstable

[a]IC_{50} values are reported as mean values of three or more determinations. [b]IC_{50} values from ref 13.

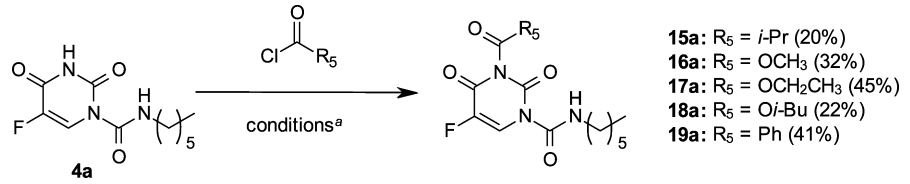
different substituents at carbons C5 and C6 and at nitrogen N3 of the uracil ring, as well as the effect of the alkyl side chain on AC inhibition (Figure 2). In addition, we conducted a quantum-mechanical study on the new molecules to explore

dx.doi.org/10.1021/jm301879g | J. Med. Chem. XXXX, XXX, XXX–XXX

Scheme 2. Syntheses of 3-Alkyluracil Derivatives 8a,b, 9a−10a

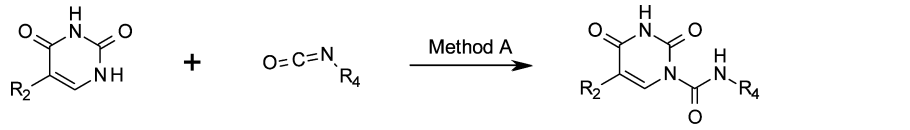


Scheme 3. Synthetic Pathways for the Preparation of Uracil Derivatives 15a−19a[a]



[a]**15a**: TEA, DCM. **16a−17a**: Py. **18a−19a**: Py, DCM.

Scheme 4. Synthesis of 2,4-Dioxopyrimidine-1-carboxamides 20b−24b, 23a, and 23e



the effect of uracil substitution on orbital stabilization energies and inhibitory potency.

CHEMISTRY

We explored and optimized various methods reported in the literature for the synthesis of ureas to access differently substituted 2,4-dioxopyrimidine-1-carboxamides.[14] The preparation of most 2,4-dioxopyrimidine-1-carboxamide derivatives was accomplished by coupling differently substituted uracils with alkyl isocyanates. Urea formation occurred regioselectively at position N1 under all conditions employed. Coupling reactions were conducted in pyridine at room temperature with a stoichiometric amount of DMAP (method A), in DMSO at 50 °C (method B), or in pyridine under microwave irradiation at 100 °C (method C). Dimethylaminopyridine was necessary for acylation reactions that proceeded incompletely or did not occur in pyridine or DMSO alone.[14b]

We first focused on the synthesis of carboxamide derivatives differently substituted at position 5. Table 1 shows the reaction conditions adopted for the coupling of 5-substituted uracils with *n*-hexyl isocyanate to afford the compound series **4f−o**. Non-commercially available uracils such as 5-(benzyl(methyl)-amino)uracil (**5i**), 5-(morpholin-4-yl)uracil (**5j**), 5-(4-methyl-piperazin-1-yl)uracil (**5k**), and 5-phenyluracil (**5l**) were prepared as described in Scheme 1. Compounds **5i−k** were obtained by nucleophilic substitution reaction of the appropriate amine on 5-bromouracil (**5n**) under microwave irradiation at 120 °C.[15] The synthesis of 5-phenyl-1*H*-pyrimidine (**5l**) was accomplished in three steps starting from 5-iodouracil (**5m**). This compound was transformed into the *N*1-benzhydryl derivative (**6m**) by treatment with benzhydryl bromide in the presence of bis(trimethylsilyl)acetamide (BSA) and a catalytic amount of iodine (I_2).[16] Subsequent reaction with phenylboronic acid under standard Suzuki coupling conditions, followed by removal of the protecting group with 10% triflic acid solution in TFA, afforded the desired 5-phenyluracil (**5l**) in good yield.

N-Hexyl-5-methylamino-2,4-dioxopyrimidine-1-carboxamide (**4p**, Table 2, Scheme S1) and *N*-hexyl-2,4-dioxohexahydropyrimidine-1-carboxamide (**7**, Table 2, Scheme S2) were obtained by catalytic hydrogenation from **4i** and **4b**, respectively. The N3-substituted-1-carboxamide derivatives **8a,b**, **9a−10a** were prepared following a three-step sequence starting from *N*1-Boc uracils **11a,b** (Scheme 2).[17] These compounds were first reacted with the appropriate alkyl iodides in the presence of cesium carbonate to give N3-substituted 2,4-dioxopyrimidine-1-carboxylates. The *tert*-butyloxycarbonyl group was then removed with potassium carbonate in methanol to afford 3-alkyluracils **12a,b**, **13a−14a** which were then converted into the corresponding carboxamides **8a,b**, **9a−10a** following method A. Syntheses of N3-substituted uracils **15a−19a** were accomplished with minor modifications of literature procedures, as reported in Scheme 3, by direct N3-acylation of *N*1-carboxamide derivatives. No decomposition of the ureidic function occurred in agreement with what observed with *N*1-alkoxycarbonyluracils.[18] Compound **15a** was prepared starting from carmofur (**4a**) by reaction with isobutyryl chloride in the presence of triethylamine. The carbamoyl derivatives **16a−17a**

dx.doi.org/10.1021/jm301879g | J. Med. Chem. XXXX, XXX, XXX−XXX

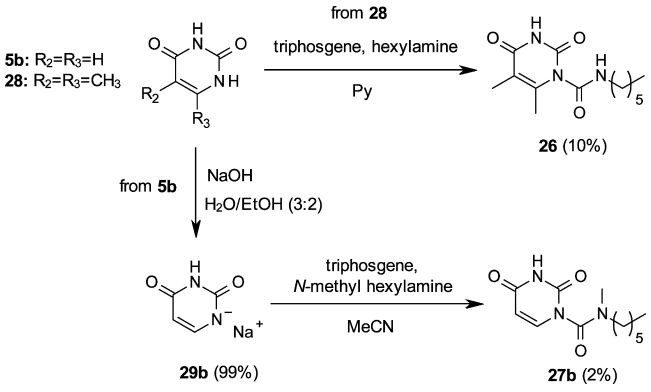
were obtained by treatment of **4a** with the appropriate chloroformate in the presence of pyridine as base. The syntheses of 3-isobutyl carboxylate **18a** and 3-benzoyluracil derivative **19a** were carried out by treatment of **4a** with isobutyl chloroformate and benzoyl chloride in pyridine as solvent and base.

The small series of compounds (**20b**−**24b**, **23a**, and **23e**) bearing *N*1-carboxamide alkyl chains of different length was prepared as shown in Scheme 4. Differently substituted uracils (**5a**,**b**, **5e**) were coupled with various alkyl isocyanates following method A to afford the desired carboxamides in good yields.

Derivative **23a** was functionalized at position N3 by reaction with methyl chloroformate, as previously described for the preparation of **16a** (Scheme 3), to yield methyl 3-(octylcarbamoyl)-2,6-dioxopyrimidine-1-carboxylate (**25a**, Table 4, Scheme S3).

An alternative synthetic procedure to access 1-carbamoyluracils was adopted for compounds **26** and **27b** (Scheme 5). The

Scheme 5. Synthesis of 5,6-Dimethyluracil Derivative 26 and *N*-Methylurea 27b



5,6-dimethyl substituted derivative **26** was obtained by reaction of 5,6-dimethyluracil (**28**) with triphosgene followed by treatment in situ with hexylamine. The synthesis of the *N*-methylurea **27b** was accomplished with a similar procedure starting from the sodium salt **29b** of the uracil **5b** (Scheme 5).

In the characterization process of the final 2,4-dioxopyrimidine-1-carboxamide derivatives, we found that few of them showed limited stability in DMSO-d_6 solution (Table S1), slowly releasing the corresponding uracil.[19] In addition, we observed that *N*-hexyl-2,4-dioxo-5,6-dimethylpyrimidine-1-carboxamide (**26**) and *N*-hexyl-2,4-dioxo-6-chloropyrimidine-1-carboxamide (**30**, Scheme S4) decomposed both in solution and as powder, revealing a marked chemical instability.

RESULTS AND DISCUSSION

The objective of the present study was to investigate substituted 2,4-dioxopyrimidine-1-carboxamides as a novel class of AC inhibitors. A preliminary SAR study on carmofur analogues (**4b**−**e**, Table 2) bearing substituents with different stereoelectronic properties at position 5 had shown that an electron-withdrawing group is crucial to achieve potent inhibition of AC activity. Accordingly, replacement of fluorine with electron-withdrawing substituents, such as chlorine and trifluoromethyl, led to the identification of two novel double-digit nanomolar AC inhibitors (**4d**,**e**). The results obtained with this small set of compounds suggested that 2,4-dioxopyrimidine-1-carboxamide might provide a promising scaffold for the discovery of potent AC inhibitors. Encouraged by these results, we decided to expand this series of compounds to elucidate the structural features that may be critical for AC inhibition.

The new substituted 2,4-dioxopyrimidine-1-carboxamides were tested for their ability to inhibit the hydrolysis of *N*-lauroylceramide by recombinant rat AC (r-AC). IC_{50} values are reported in Tables 2−4.

The limited stability of some 2,4-dioxopyrimidine-1-carboxamide derivatives in DMSO-d_6 prompted us to ask first whether the compounds were sufficiently stable under the conditions of our assay to obtain meaningful IC_{50} values. A set of molecules representative of the substitution pattern explored in our SAR analysis was selected and tested for stability in buffer over the 30 min period of the assay (Table S2).[20] Most compounds tested were stable enough to be included in the study except **4o**, which was extensively hydrolyzed (22% of the initial amount remained after 30 min) and was not further investigated. The 6-substituted derivatives **26** and **30** were also excluded because of their chemical instability.

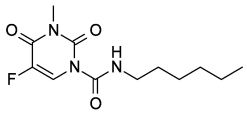
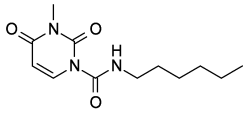
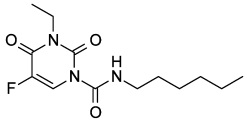
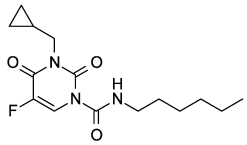
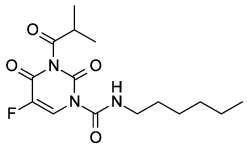
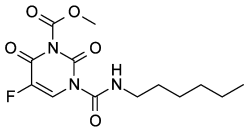
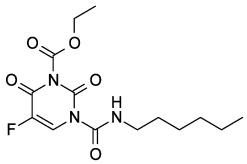
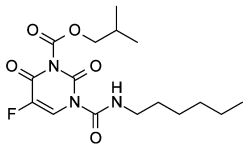
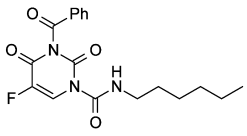
We initially focused our attention on substitutions at position 5 to expand the initial set of derivatives **4a**−**e** (Table 2). In particular, the potency of **4e** (IC_{50} = 0.012 μM) prompted us to synthesize the 5-iodo, 5-bromo, and 5-cyano derivatives (**4m**−**o**) in order to further investigate the effect of electron-withdrawing groups at that position. The introduction of iodine led to a 5-fold decrease in activity (IC_{50} = 0.147 μM) compared to carmofur (**4a**), while the 5-bromo derivative was equipotent to the 5-chloro-substituted carboxamide **4d**, with an IC_{50} of 0.051 μM. Next, we introduced a set of electron-donating groups at position 5 of the uracil ring. The compounds obtained (**4f**−**h**, **4p**) were 14- to 100-fold less potent than carmofur (**4a**). Replacement of fluorine with an alkyl group was detrimental for activity. The 5-methyl derivative **4c** (IC_{50} = 1.46 μM) showed a 50-fold decrease in potency compared to carmofur, whereas a less pronounced decrease was observed with the 5-ethyl analogue **4f** (IC_{50} = 0.733 μM) and the 5-hydroxymethyl derivative **4g** (IC_{50} = 0.417 μM). The introduction of substituents containing a heteroatom directly bound to the uracil ring led to a considerable loss in potency. The 5-methoxy derivative **4h** showed low micromolar AC inhibition (IC_{50} = 1.1 μM), as did the *N*-methylamino analogue **4p** (IC_{50} = 3.4 μM). Replacing the *N*-methylamino group with an *N*-methylbenzylamino moiety led to a further decrease in potency (**4i**, IC_{50} = 5.5 μM), probably due to increased steric hindrance. We also introduced heterocyclic and aromatic rings at position 5. Consistent with the data obtained with analogues bearing a heteroatom at position 5, introduction of morpholine or *N*-methylpiperazine caused a decrease in potency, albeit less pronounced than that observed with the *N*-methylamino derivative **4p**, as compounds **4j** and **4k** yielded IC_{50} values of 0.875 and 1.4 μM, respectively. Surprisingly, introduction of a phenyl ring led to the most potent compound within this small series (**4l**, IC_{50} = 0.177 μM), indicating that a planar group is well accepted even if it is sterically demanding.

To complete the SAR study around positions 5 and 6, we examined the role of the C5−C6 double bond. Compound **7**, the dihydro derivative of **4b**, showed no inhibitory activity toward AC, highlighting the functional importance of a fully conjugated 2,4-dioxopyrimidine-1-carboxamide system.

As the next step in our SAR study, we investigated the effect of substituents at position N3 of the uracil ring introducing alkyl, acyl, and carbamoyl groups at this position (Table 3). N3-

dx.doi.org/10.1021/jm301879g | J. Med. Chem. XXXX, XXX, XXX−XXX

Table 3. Inhibitory Potencies (IC_{50}) of Compounds on Rat AC Activity[a]

Compounds	Structure	IC_{50} (μM) ± S.E.M
8a		0.013 ± 0.001
8b		0.053 ± 0.004
9a		0.018 ± 0.005
10a		0.061 ± 0.005
15a		0.021 ± 0.003
16a		0.007 ± 0.00007
17a		0.012 ± 0.0003
18a		0.016 ± 0.0006
19a		0.020 ± 0.004

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

Methylation was first explored on 5-fluoro and unsubstituted 2,4-dioxopyrimidine-1-carboxamides (**4a**, **4b**) and was found to be highly beneficial for the activity, resulting in the two double-digit nanomolar inhibitors **8a** (IC_{50} = 0.013 μM) and **8b** (IC_{50} = 0.053 μM). Notably, an 8-fold increase in potency was observed for compound **8b** vs **4b**, and the 5-fluoro derivative **8a** turned out to be equipotent to **4e**. Considering that the 5-unsubstituted derivative **8b** was 4-fold less potent than **8a**, we turned our attention to 5-fluoro substituted compounds. The introduction of progressively bulkier alkyl groups led only to a slight decrease in inhibitory activity, as the *N*3-ethyl substituted derivative **9a** had an IC_{50} of 0.018 μM, and compound **10a**, bearing a methylcyclopropyl moiety, had an IC_{50} of 0.061 μM. Comparable high potencies were obtained with acyl substituents: **15a**, bearing a 2-methylpropanoyl group, and the benzoyl-substituted **19a** had an IC_{50} value of 0.02 μM. The effect of the N3-substitution was further explored by introducing a carbamoyl functionality, as in compounds **16a**–**18a**. Of particular interest was the result obtained with the methylcarbamoyl derivative **16a**, which showed an IC_{50} of 0.007 μM. High inhibitory potencies were retained with the more sterically demanding carbamoyl derivatives **17a** and **18a**, which displayed IC_{50} values of 0.012 and 0.016 μM, respectively.

Next, we examined the role of the alkyl side chain of the *N*1-carboxamide function, focusing on the influence of chain length. We chose the moderately potent 5-unsubstituted derivative **4b** as reference compound to better appreciate possible changes in potency by variations in the chain length. Unsubstituted uracil derivatives **20b**–**24b**, where the length of the aliphatic side chain was progressively increased from four to nine methylene units, were tested for their inhibitory activity; results are reported in Table 4. With respect to compound **4b** (IC_{50} = 0.426 μM), which bears a six-carbon alkyl chain, a decrease in chain length was associated with a drop in potency, as *N*-butyl-2,4-dioxopyrimidine-1-carboxamide **20b** (IC_{50} = 8.5 μM) was 20-fold less potent than **4b**. Increasing the length of the alkyl chain from four to eight methylene units, we observed progressively higher potencies, with the *N*-octyl derivative **23b** showing the highest activity (IC_{50} = 0.283 μM) within this set of compounds. Further extension of the chain length to nine methylene units resulted, however, in a loss in potency (compound **24b**, IC_{50} = 0.464 μM).

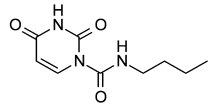
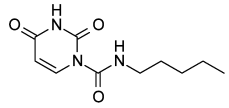
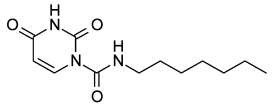
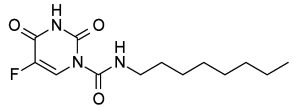
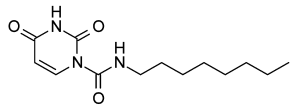
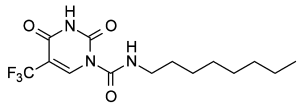
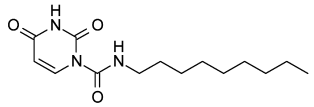
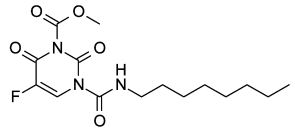
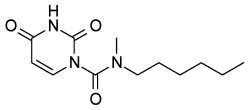
The role of the 1-carboxamide NH group was investigated by preparing the *N*-methyl derivative **27b** (Table 4). This compound showed no inhibitory activity toward AC.

Finally, we prepared and tested the 2,4-dioxopyrimidine-1-carboxamides **23a**, **23e**, and **25a** bearing at positions 5, N1, and N3 the best substituents identified in the previous SAR (Table 4). The combination of the electron-withdrawing fluorine or trifluoromethyl group at position 5 with the *n*-octyl chain at N1 led to potent AC inhibitors. The 5-fluoro derivative **23a** (IC_{50} = 0.046 μM) turned out to be 6-fold more potent than the corresponding unsubstituted analogue **23b**, while the 5-trifluoromethyl compound **23e** (IC_{50} = 0.007 μM) showed a 40-fold improvement in potency compared to **23b**. Notably, in line with our previous observations, carbamoylation of compound **23a** at position N3 delivered the single-digit nanomolar inhibitor **25a**, which displayed an IC_{50} of 0.004 μM.

The critical importance of a fully conjugated 2,4-dioxopyrimidine-1-carboxamide system for displaying AC inhibitory activity, and the opposite effect on potency of electron-withdrawing and electron-donating substituents at position 5, prompted us to undertake a quantum mechanical study on all the reported compounds to test whether any correlation could be established between the potency of the compounds and electronic descriptors of the substituted 2,4-dioxopyrimidine-1-carboxamide derivatives. The SAR exploration around position 5 found a computational counterpart in the second order perturbation theory analysis based on the natural bond orbital (NBO) approach.[21] Such technique was useful to understand how the orbital deviation from the ideal Lewis structure was modulated by different substituents and its relationship with activity (a complete list of calculated NBO energies is reported

dx.doi.org/10.1021/jm301879g | J. Med. Chem. XXXX, XXX, XXX–XXX

Table 4. Inhibitory Potencies (IC_{50}) of Compounds on Rat AC Activity[a]

Compounds	Structure	IC_{50} (μM) ± S.E.M
20b		8.5 ± 1.3
21b		2.1 ± 0.16
22b		0.325 ± 0.029
23a		0.046 ± 0.005
23b		0.283 ± 0.018
23e		0.007 ± 0.003
24b		0.464 ± 0.020
25a		0.004 ± 0.001
27b		No inhibition

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

in Table S3).[22] As illustrated in Figure 3, we observed that the stabilization energy of the antibonding (non-Lewis) exocyclic N1-CO orbital linearly correlated with inhibitory potency (R^2 = 0.87). In other words, a progressively more stable and populated antibonding N1-CO orbital designates a compound that is a more potent AC inhibitor. These results also explained the importance of the uracil ring conjugated system: in the case of the saturated derivative **7**, the stabilization energy (41.36 kcal/mol) was considerably lower than the energy values associated with pharmacologically active compounds. Through a partial least squares (PLS) regression technique (reported in detail in the Supporting Information), it was possible to confirm that the potency (pIC_{50}) of the 2,4-dioxopyrimidine-1-carboxamide 5-substituted derivatives **4a−n** and **4p** and the orbital energies of the 39 valence NBOs of the common scaffold are highly correlated. The insights gathered from this analysis were in line with the stabilization energy data, assigning the most relevant contributions to the orbitals in close proximity to N1.

We next extended the analysis to N3-substituted derivatives (**8a,b**, **9a−10a**, **15a−19a**; see Figure S2). With the only exception of compound **19a**, the activity of which was lower than predicted by the orbital stabilization energy, a satisfactory degree of correlation was maintained between stabilization energy of the exocyclic N1-CO antibonding orbitals and inhibitory potency. The squared correlation coefficients between the stabilization energy of the N1-CO antibonding orbitals and the potency of derivatives are R^2 = 0.52 and R^2 = 0.73, including and excluding **19a**, respectively (see Figure S2). It is reasonable to hypothesize that because of the bulky nature of the benzoyl substituent, the potency of **19a** is reduced by a possible steric clash at the binding site of AC. A more comprehensive PLS model confirmed that when introducing N3-substituted derivatives, the overall potency variation is still mainly accounted for by contributions of the orbitals in close proximity to N1. However, it is interesting to note that in this case the orbitals surrounding N3 also play an important role.

As observed with potency, the stability of 5-substitued compounds is linearly related to the stabilization energy of the antibonding N1-CO orbital (see Figure S5). For the derivatives substituted at position 6 or N3, other electronic properties of the uracil scaffold, besides the stabilization energy, are likely to be taken into account to explain their stability. Nonetheless, it appeared that energy values below 45 kcal/mol guarantee chemical stability. In this light, the most active compounds in the series could be regarded as the result of an optimal trade-off between potency and stability.

Compounds **4l**, **23b**, and **8a**, which showed good AC inhibition and stability, have been tested for their effect on the proliferation of the human colon adenocarcinoma cell line SW430. Cell viability was determined after a 72 h treatment schedule (see Supporting Information). Compounds **4l**, **23b**, and **8a** decreased SW403 cell viability with EC_{50} values of 20, 24, and 22 μM, respectively (Table 5).[23]

CONCLUSIONS

We have identified and characterized 2,4-dioxopyrimidine-1-carboxamides as a novel class of potent small-molecule AC inhibitors. A systematic SAR study around the uracil scaffold allowed a first elucidation of critical structural features associated with AC inhibition. Our study confirmed the importance of an electron-withdrawing group at position 5 of the uracil ring to access potent inhibitors, as previously observed. Additionally, we found that substitution at position N3 is beneficial for activity, leading to highly potent compounds, and that a 1-carboxamide alkyl chain of six to eight methylene units is important to achieve potent AC inhibition. Finally, our SAR study revealed that the C5−C6 double bond in the uracil ring and unsubstituted nitrogen in the 1-carboxamide moiety are mandatory structural features for activity. Computational studies provided a theoretical explanation for the potency of 2,4-dioxopyrimidine-1-carboxamides as AC inhibitors in terms of the electronic properties of the substituted uracil ring.

A notable result of this study is the identification of the first single-digit nanomolar inhibitors (**16a**, **23e**, and **25a**) of AC activity. Selected derivatives in this series may represent useful probes to characterize the functional roles of AC and assess the therapeutic potential of AC inhibitors in cancer and other

dx.doi.org/10.1021/jm301879g | J. Med. Chem. XXXX, XXX, XXX−XXX

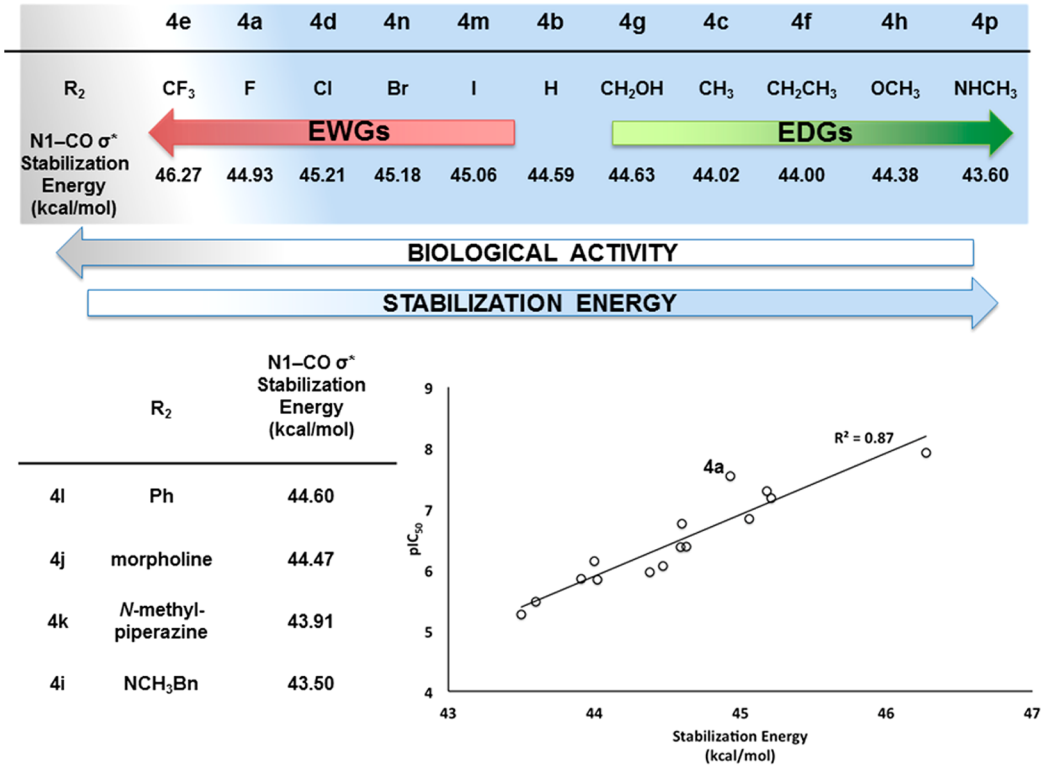


Figure 3. Correlation between the stabilization energies of the exocyclic N1-CO antibonding orbital and potencies (expressed as pIC_{50}) of the 5-substituted derivatives. For each compound, the stabilization energy is reported explicitly. Note that no activity data is associated with compound **4o**, which could not be considered in the plot.

Table 5. Antiproliferation Activity of Representative Compounds on SW403 Cells[a]

Compound	4l	23b	8a
EC_{50} (μM)	24	20	22

[a]Viability of SW403 cells following a 72 h treatment with the reported compounds was measured by MTT reduction as described in the Supporting Information (n=4 for each drug concentration). Results are shown as EC_{50} values (μM).

disorders in which modulation of ceramide levels is clinically relevant.

EXPERIMENTAL SECTION

a. Chemicals, Materials, and Methods. All the commercial available reagents and solvents were used as purchased from vendors without further purification. Dry solvents (pyridine, CH_2Cl_2, DMSO) were purchased from Sigma-Aldrich. Automated column chromatography purifications were done using a Teledyne ISCO apparatus (CombiFlash Rf) with prepacked silica gel columns of different sizes (from 4 to 40 g). Mixtures of increasing polarity of cyclohexane and ethyl acetate (EtOAc) were used as eluents. Flash column chromatography was performed manually on prepacked silica cartridges (2 or 5 g) from Biotage or on glass columns using Merck silica gel 60 (230−400 mesh) as stationary phase. Purifications by preparative HPLC−MS were run on a Waters Autopurification system consisting of a model 3100 single quadrupole mass spectrometer equipped with an electrospray ionization interface and a 2998 photodiode array detector. HPLC system included a model 2747 sample manager, model 2545 binary gradient module, system fluidic organizer, and model 515 HPLC pump. The PDA range was 210−400 nm. Purifications were performed on a XBridge Prep C_{18} OBD column (100 mm × 19 mm i.d., particle size 5 μm) with a XBridge Prep C_{18} (10 mm × 19 mm i.d., particle size 5 μm) Guard cartridge. Mobile phase was 10 mM NH_4OAc in MeCN−H_2O (95:5) at pH 5. Electrospray ionization in positive and negative modes was used.

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 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 ^{13}C) equipped with a BBI probe and Z-gradients. Spectra were acquired at 300 K, using deuterated dimethylsulfoxide (DMSO-d_6), deuterated chloroform ($CDCl_3$), or deuterated acetone (acetone-d_6) as solvents. UPLC−MS analyses were run on a Waters ACQUITY UPLC−MS system consisting of a SQD (single quadrupole detector) mass spectrometer equipped with an electrospray ionization interface and a photodiode array detector. PDA range was 210−400 nm. Analyses were performed on an ACQUITY UPLC HSS T3 C_{18} column (50 mm × 2.1 mm i.d., particle size 1.8 μm) with a VanGuard HSS T3 C_{18} precolumn (5 mm × 2.1 mm i.d., particle size 1.8 μm). Mobile phase was either 10 mM NH_4OAc in H_2O at pH 5 adjusted with AcOH (A) and 10 mM NH_4OAc in MeCN−H_2O (95:5) at pH 5 (B). Electrospray ionization in positive and negative modes was applied. All final compounds, **4a−p**, **7**, **8a,b**, **9a−10a**, **15a−19a**, **20b−24b**, **23a**, **23e**, **25a**, **26**, **27b**, and **30**, showed ≥95% purity by NMR and UPLC−MS analysis.

General Procedure for the Synthesis of 2,4-Dioxopyrimidine-1-carboxamides 4i−n, 8a,b, 9a−10a, 20b−24b, 23a, and 23e via Method A (Table 1, Schemes 3 and 5). The properly substituted uracil (1.0 equiv) was dissolved in dry pyridine. DMAP (1.1 equiv) was added, and the reaction mixture was stirred under nitrogen atmosphere at room temperature for 30 min. The appropriate alkyl isocyanate (1.1−1.8 equiv) was then added, and the resulting mixture was stirred for 12 h. The solvent was evaporated under reduced pressure, and the crude was purified by silica gel column chromatography, eluting with a mixture cyclohexane/EtOAc.

5-(Benzyl(methyl)amino)-*N*-hexyl-2,4-dioxopyrimidine-1-carboxamide (4i). The reaction was carried out following method A,

dx.doi.org/10.1021/jm301879g | J. Med. Chem. XXXX, XXX, XXX−XXX

dissolving **5i** (0.12 g, 0.52 mmol) in dry pyridine (6 mL) and employing DMAP (0.07 g, 0.57 mmol) and hexyl isocyanate (0.08 mL, 0.57 mmol). The crude was purified by silica gel column chromatography (cyclohexane/EtOAc 60:40) to afford compound **4i** (0.14 g, 75%) as a white powder. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.84 (t, J = 6.8 Hz, 3H), 1.20−1.35 (m, 6H), 1.48−1.58 (m, 2H), 2.58 (s, 3H), 3.31 (td, J = 5.7, 7.0 Hz, 2H), 4.21 (s, 2H), 7.19−7.32 (m, 5H), 7.65 (s, 1H), 8.85 (s, 1H), 9.11 (t, J = 5.7 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 13.99, 22.50, 26.52, 29.19, 31.39, 38.99, 41.23, 57.44, 122.21, 127.46, 128.11, 128.36, 128.88, 137.04, 150.22, 150.42, 160.32. MS (ESI) m/z: 359 $[M - H]^+$. MS (ESI) m/z: 357 $[M - H]^-$.

***N*-Hexyl-5-morpholino-2,4-dioxopyrimidine-1-carboxamide (4j).** The reaction was carried out following method A, dissolving **5j** (0.06 g, 0.32 mmol) in dry pyridine (3 mL) and employing DMAP (0.07 g, 0.35 mmol) and hexyl isocyanate (0.05 mL, 0.35 mmol). The crude product was purified by preparative HPLC−MS to afford compound **4j** (0.06 g, 60%) as a white powder. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.77−0.95 (m, 3H), 1.26−1.43 (m, 6H), 1.51−1.66 (m, 2H), 2.90−3.07 (m, 4H), 3.36 (td, J = 5.7, 7.1 Hz, 2H), 3.73−3.94 (m, 4H), 7.83 (s, 1H), 8.32 (s, 1H), 9.10 (t, J = 5.7 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 13.98, 22.50, 26.50, 29.18, 31.37, 41.28, 50.32, 66.54, 122.44, 128.33, 150.06, 150.14, 159.59. MS (ESI) m/z: 347 $[M - Na]^+$. MS (ESI) m/z: 323 $[M - H]^-$.

***N*-Hexyl-5-(4-methylpiperazin-1-yl)-2,4-dioxopyrimidine-1-carboxamide (4k).** The reaction was carried out following method A, dissolving **5k** (0.15 g, 0.71 mmol) in dry pyridine (2 mL) and employing DMAP (0.10 g, 0.79 mmol) and hexyl isocyanate (0.11 mL, 0.79 mmol). The crude was purified by silica gel column chromatography (cyclohexane/EtOAc 70:30) to afford compound **4k** (0.04 g, 17%) as a white powder. ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.83−0.94 (m, 3H), 1.20−1.37 (m, 6H), 1.45−1.55 (m, 2H), 2.27 (br s, 3H), 2.94 (br s, 4H), 3.23−3.36 (m, 6H), 7.55 (s, 1H), 9.22 (t, J = 5.6 Hz, 1H), 11.79 (br s, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 14.35, 22.45, 26.35, 29.14, 31.31, 40.80, 45.88, 49.37, 54.65, 121.17, 128.26, 150.72, 150.86, 160.61. MS (ESI) m/z: 338 $[M - H]^+$. MS (ESI) m/z: 336 $[M - H]^-$.

***N*-Hexyl-2,4-dioxo-5-phenylpyrimidine-1-carboxamide (4l).** The reaction was carried out following method A, dissolving **5l** (0.05 g, 0.26 mmol) in dry pyridine (2.6 mL) and employing DMAP (0.04 g, 0.28 mmol) and hexyl isocyanate (0.07 mL, 0.47 mmol). The crude was purified by silica gel column chromatography (cyclohexane/EtOAc 70:30) to afford compound **4l** (0.04 g, 49%) as a white powder. ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.87 (t, J = 6.5 Hz, 3H), 1.16−1.37 (m, 6H), 1.43−1.61 (m, 2H), 3.23−3.32 (m, 2H), 7.30−7.46 (m, 3H), 7.48−7.57 (m, 2H), 8.28 (s, 1H), 9.19 (t, J = 5.6 Hz, 1H), 11.94 (s, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 14.14, 22.67, 26.66, 29.31, 31.53, 41.50, 77.16, 116.87, 128.53, 128.76, 131.49, 136.05, 149.92, 151.03, 161.31. MS (ESI) m/z: 333 $[M - NH_4]^+$. MS (ESI) m/z: 314 $[M - H]^-$.

***N*-Hexyl-5-iodo-2,4-dioxopyrimidine-1-carboxamide (4m).** The reaction was carried out following method A, dissolving **5m** (0.10 g, 0.42 mmol) in dry pyridine (2 mL) and employing DMAP (0.06 g, 0.46 mmol) and hexyl isocyanate (0.07 mL, 0.46 mmol). The crude was purified by silica gel column chromatography (cyclohexane/EtOAc 90:10) to afford compound **4m** (0.03 g, 19%) as a white powder. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.77−0.95 (m, 3H), 1.22−1.46 (m, 6H), 1.55−1.66 (m, 2H), 3.41 (td, J = 5.6, 7.1 Hz, 2H), 8.49 (s, 1H), 8.86 (s, 1H), 8.96 (t, J = 5.6 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 13.98, 22.50, 26.46, 29.08, 31.34, 41.42, 134.64, 143.41, 148.71, 150.97, 159.06. MS (ESI) m/z: 366 $[M - H]^+$.

***N*-Hexyl-5-bromo-2,4-dioxopyrimidine-1-carboxamide (4n).** The reaction was carried out following method A, dissolving **5n** (0.30 g, 1.57 mmol) in dry pyridine (7.8 mL) and employing DMAP (0.21 g, 1.72 mmol) and hexyl isocyanate (0.27 mL, 1.88 mmol). The crude was purified by silica gel column chromatography (cyclohexane/EtOAc from 70:30 to 100% EtOAc) to afford compound **4n** (0.31 g, 62%) as a white powder. ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.87 (t, J = 6.5 Hz, 3H), 1.24−1.32 (m, 6H), 1.40−1.61 (m, 2H), 3.26 (td, J = 5.7, 7.0 Hz, 2H), 8.47 (s, 1H), 9.07 (t, J = 5.7 Hz, 1H), 12.20 (s, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 14.12, 22.65, 26.61, 29.23, 31.49, 41.63, 100.15, 138.41, 148.91, 150.70, 158.08. MS (ESI) m/z: 317 $[M - H]^-$.

5-Fluoro-*N*-hexyl-3-methyl-2,4-dioxopyrimidine-1-carboxamide (8a). The reaction was carried out following method A, dissolving **12a** (0.04 g, 0.31 mmol) in dry pyridine (1.6 mL) and employing DMAP (0.04 g, 0.34 mmol) and hexyl isocyanate (0.08 mL, 0.56 mmol). The crude was purified by silica gel column chromatography (cyclohexane/EtOAc 70:30) to afford compound **8a** (0.06 g, 71%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.90 (t, J = 6.4 Hz, 3H), 1.21−1.44 (m, 6H), 1.56−1.69 (m, 2H), 3.34−3.44 (m, 2H), 3.40 (s, 3H), 8.47 (d, J = 6.6 Hz, 1H), 9.23 (m, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 14.12, 22.65, 26.64, 28.71, 29.28, 31.51, 41.61, 121.11 (d, J = 37.4 Hz), 140.62 (d, J = 238.2 Hz), 149.61, 151.01, 156.69 (d, J = 26.4 Hz). MS (ESI) m/z: 272 $[M - H]^+$.

***N*-Hexyl-3-methyl-2,4-dioxopyrimidine-1-carboxamide (8b).** The reaction was carried out following method A, dissolving **12b** (0.08 g, 0.60 mmol) in dry pyridine (3 mL) and employing DMAP (0.08 g, 0.66 mmol) and hexyl isocyanate (0.16 mL, 1.08 mmol). The crude was purified by silica gel column chromatography (cyclohexane/EtOAc 60:40) to afford compound **8b** (0.12 g, 79%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.89 (t, J = 6.5 Hz, 3H), 1.26−1.45 (m, 6H), 1.53−1.68 (m, 2H), 3.35 (s, 3H), 3.36−3.42 (m, 2H), 5.93 (d, J = 8.5 Hz, 1H), 8.39 (d, J = 8.5 Hz, 1H), 9.29 (m, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 14.13, 22.73, 26.68, 28.00, 29.30, 31.53, 41.41, 103.42, 136.70, 150.39, 152.64, 162.09. MS (ESI) m/z: 276 $[M - Na]^+$.

3-Ethyl-5-fluoro-*N*-hexyl-2,4-dioxopyrimidine-1-carboxamide (9a). The reaction was carried out following method A, dissolving **13a** (0.04 g, 0.30 mmol) in dry pyridine (1.3 mL) and employing DMAP (0.04 g, 0.33 mmol) and hexyl isocyanate (0.07 mL, 0.50 mmol). The crude was purified by silica gel column chromatography (petroleum ether/EtOAc 85:15) to afford compound **9a** (0.02 g, 25%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.90 (t, J = 6.8 Hz, 3H), 1.26 (t, J = 7.1 Hz, 3H), 1.29−1.43 (m, 6H), 1.56−1.68 (m, 2H), 3.28−3.50 (m, 2H), 4.05 (q, J = 7.1 Hz, 2H), 8.45 (d, J = 6.6 Hz, 1H), 9.22−9.29 (m, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 12.74, 14.14, 22.65, 26.66, 29.29, 31.52, 37.74, 41.61, 121.11 (d, J = 37.4 Hz), 140.72 (d, J = 238.6 Hz), 149.73, 150.69, 156.40 (d, J = 29.7 Hz). MS (ESI) m/z: 157 $[M - CONH(CH_2)_5CH_3]^-$.

3-(Cyclopropylmethyl)-5-fluoro-*N*-hexyl-2,4-dioxopyrimidine-1-carboxamide (10a). The reaction was carried out following method A, dissolving **14a** (0.05 g, 0.26 mmol) in dry pyridine (1.4 mL) and employing DMAP (0.04 g, 0.29 mmol) and hexyl isocyanate (0.07 mL, 0.48 mmol). The crude was purified by silica gel column chromatography (cyclohexane/EtOAc 85:15) to afford compound **10a** (0.06 g, 74%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.39−0.45 (m, 2H), 0.47−0.56 (m, 2H), 0.89 (t, J = 6.9 Hz, 3H), 1.20−1.27 (m, 1H), 1.28−1.43 (m, 6H), 1.53−1.70 (m, 2H), 3.39 (td, J = 5.6, 7.2 Hz, 2H), 3.88 (d, J = 7.3 Hz, 2H), 8.46 (d, J = 6.6 Hz, 1H), 9.26 (m, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 4.07, 9.63, 14.12, 22.65, 26.65, 29.28, 31.51, 41.60, 46.96, 121.16 (d, J = 37.5 Hz), 140.76 (d, J = 238.3 Hz), 149.76, 151.11, 156.82 (d, J = 26.5 Hz). MS (ESI) m/z: 312 $[M - H]^+$, 334 $[M - Na]^+$.

***N*-Butyl-2,4-dioxopyrimidine-1-carboxamide (20b).** The reaction was carried out following method A, dissolving **5b** (0.99 g, 8.75 mmol) in dry pyridine (20 mL) and employing DMAP (1.17 g, 9.62 mmol) and butyl isocyanate (1.08 mL, 9.62 mmol). The crude was crystallized in refluxing ethanol (20 mL) and filtered at room temperature to afford compound **20b** (1.05 g, 57%) as a white powder. ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.89 (t, J = 7.3 Hz, 3H), 1.26−1.38 (m, 2H), 1.46−1.56 (m, 2H), 3.27 (td, J = 5.7, 7.0 Hz, 2H), 5.79 (d, J = 8.4 Hz, 1H), 8.20 (d, J = 8.4 Hz, 1H), 9.11 (t, J = 5.7 Hz, 1H), 11.71 (s, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 13.54, 19.39, 30.76, 40.01, 103.47, 138.72, 149.93, 151.47, 162.76. MS (ESI) m/z: 210 $[M - H]^-$.

***N*-Pentyl-2,4-dioxopyrimidine-1-carboxamide (21b).** The reaction was carried out following method A, dissolving **5b** (0.10 g, 0.89 mmol) in dry pyridine (3 mL) and employing DMAP (0.12 g, 0.98 mmol) and pentyl isocyanate (0.13 mL, 0.98 mmol). The crude was purified by silica gel column chromatography (cyclohexane/EtOAc 90:10) to afford compound **21b** (0.02 g, 10%) as a white powder. ^{1}H

NMR (400 MHz, $CDCl_3$) δ 0.94 (t, J = 6.7 Hz, 3H), 1.30−1.41 (m, 4H), 1.60−1.70 (m, 2H), 3.41 (td, J = 5.6, 7.1 Hz, 2H), 5.91 (dd, J = 8.5, 2.0 Hz, 1H), 8.25 (s, 1H), 8.44 (d, J = 8.5 Hz, 1H), 9.06 (br s, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 13.93, 22.27, 28.97, 28.86, 41.26, 103.82, 138.90, 150.80, 150.82, 161.72.

***N*-Heptyl-2,4-dioxopyrimidine-1-carboxamide (22b).** The reaction was carried out following method A, dissolving **5b** (0.37 g, 3.34 mmol) in dry pyridine (11 mL) and employing DMAP (0.45 g, 3.67 mmol) and heptyl isocyanate (0.70 mL, 4.35 mmol). The crude was triturated in dichloromethane to afford compound **22b** (0.58 g, 68%) as a white powder. ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.86 (t, J = 6.7 Hz, 3H), 1.25−1.28 (m, 8H), 1.47−1.53 (m, 2H), 3.23−3.29 (m, 2H), 5.79 (d, J = 8.4 Hz, 1H), 8.20 (d, J = 8.4 Hz, 1H), 9.11 (t, J = 5.7 Hz, 1H), 11.71 (s, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 13.75, 21.72, 25.93, 28.10, 28.85, 30.98, 40.41, 102.90, 139.00, 150.43, 151.91, 163.19. MS (ESI) m/z: 254 $[M - H]^+$, 271 $[M - NH_4]^+$. MS (ESI) m/z: 252 $[M - H]^-$.

5-Fluoro-*N*-octyl-2,4-dioxopyrimidine-1-carboxamide (23a). The reaction was carried out following method A, dissolving **5a** (0.05 g, 0.38 mmol) in dry pyridine (3.8 mL) and employing DMAP (0.05 g, 0.42 mmol) and octyl isocyanate (0.08 mL, 0.46 mmol). The crude was purified by silica gel column chromatography (cyclohexane/EtOAc 80:20) to afford compound **23a** (0.05 g, 46%) as a white powder. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88 (t, J = 7.1 Hz, 3H), 1.21−1.39 (m, 10H), 1.51−1.67 (m, 2H), 3.39 (td, J = 7.1, 5.5 Hz, 2H), 8.48 (d, J = 6.8 Hz, 1H), 8.93−9.05 (m, 1H), 9.10 (s, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 14.20, 22.75, 26.94, 29.27, 31.89, 41.63, 123.40 (d, J = 37.3 Hz), 140.89 (d, J = 241.3 Hz), 149.13, 150.03, 156. 156.43. MS (ESI) m/z: 284 $[M - H]^-$.

***N*-Octyl-2,4-dioxopyrimidine-1-carboxamide (23b).** The reaction was carried out following method A, dissolving **5b** (0.08 g, 0.72 mmol) in dry pyridine (7.2 mL) and employing DMAP (0.09 g, 0.78 mmol) and octyl isocyanate (0.23 mL, 1.30 mmol). The crude was triturated in a mixture of dichloromethane/MeOH/EtOAc to afford compound **23b** (0.14 g, 73%) as a white powder. ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.85 (t, J = 6.7 Hz, 3H), 1.24−1.31 (m, 10H), 1.49−1.52 (m, 2H), 3.23−3.28 (m, 2H), 5.79 (d, J = 8.4 Hz, 1H), 8.20 (d, J = 8.3 Hz, 1H), 9.11 (t, J = 5.7 Hz, 1H), 11.71 (s, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 13.91, 22.04, 26.19, 28.57, 28.63, 31.18, 40.33, 103.43, 138.73, 149.93, 151.49, 162.72. MS (ESI) m/z: 266 $[M - H]^-$.

***N*-Octyl-2,4-dioxo-5-(trifluoromethyl)pyrimidine-1-carboxamide (23e).** The reaction was carried out following method A, dissolving **5e** (0.10 g, 0.55 mmol) in dry pyridine (5.5 mL) and employing DMAP (0.08 g, 0.61 mmol) and octyl isocyanate (0.28 mL, 1.64 mmol). The crude was purified by silica gel column chromatography (cyclohexane/EtOAc from 70:30 to cyclohexane/EtOAc 60:40) and then washed with *n*-heptane to afford compound **23e** (0.04 g, 22%) as a white powder. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88 (d, J = 6.8 Hz, 3H), 1.18−1.44 (m, 10H), 1.53−1.70 (m, 2H), 3.41 (td, J = 7.1, 5.5 Hz, 2H), 8.90−8.97 (m, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 14.20, 22.75, 26.92, 29.20, 29.26, 31.89, 41.80, 121.16 (q, J = 268.7 Hz), 139.73, 148.25, 150.83, 156.86. MS (ESI) m/z: 334 $[M - H]^-$.

***N*-Nonyl-2,4-dioxopyrimidine-1-carboxamide (24b).** The reaction was carried out following method A, dissolving **5b** (0.07 g, 0.62 mmol) in dry pyridine (3.1 mL) and employing DMAP (0.08 g, 0.69 mmol) and nonyl isocyanate (0.16 mL, 1.30 mmol). The crude was triturated in dichloromethane to afford compound **24b** (0.10 g, 57%) as a white powder. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88 (t, J = 6.8 Hz, 3H), 1.26−1.32 (m, 12H), 1.57−1.61(m, 2H), 3.38 (td, J = 5.6, 7.2 Hz, 2H), 5.88 (dd, J = 2.0, 8.5 Hz, 1H), 8.37−8.39 (m, 1H), 8.42 (d, J = 8.5 Hz, 1H), 9.03 (t, J = 5.8 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 14.24, 22.80, 26.99, 29.32, 29.35, 29.59, 31.98, 41.44, 103.97, 139.07, 149.78, 151.46, 162.06. MS (ESI) m/z: 282 $[MH]^+$. MS (ESI) m/z: 280 $[M - H]^-$.

General Procedure for the Synthesis of 2,4-Dioxopyrimidine-1-carboxamides 4f and 4h via Method B (Table 1). The properly 5-substituted uracil (1.0 equiv) was dissolved in dry DMSO, and hexyl isocyanate (1.1−1.8 equiv) was added. The reaction mixture was heated to 50 °C and stirred under nitrogen atmosphere for 4−12 h. The mixture was poured into water (50 mL). The product precipitated, and it was filtered. The desired carboxamide was then washed with cyclohexane (3 × 10 mL) and dried under reduced pressure.

5-Ethyl-*N*-hexyl-2,4-dioxopyrimidine-1-carboxamide (4f). The reaction was carried out following method B, dissolving 5-ethyluracil (0.10 g, 0.71 mmol) in dry DMSO (4 mL) and employing hexyl isocyanate (0.11 mL, 0.78 mmol). The crude was purified by silica gel column chromatography (EtOAc/cyclohexane 90:10) to afford compound **4f** (0.03 g, 16%) as a white powder. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88 (t, J = 6.3 Hz, 3H), 1.16 (t, J = 7.4 Hz, 3H), 1.21−1.50 (m, 6H), 1.54−1.68 (m, 2H), 2.42 (q, J = 7.4 Hz, 2H), 3.37 (td, J = 5.7, 7.0 Hz, 2H), 8.21 (s, 1H), 9.15 (br s, 1H), 9.54 (s, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 12.67, 14.31, 20.19, 22.56, 26.58, 29.23, 31.43, 41.26, 117.92, 134.02, 150.12, 151.53, 163.31. MS (ESI) m/z: 266 $[M - H]^-$.

***N*-Hexyl-5-methoxy-2,4-dioxopyrimidine-1-carboxamide (4h).** The reaction was carried out following method B, dissolving 5-methoxyuracil (0.05 g, 0.33 mmol) in dry DMSO (1.6 mL) and employing hexyl isocyanate (0.09 mL, 0.59 mmol). The crude was triturated with cyclohexane to afford compound **4h** (0.05 g, 56%) as a white powder. ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.86 (t, J = 6.9 Hz, 3H), 1.26−1.31 (m, 6H), 1.47−1.54 (m, 2H), 3.27 (td, J = 5.7, 7.0 Hz, 2H), 3.66 (s, 3H), 7.71 (s, 1H), 9.22 (t, J = 5.6 Hz, 1H), 11.94 (s, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 13.86, 21.97, 25.87, 28.67, 30.83, 40.35, 56.49, 116.17, 136.52, 150.13, 150.24, 158.78. MS (ESI) m/z: 270 $[M - H]^+$, 287 $[M - NH_4]^+$, 308 $[M - K]^+$. MS (ESI) m/z: 268 $[M - H]^-$.

General Procedure for the Synthesis of 2,4-Dioxopyrimidine-1-carboxamides 4g and 4o[24] via Method C (Table 1). The properly 5-substituted uracil (1.0 equiv) was dissolved in dry pyridine, and hexyl isocyanate (1.1−1.5 equiv) was added. The reaction mixture was heated under microwave irradiation at 100 °C for 10 min. The solvent was evaporated under reduced pressure, and the crude products were purified by silica gel column chromatography, eluting with dichloromethane or dichloromethane/acetone mixture.

***N*-Hexyl-5-(hydroxymethyl)-2,4-dioxopyrimidine-1-carboxamide (4g).** The reaction was carried out following method C, dissolving 5-hydroxymethyluracil (0.07 g, 0.49 mmol) in dry pyridine (2.5 mL) and employing hexyl isocyanate (0.08 mL, 0.54 mmol).The crude was purified by silica gel column chromatography (dichloromethane/acetone 60:40) to afford compound **4g** (0.04 g, 27%) as a white powder. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.90 (t, J = 6.9 Hz, 3H), 1.31−1.39 (m, 6H), 1.56−1.64 (m, 2H), 2.40 (t, J = 6.5 Hz, 1H), 3.39 (td, J = 5.6, 7.1, 2H), 4.48 (d, J = 6.6 Hz, 2H), 8.33 (s, 1H), 8.45 (s, 1H), 9.00 (s, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 14.13, 22.66, 26.63, 29.27, 31.51, 41.47, 58.82, 115.26, 136.19, 149.74, 151.09, 162.49. MS (ESI) m/z: 292 $[M - Na]^+$, 308 $[M - K]^+$. MS (ESI) m/z: 268 $[M - H]^-$.

Synthesis of *N*-Hexyl-5-methylamino-2,4-dioxopyrimidine-1-carboxamide (4p). Compound **4i** (0.10 g, 0.28 mmol) was dissolved in EtOAc (30 mL) and hydrogenated in an H-Cube apparatus (using 10% Pd/C as catalyst, 1 bar hydrogen pressure, at 40 °C). The reaction mixture was concentrated under reduced pressure. The crude was purified by silica gel column chromatography (cyclohexane/EtOAc 85:15) to afford compound **4p** (0.02 g, 27%) as a white powder. ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.78−0.99 (m, 3H), 1.17−1.36 (m, 6H), 1.45−1.55 (m, 2H), 2.57 (s, 3H), 3.27 (td, J = 5.7, 6.9 Hz, 2H), 5.01 (s, 1H), 7.05 (s, 1H), 9.32 (t, J = 5.7 Hz, 1H), 11.87 (s, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 13.85, 21.96, 25.89, 28.71, 29.70, 30.82, 40.21, 106.74, 126.49, 150.01, 150.68, 160.01. MS (ESI) m/z: 267 $[M - H]^-$.

Synthesis of *N*-Hexyl-2,4-dioxohexahydropyrimidine-1-carboxamide (7). The reaction was carried out following the procedure described for compound **4p**, dissolving **4b** (0.03 g, 0.12 mmol) in THF (7 mL) and using 10% Pd/C as catalyst and 40 bar hydrogen pressure at 50 °C. The solvent was removed under reduced pressure to afford compound **7** (0.03 g, quantitative) as a white powder. ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.86 (t, J = 6.6 Hz, 3H), 1.22−1.34 (m, 6H), 1.41−1.51 (m, 2H), 2.57 (t, J = 6.6 Hz, 2H), 3.18 (td, J = 5.6, 6.8 Hz,

dx.doi.org/10.1021/jm301879g | J. Med. Chem. XXXX, XXX, XXX−XXX

2H), 3.87 (t, J = 6.6 Hz, 2H), 8.71 (t, J = 5.3 Hz, 1H), 10.67 (s, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 13.84, 21.97, 25.94, 28.94, 30.35, 30.85, 37.35, 39.85, 152.98, 153.77, 170.75. MS (ESI) m/z: 240 [M − H]$^-$.

Synthesis of 5-Fluoro-*N*-hexyl-3-(2-methylpropanoyl)-2,4-dioxopyrimidine-1-carboxamide (15a). 5-Fluoro-*N*-hexyl-2,4-dioxopyrimidine-1-carboxamide (**4a**, 0.04 g, 0.15 mmol) was dissolved in dry dichloromethane (0.8 mL). To the resulting solution, cooled to 0 °C, triethylamine (0.09 mL, 0.62 mmol) was added, followed by isobutyryl chloride (0.05 mL, 0.50 mmol). The mixture was allowed to warm to room temperature and stirred under nitrogen atmosphere for 45 min. Water was added and the aqueous phase extracted with dichloromethane (3 × 15 mL). The combined organic layers were washed with saturated $NaHCO_3$ solution and brine, dried over Na_2SO_4, and the solvent was removed under reduced pressure. The crude was purified by silica gel column chromatography (cyclohexane/EtOAc 85:15) to afford compound **15a** (0.01 g, 20%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.89 (t, J = 7.0 Hz, 3H), 1.24−1.40 (m, 6H), 1.31 (d, J = 7.0 Hz, 6H), 1.57−1.64 (m, 2H), 3.05 (hept, J = 7.0 Hz, 1H), 3.38 (td, J = 5.5, 7.2 Hz, 2H), 8.48 (d, J = 6.7 Hz, 1H), 8.86 (t, J = 5.6 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 14.11, 17.94, 22.63, 26.60, 29.21, 31.48, 40.02, 41.74, 122.78 (d, J = 37.4 Hz), 140.55 (d, J = 243.3 Hz), 148.92, 149.40, 155.64 (d, J = 28.5 Hz), 177.21. MS (ESI) m/z: 256 [M − COC(CH$_3$)$_2$]$^-$.

Synthesis of Methyl 5-Fluoro-3-(hexylcarbamoyl)-2,6-dioxopyrimidine-1-carboxylate (16a). 5-Fluoro-*N*-hexyl-2,4-dioxopyrimidine-1-carboxamide (**4a**, 0.05 g, 0.20 mmol) was dissolved in dry dichloromethane (4 mL), and pyridine (0.04 mL, 0.43 mmol) was added, followed by methyl chloroformate (0.02 mL, 0.23 mmol) at 0 °C. The mixture was stirred at room temperature for 18 h, and then the solvent was removed under reduced pressure. The crude was purified by silica gel column chromatography (cyclohexane/EtOAc 90:10) to afford compound **16a** (0.02 g, 32%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.86−0.92 (m, 3H), 1.22−1.43 (m, 6H), 1.55−1.65 (m, 2H), 3.33−3.43 (m, 2H), 4.09 (s, 3H), 8.48 (d, J = 6.7 Hz, 1H), 8.79 (t, J = 5.5 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 14.33, 22.35, 26.52, 28.98, 31.50, 41.69, 56.85, 122.71 (d, J = 37.5 Hz), 140.17 (d, J = 242.8 Hz), 148.38, 148.46, 148.51, 153.88 (d, J = 29.3 Hz). MS (ESI) m/z: 316 [M − H]$^+$, 333 [M − NH$_4$]$^+$.

Ethyl 5-Fluoro-3-(hexylcarbamoyl)-2,6-dioxopyrimidine-1-carboxylate (17a). The reaction was carried out following the procedure described for compound **16a**, dissolving **4a** (0.05 g, 0.20 mmol) in dry dichloromethane (2.0 mL) and employing pyridine (0.09 mL, 1.14 mmol) and ethyl chloroformate (0.05 mL, 0.57 mmol). The crude was purified by silica gel column chromatography (cyclohexane/EtOAc 80:20) to afford compound **17a** (0.03 g, 46%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.89 (t, J = 7.5 Hz, 3H), 1.23−1.41 (m, 6H), 1.44 (t, J = 7.1 Hz, 3H), 1.55−1.65 (m, 2H), 3.38 (td, J = 5.6, 7.1 Hz, 2H), 4.53 (q, J = 7.1 Hz, 2H), 8.48 (d, J = 6.8 Hz, 1H), 8.82 (t, J = 5.7 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 13.87, 14.13, 22.64, 26.60, 29.21, 31.49, 41.78, 67.17, 122.77 (d, J = 37.4 Hz), 140.38 (d, J = 242.7 Hz), 147.92, 148.57, 148.70, 155.16 (d, J = 31.5 Hz). MS (ESI) m/z: 347 [M − NH$_4$]$^+$.

Methyl 5-Fluoro-3-(octylcarbamoyl)-2,6-dioxopyrimidine-1-carboxylate (25a). The reaction was carried out following the procedure described for compound **16a**, dissolving **23a** (0.20 g, 0.70 mmol) in dry dichloromethane (7.0 mL) and employing pyridine (0.22 mL, 2.80 mmol) and methyl chloroformate (0.05 mL, 0.57 mmol). The crude was purified by silica gel column chromatography (cyclohexane/EtOAc 85:15) to afford compound **25a** (0.16 g, 66%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.74−1.00 (m, 3H), 1.11−1.43 (m, 11H), 1.47−1.69 (m, 2H), 1.55−1.65 (m, 2H), 3.37 (td, J = 5.6, 7.1 Hz, 2H), 4.08 (q, J = 7.1 Hz, 2H), 8.47 (d, J = 6.7 Hz, 1H), 8.78 (t, J = 5.6 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 14.17, 22.73, 26.88, 29.18, 29.23, 31.87, 41.76, 56.96, 122.85 (d, J = 37.5 Hz), 140.30 (d, J = 242.6 Hz), 148.52, 148.60, 148.65, 154.01 (d, J = 29.3 Hz). MS (ESI) m/z: 344 [M − H]$^+$.

Synthesis of Isobutyl 5-Fluoro-3-(hexylcarbamoyl)-2,6-dioxopyrimidine-1-carboxylate (18a). 5-Fluoro-*N*-hexyl-2,4-dioxopyrimidine-1-carboxamide (**4a**, 0.10 g, 0.39 mmol) was dissolved in dry pyridine (4 mL). To the resulting solution, isobutyl chloroformate (0.06 mL, 0.47 mmol) was added. The reaction was stirred at room temperature under nitrogen for 3 h, and then the solvent was removed under reduced pressure. The crude was purified by silica gel column chromatography (cyclohexane/EtOAc 90:10) to afford compound **18a** (0.03 g, 22%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.86−0.92 (m, 3H), 1.01 (d, J = 6.7 Hz, 6H), 1.20−1.42 (m, 6H), 1.55−1.65 (m, 2H), 2.11 (hept, J = 6.7 Hz, 1H), 3.31−3.52 (m, 2H), 4.26 (d, J = 6.7 Hz, 2H), 8.48 (d, J = 6.7 Hz, 1H), 8.83 (t, J = 5.5 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 14.12, 18.88, 22.64, 26.59, 27.81, 29.21, 31.48, 41.78, 76.84, 122.77 (d, J = 37.62 Hz), 140.42 (d, J = 243.18 Hz), 148.09, 148.36 (d, J = 50.64 Hz), 148.64, 154.33. MS (ESI) m/z: 358 [M − H]$^+$, 375 [M − NH$_4$]$^+$, 396 [M − K]$^+$.

3-Benzoyl-5-fluoro-*N*-hexyl-2,4-dioxopyrimidine-1-carboxamide (19a). The reaction was carried out following the procedure described for compound **18a**, dissolving **4a** (0.05 g, 0.20 mmol) in dry pyridine (4 mL) and employing benzoyl chloride (0.03 mL, 0.25 mmol). The crude was purified by silica gel column chromatography (cyclohexane/EtOAc 90:10) to afford compound **19a** (0.03 g, 41%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.87 (t, J = 6.9 Hz, 3H), 1.24−1.39 (m, 6H), 1.49−1.65 (m, 2H), 3.36 (td, J = 5.7, 7.0 Hz, 2H), 7.47−7.61 (m, 2H), 7.65−7.79 (m, 1H), 7.89−8.01 (m, 2H), 8.58 (d, J = 6.7 Hz, 1H), 8.82 (t, J = 5.7 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 13.82, 22.48, 26.55, 29.07, 31.41, 40.32, 41.74, 123.05 (d, J = 37.38 Hz), 130.32, 130.62, 135.89, 140.38 (d, J = 243.88 Hz), 148.65, 149.33 (d, J = 12.50 Hz), 155.46, 165.98. MS (ESI) m/z: 378 [M − Na]$^+$.

Synthesis of *N*-Hexyl-*N*-methyl-2,4-dioxopyrimidine-1-carboxamide 27b. Sodium salt **29b** (3.54 g, 26.38 mmol) was suspended in dry acetonitrile (50 mL). Triphosgene (2.53 g, 7.91 mmol) was added, and the suspension was stirred at room temperature for 20 min. *N*-Methylhexylamine (4.0 mL, 26.4 mmol) was added dropwise, and the reaction mixture was stirred at room temperature for 12 h. The suspension was filtered, and the filtrate was evaporated under reduced pressure. The crude was purified by silica gel column chromatography (EtOAc/cyclohexane 90:10) to afford compound **27b** (0.12 g, 2%) as a white powder. ^{1}H NMR (400 MHz, DMSO-d_6) showed a 66:34 ratio of two rotamers with the following chemical shifts: major rotamer, δ 0.87 (t, J = 6.7 Hz, 3H), 1.09−1.37 (m, 6H), 1.45−1.56 (m, 2H), 2.97 (s, 3H), 3.10−3.20 (m, 1H), 3.43−3.53 (m, 1H), 5.69 (d, J = 8.0 Hz, 1H), 7.64 (d, J = 8.0 Hz, 1H), 11.49 (t, J = 5.7 Hz, 1H); minor rotamer, δ 0.83 (t, J = 6.7 Hz, 3H), 1.09−1.37 (m, 6H), 1.45−1.56 (m, 2H), 2.87 (s, 3H), 3.23−3.32 (m, 2H), 5.70 (d, J = 8.0 Hz, 1H), 7.69 (d, J = 8.0 Hz, 1H), 11.49 (t, J = 5.7 Hz, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) showed the presence of two rotamers with the following chemical shifts: major rotamer, δ 13.85, 21.97, 25.49, 26.08, 30.91, 35.62, 48.88, 102.42, 141.49, 148.41, 151.33, 163.34; minor rotamer, δ 13.84, 21.87, 25.41, 27.05, 30.73, 34.32, 50.17, 102.38, 141.59, 148.78, 151.11, 163.27. MS (ESI) m/z: 252 [M − H]$^-$.

b. Pharmacology. Recombinant Acid Ceramidase Expression. Rat AC was cloned from a brain cDNA library using primers based on the sequence obtained from the National Center for Biotechnology Information (NCBI) database: 5′rAC (5′-GACCATG-CTGGGCCGTAGT-3′) and 3′rAC (5′-CCAGCCTATACAAG-GGTCT-3′). The PCR (High Fidelity PCR Master, Roche) product was subcloned into a pEF6-V5/His vector (Invitrogen) to construct a mammalian expression vector encoding V5/His-tagged rat AC. HEK 293 cells were transfected with pEF6-rAC-V5/His using Super-Fect reagent (Qiagen) and screened with G418 (0.3 mg/mL).

Acid Ceramidase (AC) Activity. AC activity was analyzed as previously described.[25] Briefly, cells were suspended in 20 mM Tris-HCl (pH 7.5) containing 0.32 M sucrose, sonicated, and centrifuged at 800g for 15 min at 4 °C. The supernatants were centrifuged again at 12000g for 30 min at 4 °C. The pellets were suspended in phosphate buffered saline (PBS) and subjected to two freeze−thaw cycles at −80 °C. The suspensions were centrifuged at 105000g for 1 h at 4 °C. The supernatants containing recombinant AC were kept at −80 °C until use. Protein concentration was measured using the bicinchoninic acid (BCA) assay (Pierce). Recombinant rat AC (50 μg) was preincubated with inhibitors (final DMSO concentration 1%) in assay buffer (100

dx.doi.org/10.1021/jm301879g | J. Med. Chem. XXXX, XXX, XXX−XXX

mM sodium phosphate, 0.1% Nonidet P-40, 150 mM NaCl, 3 mM DTT, 100 mM sodium citrate, pH 4.5) for 30 min at 37 °C. Reactions were started by the addition of 100 μM *N*-lauroylceramide (Nu-Chek Prep, Elysian, MN) and carried on for 30 min at 37 °C. Reactions were stopped by addition of a mixture of chloroform/methanol (2:1, vol/vol) containing 1 nmol of heptadecanoic acid (HDA, NuChek Prep). The organic phases were collected, dried under nitrogen, and analyzed by liquid chromatography/mass spectrometry in the negative-ion mode monitoring the reaction product (lauric acid, m/z = 199) using HDA as internal standard. Lipids were eluted on an XDB Eclipse C18 column isocratically at 2.2 mL·min^{-1} for 1 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 50 °C. Electrospray ionization was in the negative mode. Capillary voltage was 4 kV, and fragmentor voltage was 100 V. Nitrogen was used as drying gas at a flow rate of 13 L·min^{-1} and at a temperature of 350 °C. Nebulizer pressure was set at 60 psi. We monitored $[M - H]^-$ in the selected-ion monitoring mode. Calibration curves were generated with authentic lauric acid (Nu Check Prep).

c. Computational Methods. Electronic structure calculations were performed with Gaussian 09 software.[26] All the calculations adopted the B3LYP hybrid density functional, and a 6-311+g(d) basis set was employed.[27] All the optimizations were carried out in polarizable conductor calculation model (CPCM), and the chosen solvent was water.[28] The structures were optimized with tight convergence parameters and ultrafine grid. Finally, natural bond orbital (NBO) analysis and second-order perturbation theory analysis of Fock matrix in NBO basis were carried out to better understand the effect of the local changes in the electronic structure induced by different substituents.[21] This last part was carried out through the NBO 3.1 software included in the Gaussian package. All the structures were prepared using Gaussview 5.0.[26] In the alkyl side chain, methylene units beyond the first were omitted.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Detailed experimental procedures, analytical and spectroscopical data of intermediates and final compounds, analytical stability data, additional computational details on the NBO and PLS studies, and MTT cell viability assay conditions. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*For D.P.: e-mail, piomelli@uci.edu. For T.B.: phone, +39-010-71781533; fax, +39-010-71781228; e-mail, tiziano.bandiera@iit.it.

Author Contributions

‡D.P. and C.P. contributed equally to design and perform research.

Notes

The authors declare the following competing financial interests. Two patent applications protecting the class of compounds disclosed in this paper were filed by the following authors: Piomelli, D.; Realini, N.; Mor, M.; Pagliuca, C.; Pizzirani, D.; Scarpelli, R.; Bandiera, T.

■ ACKNOWLEDGMENTS

The authors thank Giuliana Ottonello for analytical stability data, Sine Mandrup Bertozzi for reverse phase HPLC purifications, Luca Goldoni for NMR technical support, and Silvia Venzano for handling of compounds.

■ ABBREVIATIONS USED

AC, acid ceramidase; OEA, oleoylethanolamide; 5-FU, 5-fluorouracil; DMAP, 4-dimethylaminopyridine; DMSO, dimethyl sulfoxide; BSA, bis(trimethylsilyl)acetamide; TFA, trifluoroacetic acid; BOC, *tert*-butyloxycarbonyl; NBO, natural bond orbital; PLS, partial least squares; HDA, heptadecanoic acid

■ REFERENCES

(1) (a) Kitatani, K.; Idkowiak-Baldys, J.; Hannun, Y. A. The sphingolipid salvage pathway in ceramide metabolism and signaling. *Cell. Signallling* **2008**, *20* (6), 1010−1018. (b) Wymann, M. P.; Schneiter, R. Lipid signalling in disease. *Nat. Rev. Mol. Cell Biol.* **2008**, *9* (2), 162−176. (c) Gangoiti, P.; Camacho, L.; Arana, L.; Ouro, A.; Granado, M. H.; Brizuela, L.; Casas, J.; Fabrias, G.; Abad, J. L.; Delgado, A.; Gomez-Munoz, A. Control of metabolism and signaling of simple bioactive sphingolipids: Implications in disease. *Prog. Lipid Res.* **2010**, *49* (4), 316−334.

(2) (a) Bielawska, A.; Linardic, C. M.; Hannun, Y. A. Ceramide-mediated biology. Determination of structural and stereospecific requirements through the use of *N*-acyl-phenylaminoalcohol analogs. *J. Biol. Chem.* **1992**, *267* (26), 18493−18497. (b) Hannun, Y. A. Functions of ceramide in coordinating cellular responses to stress. *Science* **1996**, *274* (5294), 1855−1859.

(3) (a) Ogretmen, B.; Hannun, Y. A. Biologically active sphingolipids in cancer pathogenesis and treatment. *Nat. Rev. Cancer* **2004**, *4* (8), 604−616. (b) Huang, W. C.; Chen, C. L.; Lin, Y. S.; Lin, C. F. Apoptotic sphingolipid ceramide in cancer therapy. *J. Lipids* **2011**, *2011*, 565316.

(4) Nussbaumer, P. Medicinal chemistry aspects of drug targets in sphingolipid metabolism. *ChemMedChem* **2008**, *3* (4), 543−551.

(5) Mao, C.; Obeid, L. M. Ceramidases: regulators of cellular responses mediated by ceramide, sphingosine, and sphingosine-1-phosphate. *Biochim. Biophys. Acta* **2008**, *1781* (9), 424−434.

(6) (a) Zeidan, Y. H.; Jenkins, R. W.; Korman, J. B.; Liu, X.; Obeid, L. M.; Norris, J. S.; Hannun, Y. A. Molecular targeting of acid ceramidase: implications to cancer therapy. *Curr. Drug Targets* **2008**, *9* (8), 653−661. (b) Radin, N. S. Killing tumours by ceramide-induced apoptosis: a critique of available drugs. *Biochem. J.* **2003**, *371* (Part 2), 243−256. (c) Mahdy, A. E.; Cheng, J. C.; Li, J.; Elojeimy, S.; Meacham, W. D.; Turner, L. S.; Bai, A.; Gault, C. R.; McPherson, A. S.; Garcia, N.; Beckham, T. H.; Saad, A.; Bielawska, A.; Bielawski, J.; Hannun, Y. A.; Keane, T. E.; Taha, M. I.; Hammouda, H. M.; Norris, J. S.; Liu, X. Acid ceramidase upregulation in prostate cancer cells confers resistance to radiation: AC inhibition, a potential radiosensitizer. *Mol. Ther.* **2009**, *17* (3), 430−438.

(7) (a) Seelan, R. S.; Qian, C.; Yokomizo, A.; Bostwick, D. G.; Smith, D. I.; Liu, W. Human acid ceramidase is overexpressed but not mutated in prostate cancer. *Genes, Chromosomes Cancer* **2000**, *29* (2), 137−146. (b) Norris, J. S.; Bielawska, A.; Day, T.; El-Zawahri, A.; ElOjeimy, S.; Hannun, Y.; Holman, D.; Hyer, M.; Landon, C.; Lowe, S.; Dong, J. Y.; McKillop, J.; Norris, K.; Obeid, L.; Rubinchik, S.; Tavassoli, M.; Tomlinson, S.; Voelkel-Johnson, C.; Liu, X. Combined therapeutic use of AdGFPFasL and small molecule inhibitors of ceramide metabolism in prostate and head and neck cancers: a status report. *Cancer Gene Ther.* **2006**, *13* (12), 1045−1051. (c) Bedia, C.; Casas, J.; Andrieu-Abadie, N.; Fabrias, G.; Levade, T. Acid ceramidase expression modulates the sensitivity of A375 melanoma cells to dacarbazine. *J. Biol. Chem.* **2011**, *286* (32), 28200−28209. (d) Raisova, M.; Goltz, G.; Bektas, M.; Bielawska, A.; Riebeling, C.; Hossini, A. M.; Eberle, J.; Hannun, Y. A.; Orfanos, C. E.; Geilen, C. C. Bcl-2 overexpression prevents apoptosis induced by ceramidase inhibitors in malignant melanoma and HaCaT keratinocytes. *FEBS Lett.* **2002**, *516* (1−3), 47−52.

(8) Xia, Z.; Draper, J. M.; Smith, C. D. Improved synthesis of a fluorogenic ceramidase substrate. *Bioorg. Med. Chem.* **2010**, *18* (3), 1003−1009.

(9) (a) Bielawska, A.; Bielawski, J.; Szulc, Z. M.; Mayroo, N.; Liu, X.; Bai, A.; Elojeimy, S.; Rembiesa, B.; Pierce, J.; Norris, J. S.; Hannun, Y. A. Novel analogs of D-e-MAPP and B13. Part 2: Signature effects on bioactive sphingolipids. *Bioorg. Med. Chem.* **2008**, *16* (2), 1032−1045. (b) Bai, A.; Szulc, Z. M.; Bielawski, J.; Mayroo, N.; Liu, X.; Norris, J.; Hannun, Y. A.; Bielawska, A. Synthesis and bioevaluation of omega-*N*-amino analogs of B13. *Bioorg. Med. Chem.* **2009**, *17* (5), 1840−1848. (c) Proksch, D.; Klein, J. J.; Arenz, C. Potent inhibition of acid ceramidase by novel B-13 analogues. *J. Lipids* **2011**, *2011*, 971618. (d) Szulc, Z. M.; Mayroo, N.; Bai, A.; Bielawski, J.; Liu, X.; Norris, J. S.; Hannun, Y. A.; Bielawska, A. Novel analogs of D-e-MAPP and B13. Part 1: Synthesis and evaluation as potential anticancer agents. *Bioorg. Med. Chem.* **2008**, *16* (2), 1015−1031. (e) Grijalvo, S.; Bedia, C.; Triola, G.; Casas, J.; Llebaria, A.; Teixido, J.; Rabal, O.; Levade, T.; Delgado, A.; Fabrias, G. Design, synthesis and activity as acid ceramidase inhibitors of 2-oxooctanoyl and *N*-oleoylethanolamine analogues. *Chem. Phys. Lipids* **2006**, *144* (1), 69−84.

(10) (a) Bedia, C.; Canals, D.; Matabosch, X.; Harrak, Y.; Casas, J.; Llebaria, A.; Delgado, A.; Fabrias, G. Cytotoxicity and acid ceramidase inhibitory activity of 2-substituted aminoethanol amides. *Chem. Phys. Lipids* **2008**, *156* (1−2), 33−40. (b) Ryland, L. K.; Fox, T. E.; Liu, X.; Loughran, T. P.; Kester, M. Dysregulation of sphingolipid metabolism in cancer. *Cancer Biol. Ther.* **2011**, *11* (2), 138−149.

(11) Selzner, M.; Bielawska, A.; Morse, M. A.; Rudiger, H. A.; Sindram, D.; Hannun, Y. A.; Clavien, P. A. Induction of apoptotic cell death and prevention of tumor growth by ceramide analogues in metastatic human colon cancer. *Cancer Res.* **2001**, *61* (3), 1233−1240.

(12) Alphonse, G.; Bionda, C.; Aloy, M. T.; Ardail, D.; Rousson, R.; Rodriguez-Lafrasse, C. Overcoming resistance to gamma-rays in squamous carcinoma cells by poly-drug elevation of ceramide levels. *Oncogene* **2004**, *23* (15), 2703−2715.

(13) Realini, N.; Solorzano, C.; Pagliuca, C.; Pizzirani, D.; Armirotti, A.; Luciani, R.; Costi, M. P.; Bandiera, T.; Piomelli, D. Discovery of highly potent acid ceramidase inhibitors with in vitro tumor chemosensitizing activity. *Sci. Rep.* **2013**, *3*, 1035.

(14) (a) Andurkar, S. V.; Beguin, C.; Stables, J. P.; Kohn, H. Synthesis and structural studies of aza analogues of functionalized amino acids: new anticonvulsant agents. *J. Med. Chem.* **2001**, *44* (9), 1475−1478. (b) Höfle, G.; Steglich, W.; Vorbrüggen, H. 4-Dialkylaminopyridines as highly active acylation catalysts. *Angew. Chem., Int. Ed. Engl.* **1978**, *17*, 569−583. (c) Arnal-Herault, C.; Barboiu, M.; Pasc, A.; Michau, M.; Perriat, P.; van der Lee, A. Constitutional self-organization of adenine-uracil-derived hybrid materials. *Chemistry* **2007**, *13* (24), 6792−6800. (d) Ozaki, S.; Ike, Y.; Mizuno, H.; Ishikawa, K.; Mori, H. 5-Fluorouracil derivatives. I. The synthesis of 1-carbamoyl-5-fluorouracils. *Bull. Chem. Soc. Jpn.* **1977**, *50* (9), 2406−2412.

(15) Fang, W. P.; Cheng, Y. T.; Cheng, Y. R.; Cherng, Y. J. Synthesis of substituted uracils by the reactions of halouracils with selenium, sulfur, oxygen and nitrogen nucleophiles under focused microwave irradiation. *Tetrahedron* **2005**, *61* (12), 3107−3113.

(16) Wu, F.; Buhendwa, M. G.; Weaver, D. F. Benzhydryl as an efficient selective nitrogen protecting group for uracils. *J. Org. Chem.* **2004**, *69* (26), 9307−9309.

(17) Jaime-Figueroa, S.; Zamilpa, A.; Guzman, A.; Morgans, D. J. N-3-Alkylation of uracil and derivatives via N-1-BOC protection. *Synth. Commun.* **2001**, *31* (24), 3739−3746.

(18) Kametani, T.; Kigasawa, K.; Hiiragi, M.; Wakisaka, K.; Haga, S.; Nagamatsu, Y.; Sugi, H.; Fukawa, K.; Irino, O.; Yamamoto, T.; Nishimura, N.; Taguchi, A.; Okada, T.; Nakayama, M. Studies on the synthesis of chemotherapeutics. 10. Synthesis and antitumor activity of *N*-acyl- and *N*-(alkoxycarbonyl)-5-fluorouracil derivatives. *J. Med. Chem.* **1980**, *23* (12), 1324−1329.

(19) Compounds **4o** and **19a** were found to be unstable in DMSO-d_6 over 120 min. All the other derivatives were stable.

(20) Selected compounds, representative of the whole series, were analyzed by UPLC−MS. See Supporting Information for assay conditions and results. Compounds **16a**, **19a**, and **25a** were included in the set of compounds tested for AC inhibition, as 74%, 84%, and 78%, respectively, of the initial amounts was still present after 30 min.

(21) Reed, A. E.; Curtiss, L. A.; Weinhold, F. Intermolecular interactions from a natural bond orbital, donor−acceptor viewpoint. *Chem. Rev.* **1988**, *88* (6), 899−926.

(22) See also Supporting Information for a detailed description of the used NBO approach.

(23) The corresponding uracils **5l**, **5b**, and **12a** lacking the aliphatic chain did not cause any effect on cell viability at concentrations up to 100 μM.

(24) See Supporting Information for experimental data

(25) Solorzano, C.; Zhu, C.; Battista, N.; Astarita, G.; Lodola, A.; Rivara, S.; Mor, M.; Russo, R.; Maccarrone, M.; Antonietti, F.; Duranti, A.; Tontini, A.; Cuzzocrea, S.; Tarzia, G.; Piomelli, D. Selective *N*-acylethanolamine-hydrolyzing acid amidase inhibition reveals a key role for endogenous palmitoylethanolamide in inflammation. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106* (49), 20966−20971.

(26) Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Scalmani, G.; Barone, V.; Mennucci, B.; Petersson, G. A.; Nakatsuji, H.; Caricato, M.; Li, X.; Hratchian, H. P.; Izmaylov, A. F.; Bloino, J.; Zheng, G.; Sonnenberg, J. L.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Vreven, T.; Montgomery, J. A., Jr.; Peralta, J. E.; Ogliaro, F.; Bearpark, M.; Heyd, J. J.; Brothers, E.; Kudin, K. N.; Staroverov, V. N.; Kobayashi, R.; Normand, J.; Raghavachari, K.; Rendell, A.; Burant, J. C.; Iyengar, S. S.; Tomasi, J.; Cossi, M.; Rega, N.; Millam, J. M.; Klene, M.; Knox, J. E.; Cross, J. B.; Bakken, V.; Adamo, C.; Jaramillo, J.; Gomperts, R.; Stratmann, R. E.; Yazyev, O.; Austin, A. J.; Cammi, R.; Pomelli, C.; Ochterski, J. W.; Martin, R. L.; Morokuma, K.; Zakrzewski, V. G.; Voth, G. A.; Salvador, P.; Dannenberg, J. J.; Dapprich, S.; Daniels, A. D.; Farkas, Ö.; Foresman, J. B.; Ortiz, J. V.; Cioslowski, J.; Fox, D. J. *Gaussian 09*; Gaussian, Inc.: Wallingford, CT, 2009.

(27) Becke, A. D. Density-functional thermochemistry. 3. The role of exact exchange. *J. Chem. Phys.* **1993**, *98* (7), 5648−5652.

(28) Barone, V.; Cossi, M. Quantum calculation of molecular energies and energy gradients in solution by a conductor solvent model. *J. Phys. Chem. A* **1998**, *102* (11), 1995−2001.