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SAR and LC/MS Studies of β-Lactamic Inhibitors of Human Fatty Acid Amide Hydrolase (*h*FAAH): Evidence of a Nonhydrolytic Process

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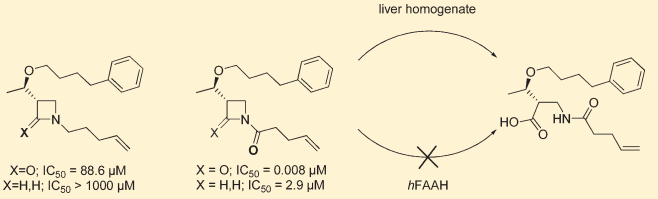
Supporting Information

ABSTRACT: The endocannabinoid hydrolyzing enzyme FAAH uses a nonclassical catalytic triad (namely, Ser-Ser-Lys instead of Ser-Asp-His) to cleave its endogenous substrates. Because inhibiting FAAH has a clear therapeutic potential, we previously developed β-lactam-type inhibitors of *h*FAAH. Here, we report the synthesis of five novel derivatives (**5**−**9**) of our lead compound 1-(pent-4-enoyl)-3(*S*)-[1(*R*)-(4-phenylbutanoyloxy)-ethyl]-azetidin-2-one (**4**, IC_{50} = 5 nM) obtained via the systematic replacement of one to three carbonyls by methylene groups. The SAR results showed that the imide, but not the lactam, function is essential to the inhibition of *h*FAAH. We also performed LC/MS analysis following incubation of our inhibitors with *h*FAAH or mouse liver. We demonstrated that *h*FAAH interacts with these β-lactam-type inhibitors but, unexpectedly, does not open the β-lactam moiety. This mechanism seems to be unique to FAAH because the β-lactam function of the inhibitors is hydrolyzed when they are incubated in the presence of the serine hydrolases expressed in the mouse liver. Finally, we confirmed these results by showing that a highly selective FAAH inhibitor (PF-750) does not prevent this hydrolysis by liver homogenates.



INTRODUCTION

The endogenous cannabinoid (CB) system has been extensively explored these last two decades due to its involvement in a lot of therapeutically promising biological effects.[1−3] Composed of two G-protein coupled receptors (GPCR) named CB_1 and CB_2 and their endogenous ligands called endocannabinoids (*N*-arachidonoylethanolamine (AEA) also named anandamide and 2-arachidonoylglycerol (2-AG), for the most studied),[4−6] the endocannabinoid system is involved in numerous physiological and pathological processes depending on the ligand, receptor, and tissue localization, which are considered.[7] Three main strategies were investigated to take advantage of the beneficial effects of CB_1 and/or CB_2 activation. First, a large number of synthetic agonists were considered, which allow the enhancement of the effects directly resulting from receptor activation.[8−12] However, most of the time psychoactive effects (due to CB_1 activation in the CNS) also occurred.[13,14] More recently, allosteric modulators of cannabinoid receptors were explored as a second strategy that would increase the effect of the endogenous ligands without displaying their characteristic side effects.[15,16] The third strategy was to design inhibitors of endocannabinoid-degrading enzymes as a potential alternative to direct agonist administration with the hope of reducing side effects associated with agonist administration. In this research line, fatty acid amide hydrolase (FAAH), the main anandamide-degrading enzyme, is the most studied.[17,18] This serine amidase possesses an unusual Ser-Ser-Lys catalytic triad, with substrate hydrolysis involving the nucleophilic attack of the Ser241 residue on the amide carbonyl group leading to an unstable acyl-enzyme intermediate. To date, small molecules featuring a wide diversity of electrophilic functions have been investigated as potent FAAH inhibitors for therapeutic applications or as pharmacological tools.[19−22] For most of them, the modes of inhibition have been studied and elucidated. Thus, different types of mechanisms have been listed, depending on the interaction between the inhibitor and the active serine: (i) a covalent interaction that leads to a stable acyl-enzyme complex, (ii) a covalent interaction that leads to a reversible tetrahedral intermediate, and (iii) a noncovalent interaction based on strong affinity. Thus, URB-597 (**1**, Chart 1)[23] and PF-750 (**2**, Chart 1)[24] were demonstrated to covalently bind FAAH by carbamylation. Indeed, the aniline and the phenol moieties, respectively, were shown by MALDI-MS mapping after trypsinization to be expelled as leaving groups, leading to stable and inactive acyl-enzyme complexes. On the contrary, OL-135 (**3**, Chart 1) covalently binds to FAAH but in a reversible manner. X-ray crystal structures of the carbamoyl intermediates formed by Ser241 acylation with **1**[25] and **2**,[26,27] as well as the tetrahedral intermediate resulting from Ser241

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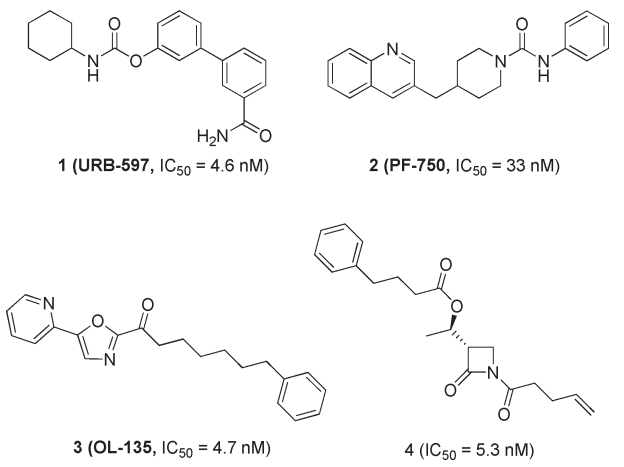
Chart 1. Chemical Structure of Previously Described FAAH-Inhibitors



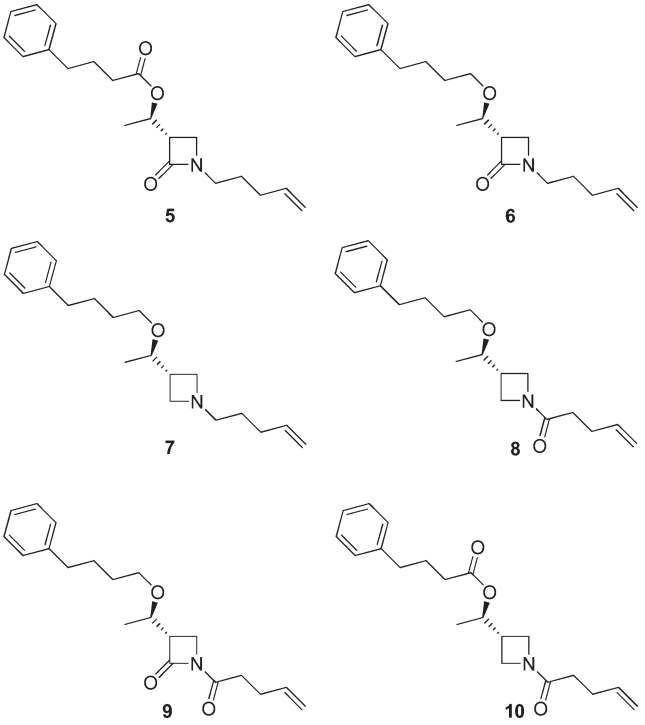
nucleophilic addition on the carbonyl function of **3**,[28,29] confirmed the covalent irreversible and reversible FAAH inhibitions, respectively.

We have recently described a novel template designed for FAAH inhibition, which is based on the β-lactam ring. From that study, a lead (i.e., 1-(pent-4-enoyl)-3(*S*)-[1(*R*)-(4-phenylbutanoyloxy)-ethyl]-azetidin-2-one **4**, Chart 1) emerged as a promising nanomolar inhibitor.[30] Preliminary experiments to determine the inhibition mode showed a fully reversible, competitive inhibition, a rather surprising result for a β-lactam-based inhibitor. Indeed, β-lactamic inhibitors of serine hydrolases usually lead to irreversible inhibition via the formation of stable acyl-enzyme complexes, resulting possibly from a suicide-type mechanism.[31] Slow reversible inhibition can also occur when the β-lactam ring is in fine hydrolyzed as a bad substrate, after being covalently attached to the active serine via its acyl-enzyme complex. Other possibilities could explain a reversible inhibition: the tetrahedral intermediate cannot evolve toward a stable acyl-enzyme complex, or the inhibitor does not interact covalently with the enzyme but has a very good affinity for the catalytic site. In order to elucidate the mode of action of FAAH β-lactamic inhibitors such as **4**, we undertook (I) a structure−activity relationship (SAR) study, based on the successive deletions of one to three carbonyls from the parent β-lactam **4**. This aims at identifying the electrophilic function(s) susceptible to interact with the FAAH active serine residue. Thus, we synthesized and evaluated five molecules (**5**−**9**) as potential FAAH inhibitors. Then, (II) we performed analyses by mass spectrometry coupled with liquid chromatography (LC/MS) to identify the products resulting from the interaction of the inhibitors with FAAH.

RESULTS

Chemistry. On the basis of the structure of our lead compound **4**, potentially six derivatives can be imagined that would possess two (**5**, **9**, **10**), one (**6**, **8**), or zero (**7**) carbonyl(s) (Chart 2) instead of the three carbonyls of **4**. Among these six analogues that we planned to synthesize, **10** was not obtained despite huge synthetic efforts, while the other ones were obtained with various difficulties. We first thought to investigate the synthetic scheme used in our previous report for the synthesis of compounds **5** to **9**.[30] Indeed, this strategy offers the possibility

Chart 2. Analogues of Compound 4



to independently modulate the two key positions N1 and C5-O thanks to the silyl ether protection of the commercial starting material ((3*S*)-[1(*R*)-(*t*butyldimethylsilyloxy)-ethyl]-azetidin-2-one (Scheme 1)). Unfortunately, drawbacks inherent to the reduction of azetidinone into azetidine and to the *O*-alkylation step ultimately guided the synthesis of compounds **8** and **9** via longer ways. Actually, their synthetic routes evolved according to the following experimental considerations: (i) the silyl ether protection did not resist to reducing conditions such as AlH_2Cl treatment, which caused the degradation of the β-lactam ring; (ii) the azetidinone reduction was not selective versus ester, amide, or imide motifs, which implied that solely the carbonyl of the lactam ring should be present on the precursors to be reduced; and (iii) the *O*-alkylation reaction did not occur when the N1 position was acylated because the anionic intermediate resulted in a rearrangement; this implied that the N1 position had to be protected with an alkyl group in the case of treatment with a strong base.

Briefly, compounds **5** and **6** were prepared from the starting material in three steps by alkylation of the N1 position, deprotection of the silyl ether group, and functionalization of the C5-O position (Scheme 1). The starting material was *N*-alkylated by reaction with 4-pentenyl bromide and KOH, in the presence of a phase transfer agent, giving compound **11** (76%).[32] Then, the silyl ether was deprotected in an acidic solution of tetrabutylammonium fluoride in tetrahydrofuran. The corresponding alcohol **12** (93%) was acylated by reaction with 4-phenylbutanoyl chloride in the presence of pyridine or alkylated using sodium hydride and 4-phenylbutyl bromide to furnish compounds **5** (80%) and **6** (92%), respectively. Azetidinone **6** was then reduced into azetidine **7** (99%) by reaction with AlH_2Cl formed in situ from a mixture of $AlCl_3$ and $LiAlH_4$.[33−36] Unfortunately, a similar sequence of reactions was not applicable for the synthesis

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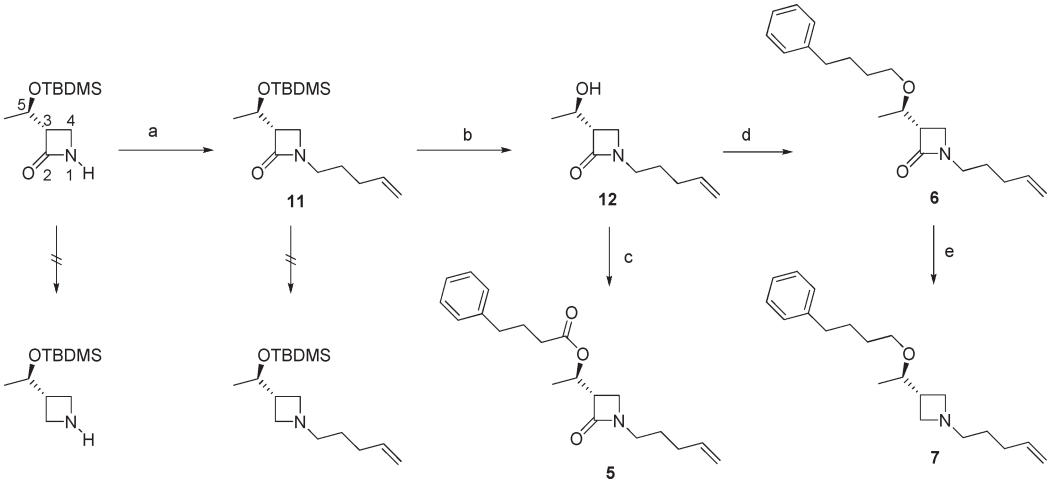
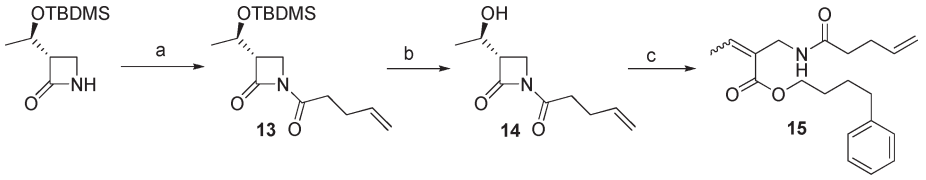
Scheme 1[a]



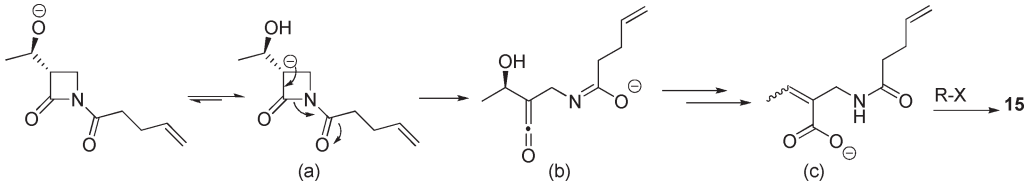
[a] Reagents and conditions: (a) 5-bromopent-1-ene, KOH, Bu_4NHSO_4, KI, THF, rt, 24 h; (b) TBAF, AcOH, THF, rt, 1 h; (c) 4-phenylbutanoyl chloride, pyridine, DCM, rt, 15 h; (d) 4-phenyl-1-butyl bromide, KI, NaH, DMF, 0 °C to rt, 4 h; (e) $LiAlH_4$, $AlCl_3$, Et_2O, 0 to 40 °C, 4 h.

Scheme 2[a]



[a] Reagents and conditions: (a) 4-pentenoyl chloride, pyridine, DCM, 45 °C, 24 h; (b) HCl, AcOH, ACN, 0 °C, 3 h; (c) 4-phenyl-1-butyl bromide, KI, NaH, DMF, 0 °C to rt, 4 h.

Scheme 3. Proposed Mechanism for the Formation of 15 in Anhydrous Conditions



of **8** and **9**. Indeed, the precursor **14**, resulting from *N*-acylation of the starting material (80%, **13**) and silyl ether deprotection with HCl (95%, **14**), could not be *O*-alkylated without β-lactam degradation (Scheme 2).

After treatment with a strong base (NaH) and 4-phenyl-1-butyl bromide, we recovered a complex mixture. The only isolated product **15**, in moderate yield (47%), resulted from the four-membered ring-opening. As proposed in Scheme 3, the driving force should be the acidity of the H3 proton due to the electron-withdrawing effect of the imide function. The N1−C2 bond of the carbanionic intermediate (Scheme 3a) might be cleaved to furnish the hydroxyketene intermediate (Scheme 3b) leading to the α,β-unsaturated carboxylate (Scheme 3c) trapped in fine by the alkyl bromide reagent (see Supporting Information). As a matter of fact, compound **12**, devoid of exocyclic carbonyl function, was readily *O*-alkylated into **6** under the same experimental conditions (see Scheme 1). We conclude that the *O*-alkylation step must occur before the *N*-acylation step. Accordingly, we decided to protect the nitrogen atom of the β-lactam ring with an alkyl group to avoid the rearrangement previously observed. The paramethoxybenzyl (PMB) group[37,38] was selected and introduced by reacting the starting material with paramethoxybenzyl bromide under phase transfer conditions (**16**, 79%). After *t*butyldimethylsilyl deprotection, the alcohol **17** (99%) was alkylated by the same procedure as that described above, to furnish compound **18** (91%) (Scheme 4). Azetidinone **18** was then reduced in azetidine **19** (73%) using monochlorohydroalane as the reductive agent. Finally, the paramethoxybenzyl group was removed by an oxidative treatment with cerium ammonium nitrate. The resulting azetidine **20** (86%) was acylated by a method of peptidic coupling using PyBOP and pentenoic acid, which resulted in compound **8** (54%).

Attempt to *N*-deprotect azetidinone **18**, similarly to azetidinone **19**, failed because the oxidized intermediate, i.e., hydroxyl group on the benzylic position, is stabilized by intramolecular hydrogen bonding and does not decompose further.[39] Thus, we

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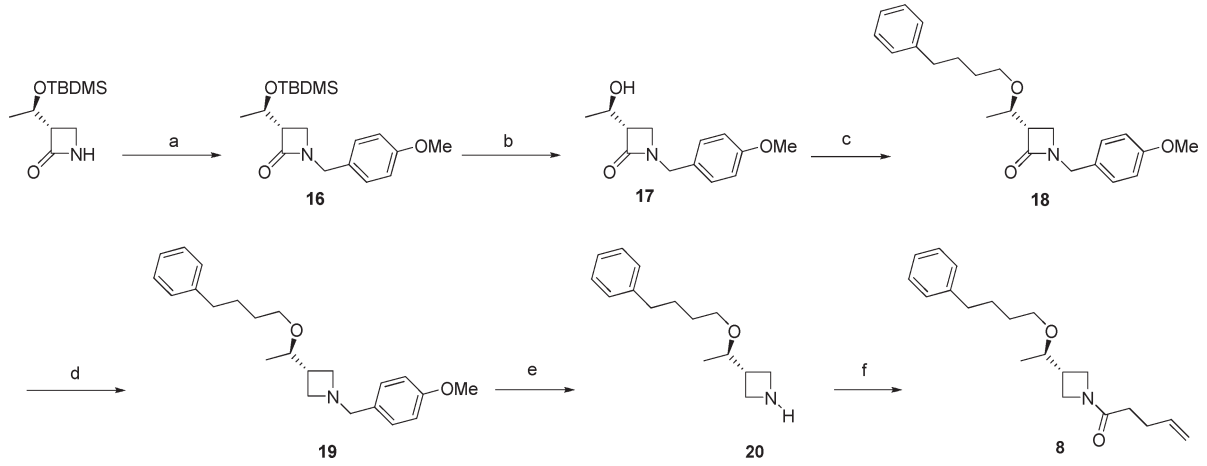
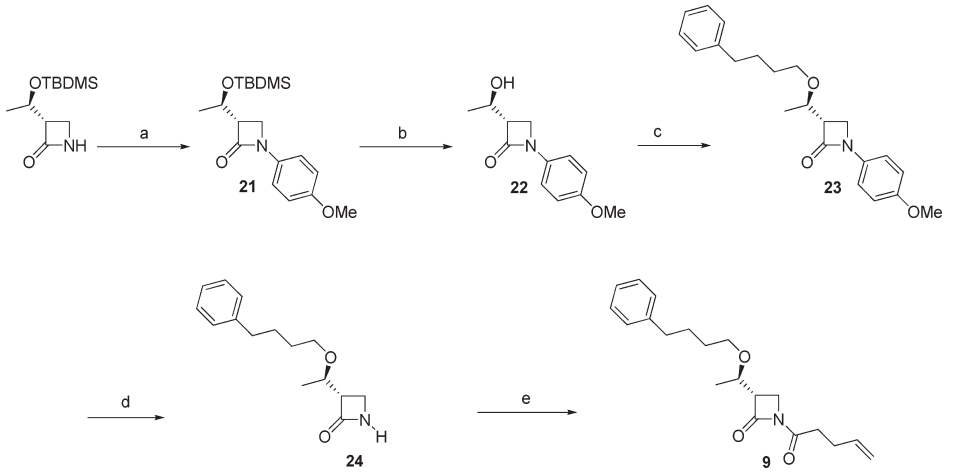
Scheme 4[a]



[a] Reagents and conditions: (a) PMBBr, KOH, Bu_4NHSO_4, KI, THF, rt, 24 h; (b) TBAF, AcOH, THF, rt, 1 h; (c) 4-phenylbutyl bromide, KI, NaH, DMF, 0 °C to rt, 4 h; (d) $LiAlH_4$, $AlCl_3$, Et_2O, 0 to 40 °C, 4 h; (e) CAN, H_2O, ACN, 70 °C, 1 h; (f) 4-pentenoic acid, DIEA, PyBOP, DMF, rt, 15 h.

Scheme 5[a]



[a] Reagents and conditions: (a) bromoanisole, *N*,*N*-dimethylethylenediamine, CuI, K_2CO_3, dioxane, 105 °C, 24 h; (b) TBAF, AcOH, THF, rt, 1 h; (c) 4-phenylbutyl bromide, KI, NaH, DMF, 0 °C to rt, 4 h; (d) CAN, H_2O, ACN, −15 °C, 15 min; (e) 4-pentenoyl chloride, pyridine, DCM, 45 °C, 24 h.

had to modify our synthetic route, and the paramethoxyphenyl (PMP) substituent was considered as the *N*-protecting group (Scheme 5).[40,41] Compound **21** (61%) was obtained by organometallic coupling between the starting material and paramethoxyphenyl bromide[42] and then *O*-deprotected as already described. The corresponding alcohol **22** (99%) was alkylated under standard conditions. Compound **23** (37%) was obtained in moderate yield and with some contamination with a diastereoisomer (partial epimerization at the C3 position) that could not be separated. PMP was easily removed using cerium ammonium nitrate, and compound **24** (80%) was *N*-acylated with 4-pentenoyl chloride in the presence of pyridine giving azetidinone **9** (47%) (Scheme 5).

Despite numerous efforts, azetidine **10** (see Chart 2) could not be prepared starting from the different available potential precursors **4**, **17**, **22**, and the commercial starting material by applying synthetic routes inspired from Schemes 1, 4, and 5.

The synthesized analogues of compound **4** and all intermediates were fully characterized by the usual spectroscopies (see Experimental Section). Of note, we observed particular features concerning azetidine ^1H and ^{13}C NMR spectra due to the flipping of the small ring. Indeed, spectra of compounds **7**, **8**, and **20** revealed split, broad, or missing signals in $CDCl_3$ or C_6D_6 at 25 °C, especially for protons or carbons at the positions 2, 4, and 5 of the β-lactamic core. Experiments with **8** in C_6D_6 at 25 °C showed that these signals were split into two but that a coalescence of signals occurred when raising the temperature to 75 °C, confirming the presence of conformers (see Supporting Information).

Biochemical Evaluations. The inhibition activity of compounds **5** to **9** was evaluated on human recombinant FAAH. Then, in order to determine whether our azetidinones are hydrolyzed by FAAH or not, incubation mixtures were analyzed by HPLC/MS using a high resolution mass spectrometer (LTQ-orbitrap) as LC detector. First, hydrolytic products from compounds

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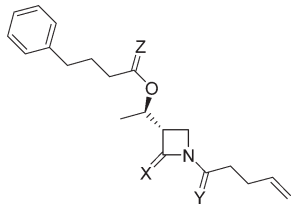
Table 1. Determination of the Inhibition Activity Towards Human FAAH



compd	X	Y	Z	pI50	IC_{50}[a]
4	O	O	O	8.27 ± 0.05	0.005
5	O	H, H	O	4.08 ± 0.03	82.8
6	O	H, H	H, H	4.05 ± 0.05	88.6
7	H, H	H, H	H, H	<3.5	>1000
8	H, H	O	H, H	5.54 ± 0.06	2.9
9	O	O	H, H	8.09 ± 0.06	0.008

[a] IC_{50} in μM (from three independent experiments performed in duplicate).

4 and **9** were analyzed after incubation in the presence of murine liver homogenate. Then, compound **9** was incubated with murine liver homogenate after a preincubation with URB-597 or PF-750 (see **1** and **2**, Chart 1), known for being irreversible and selective FAAH inhibitors. The results were then compared to similar experiments performed in the presence of human recombinant FAAH.

FAAH Inhibition. Human recombinant enzyme, developed in our laboratory,[43] was used in a competitive hydrolytic assay using [^{3}H]-AEA as the substrate. Tested compounds, enzyme, and [^{3}H]-AEA were incubated at 37 °C during 10 min. The extent of inhibition was evaluated by liquid scintillation counting of the [^{3}H]-ethanolamine resulting from the hydrolysis of the labeled AEA. Regarding the inhibition potencies summarized in Table 1, the relative importance of the three carbonyls (X, Y, and Z) can be discussed as follows: (i) a significant loss of activity occurs when the *N*-acyl chain is replaced by a *N*-alkyl chain independently of the nature of the C5-O chain (compare compounds **5** and **6** to **4**); (ii) the loss of activity is total when the carbonyl of the β-lactam ring is also deleted (compound **7**); (iii) the activity is linked to the exocyclic *N*-carbonyl and not so much to the β-lactam carbonyl (compare compounds **6** and **8**); and (iv) the deletion of the C5-O carbonyl has no influence on the activity (compare compounds **9** and **4**). On the basis of these observations, and contrary to what could be expected, we conclude that the β-lactam carbonyl is not the main electrophilic group responsible for FAAH inhibition. Indeed, its sole preservation leads to a 40-times less potent inhibitor (**6**) than the analogue in which the exocyclic *N*-carbonyl was exclusively preserved (**8**). However, the inhibition potency is completely preserved when both *N*-carbonyls are kept from the original structure, which clearly indicates that the imide function is the most important, at the expense of the ester one, for the inhibition of human FAAH.

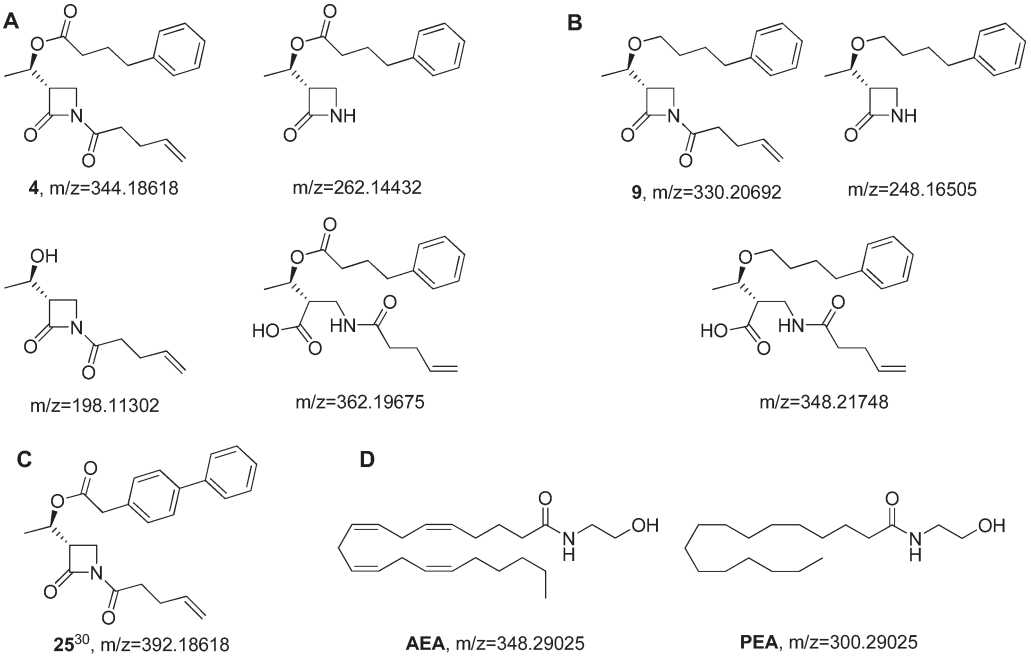
Mechanistic Studies by HPLC/MS Analysis. We used a high accuracy mass spectrometer coupled to a high performance liquid chromatography apparatus (HPLC) to study the crude mixtures obtained by incubating the β-lactams **4** and **9** (the most active compounds of Table 1) with mouse liver homogenate and with recombinant *h*FAAH, in Tris-HCl buffer at 37 °C for 90 min. The reactions were stopped with cold acetonitrile. After the addition of the internal standard (**25**, see Chart 3C) and centrifugation, the supernatants were concentrated and injected in the HPLC/MS system. In order to avoid artifacts due to matrix effects, the blank controls (for chemical hydrolysis) were performed with heat-denatured enzymes (either *h*FAAH or liver homogenate depending on the assay). For comparison, the enzymatic hydrolysis of anandamide (AEA) was similarly examined by HPLC/MS, using *N*-palmitoylethanolamine (PEA) as the internal standard (Chart 3D). All compounds (native inhibitors/substrates, hydrolysis products, and internal standards, see Chart 3) are separated on the HPLC reverse phase column and detected by MS as $[M + H]^+$ positive ions. Chart 3 gathers the species detected and the corresponding m/z values. Results are given as the area under curve (AUC) ratios of measured compounds to the respective internal standard.

Assay with Liver Homogenate. The mammalian liver contains a number of hydrolases, including FAAH. Thus, we considered murine liver homogenate as a good model to assess the stability of compounds **4** and **9** under physiological conditions. The β-lactam ring-opening by hydrolases is a general reaction that we expected to occur, as well as the ester hydrolysis in the case of compound **4**, leading to the cleavage of the C5 side chain. Moreover, hydrolysis of the exocyclic N1—C═O bond cannot be excluded. Accordingly, after the incubation of **4** and **9** with liver homogenate (10^{-5} M final concentration, 37 °C, 90 min), we mainly detected by HPLC/MS the ions at m/z = 362.19675 and m/z = 348.21748 corresponding to the hydrolytic ring-opening of **4** and **9**, respectively. For compound **4**, the ion at m/z = 198.11302, which results from the cleavage of the ester function, was also visible (see Supporting Information). Additionally, m/z = 262.14432 (Chart 3A) and m/z = 248.16505 (Chart 3B) ions, which correspond to the imide bond cleavage, were also detected but in tenuous amounts (see Supporting Information). Figure 1 shows the disappearance of the parent compounds **4** and **9** under enzymatic hydrolysis ((+) liver homogenate) by measuring the AUC for the peaks corresponding to the ions at m/z = 344.18618 and m/z = 330.20692, respectively. The data are expressed relative to the signal obtained by incubating the parent compounds in the presence of heat-inactivated liver homogenate ((−) liver homogenate) (see Supporting Information). Following 90 min of incubation in the presence of liver homogenate, more than 80% of β-lactams **9** and **4** (10^{-5} M) were hydrolyzed. Note, however, that **9** is twice as stable as **4**, probably most due to the replacement of the ester bond (at the C5-O position) with an ether bond.

With these results, we have validated the HPLC/MS method for further detection of the potential hydrolytic products of our compounds under FAAH processing. The next experiments were conducted with β-lactam **9** using anandamide (AEA) for comparison.

To determine whether the hydrolysis of compound **9** by liver homogenate is FAAH-dependent, the same experiment was performed in the presence of FAAH inhibitors **1** and **2** (see Chart 1). First of all, we confirmed the presence of FAAH activity in our liver homogenate preparation, by measuring the hydrolysis of anandamide (AEA) in the same conditions. We found a strong decrease in AEA (10^{-5} M) following a 90 min incubation, which was completely prevented when the liver homogenate was preincubated with FAAH inhibitors **1** or **2** (Figure 2A). This clearly shows that the FAAH activity in the liver is sufficient for a total hydrolysis of its substrate and that we can inhibit this activity

Chart 3. Ions Detected in the HPLC/MS Analysis[a]



[a]Structure and exact mass for the $[M + H]^+$ ions detected by HPLC/MS analysis of incubation media containing **4** (A) or **9** (B) and native (or denatured) *h*FAAH or liver homogenate. (C) Compound **25**, 1-(pent-4-enoyl)-(3*S*)-3-[1(*R*)-(biphenylacetyloxy)-ethyl]-azetidin-2-one,[30] was used as the internal standard for the experiments performed with **4** and **9**. (D) Structure and exact mass for anandamide (AEA) and *N*-palmitoylethanolamine (PEA) (used as the internal standard for experiments involving anandamide) $[M + H]^+$ ions.

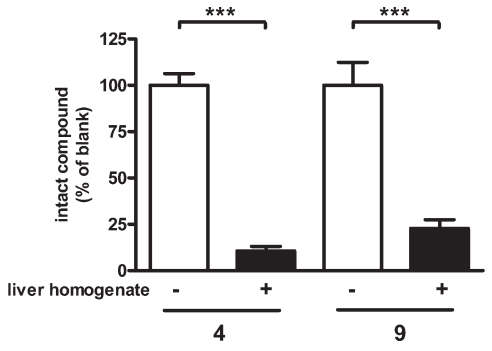


Figure 1. HPLC/MS analysis of **4** and **9** hydrolysis by liver homogenate. Detection of m/z = 344.18618 and m/z = 330.20692 ions corresponding to the $[M + H]^+$ ions of **4** and **9**, respectively, after incubation with mouse liver homogenate. The data are reported to the signal obtained for the chemical hydrolysis ((−) liver homogenate), which was determined using heat-inactivated liver homogenate. (***) $P < 0.0001$ compared to that of denatured liver (Student's t test). Data are shown as the mean ± SEM of 3 independent experiments performed in duplicate.

using irreversible FAAH inhibitors such as **1** and **2**. When analyzing the fate of β-lactam **9**, a difference between the effect of inhibitors **1** and **2** was observed, considering either the disappearance of the native compound at m/z = 330.20692 (Figure 2B) or the appearance of the hydrolytic product at m/z = 348.21748 (Figure 2C). From Figure 2B, it clearly appears that **1** completely prevents the hydrolysis of **9**, whereas **2** had no effect since **9** was hydrolyzed to the same extent as that in the control. Accordingly, the ion due to the azetidinone ring-opening (m/z = 348.21748), was detected in a large amount in the presence of **2**, whereas it was almost not detected in the presence of **1** (Figure 2C). These results, at first surprising, may be explained by the reported lower selectivity of **1** toward FAAH compared to that for **2**[24,44] and suggest that **9** is not hydrolyzed by FAAH but by other hydrolase(s) present in the liver.

Assay with Recombinant *h*FAAH. To confirm the above-mentioned results and to firmly establish that FAAH was not responsible for the hydrolysis of **9**, we performed a similar set of experiments but using recombinant human FAAH instead of liver homogenate.

First, we used AEA to confirm the suitability of our experimental conditions. As expected, an almost total AEA hydrolysis occurred in the presence of *h*FAAH, and this hydrolysis was fully blocked in the presence of the irreversible inhibitors **1** and **2** (Figure 3A). Confirming what was suggested with the liver homogenates, β-lactam **9** was not hydrolyzed at all, including in the absence of inhibitors **1** or **2** (Figure 3B). We performed additional experiments using larger amounts of *h*FAAH (150 μg/tube versus 90 μg/tube), and even in this case, no hydrolytic processing was observed (data not shown).

DISCUSSION

To date, only a few published FAAH inhibitors are described as acting in a reversible manner. Their structures feature benzothiazole,[45] (thio)hydanthoin,[46] and oxime carbamate[47] moieties, whose interaction with a FAAH catalytic pocket could not be experimentally established. Indeed, short-lived and transient phenomena most often prove to be hardly detectable by usual protein analysis by MS and X-ray diffraction methods. Note, however, that very recently, ketobenzimidazoles described as reversible inhibitors were demonstrated to act by a noncovalent mechanism.[48] Indeed,

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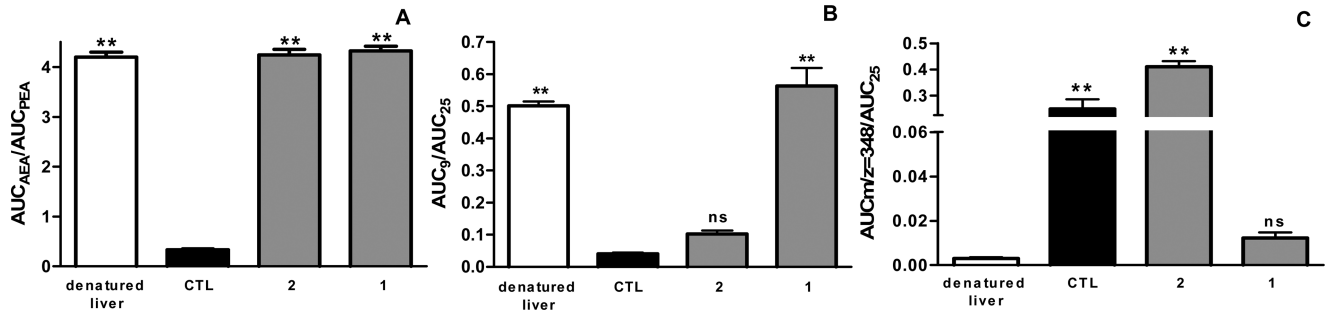

Figure 2. Study of the influence of FAAH inhibitors on the liver homogenate hydrolysis of AEA and **9**. AEA (m/z = 348.29025) (A) and **9** (m/z = 344.18618) (B) were incubated at 10^{-5} M with liver homogenates in the presence (or not) of the FAAH inhibitors **1** and **2** (5.10^{-5} M). Heat-denaturated liver homogenate was used as the control for chemical hydrolysis and used to normalize the data. PEA and **25** were used as internal standards for the HPLC/MS analysis in A and B, respectively. (C) The relative quantification of the azetidinone ring-opening product during the incubation of **9**. (**) $P < 0.01$ compared to that of the denatured liver (C) or control (A and B) (ANOVA one-way, Dunnett's post-test). Data are shown as the mean ± SEM of 3 independent experiments performed in duplicate.

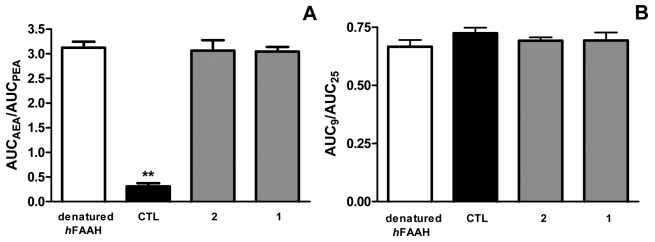

Figure 3. Study of the role of FAAH in the hydrolysis of **9**. AEA (m/z = 348.29025) (A) and **9** (m/z = 344.18618) (B) were incubated at 10^{-5} M with recombinant *h*FAAH in the presence (or not) of the FAAH inhibitors **1** and **2** (5.10^{-5} M). Heat-denaturated enzyme preparation was used as the control for chemical hydrolysis and used to normalize the data. PEA and **25** were used as internal standards for the HPLC/MS analysis in A and B, respectively. (**) $P < 0.01$ compared to that of denatured *h*FAAH (ANOVA one-way, Dunnett's post-test). Data are shown as the mean ± SEM of 2 (AEA) or 3 (**9**) independent experiments performed in duplicate.

from X-ray studies of cocrystal structures, the authors concluded that a noncovalent inhibition mechanism exists because of the absence of covalent interaction between Ser241 and the inhibitors.[49] The present study has established that our β-lactamic inhibitors of *h*FAAH are not substrates of this enzyme, although they can be degraded by other hydrolases present in mammalian liver. A SAR study based on the systematic replacement of C═O functions of the lead compound **4** with CH_2 units confirmed that the azetidinone carbonyl is not essential for activity but rather reinforces the effect of the exocyclic carbonyl. Thus, the imide function clearly appears to be essential for an efficient *h*FAAH inhibition: compounds **8** and **9** exhibit good to excellent activities as does **4**, whereas analogues **5** and **6** show significantly lower activities. Mass spectrometry is now recognized as an accurate analytical tool in medicinal chemistry.[50] Thus, we have validated a HPLC/MS assay to analyze crude mixtures from enzymatic reactions and follow (i) the disappearance of native inhibitors **4** and **9** and (ii) the appearance of their respective potential hydrolysis products. Two different sources of FAAH were considered: a mouse liver homogenate in which a wide diversity of serine hydrolases is present and a human recombinant FAAH. For the identification of the components exclusively due to the reaction with FAAH, we preincubated both enzyme sources with URB-597 (**1**) and PF-750 (**2**), two standard irreversible FAAH inhibitors. Interestingly, the assay with liver homogenate showed a dramatic difference between the inhibitors **1** and **2**. Several hydrolases from liver were inhibited in the presence of **1**, whereas **2** only inhibited FAAH. Consequently, β-lactam **9** was fully protected from hydrolases in the presence of **1** and was almost completely hydrolyzed in the presence of **2**. Additionally, a similar experiment was performed with recombinant *h*FAAH. In this case, **9** did not undergo any processing, independently of the presence of inhibitors **1** or **2**. Taken together, these results bear strong evidence for a nonhydrolytic interaction between our β-lactamic inhibitor and FAAH. Moreover, we have indirectly demonstrated that PF-750 (**2**) is a selective inhibitor of FAAH, while URB-597 (**1**) is not selective for FAAH since it inhibits other hydrolases present in a liver homogenate. This problem has recently been commented on in the literature.[24,44] Because we demonstrated that **9** is not a slow substrate for FAAH, the mechanism by which it reversibly inhibits FAAH is questionable. Indeed, the fact that a β-lactam ring is left intact in the presence of a serine hydrolase is quite unusual. The experiments we performed with liver homogenates, i.e., in the presence of a wide variety of serine hydrolases, confirm that a β-lactam ring is easily processed by the classical Ser-Asp-His catalytic triad. However, FAAH is a member of a distinct serine hydrolase family, featuring the amidase signature and possessing its own catalytic Ser-Ser-Lys triad. Thus, here, it seems that the β-lactam ring is not targeted by the active serine of this unaccustomed triad. However, although this carbonyl is not the key electrophilic function, the β-lactam ring appears to be necessary for an efficient FAAH inhibition. Indeed, azetidine **8** is a 300-fold less potent inhibitor compared to **9**, demonstrating that both the exo and the endo carbonyls of the imide function are essential. These results suggest that the ensemble of endo- and exocyclic carbonyls, which constitute the imide function, is the pharmacophore in FAAH inhibition and not the β-lactam ring alone. Indeed, the β-lactam ring is probably not attacked by the active serine because in this case, the resulting tetrahedral intermediate would evolve toward the C2−N1 bond cleavage with the release of the four-membered cyclic strain as the driving force. Thus, we hypothesize here that the β-lactam scaffold correctly presents the exocyclic carbonyl of the imide function to the active serine of *h*FAAH. Two explanations may be proposed for the mechanism of FAAH inhibition: (i) the exocyclic carbonyl, which is directed toward the active serine Ser241 (from previous modelization studies),[30] may undergo nucleophilic

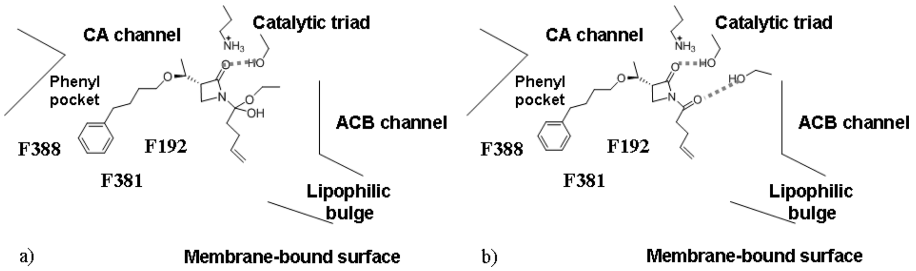

Figure 4. Schematic proposed binding mode between **9** and FAAH.

attack (Figure 4a). Then, the resulting tetrahedral intermediate is reversed, probably because of the absence of proton transfer to N1 as required to be a good leaving group; (ii) there is no nucleophilic attack at all. A high affinity between the *N*-acyl-β-lactam and the catalytic site occurs (Figure 4b). Because of the limited purity and stability of recombinant *h*FAAH and the absence of X-ray data, the real structure of the inhibitor-*h*FAAH complex formed with our β-lactamic inhibitor is not experimentally accessible for discriminating between the two mechanisms of reversible, nonprocessing inhibition.

CONCLUSIONS

We have disclosed a novel class of potent *h*FAAH reversible inhibitors, featuring an imide function, which is part of a β-lactam ring that is not hydrolyzed by the enzyme. The behavior of β-lactams **4** and **9** in the presence of *h*FAAH and other hydrolases from the liver was accurately analyzed thanks to a HPLC/MS method validated with a set of reference compounds (AEA (substrate), URB-597, and PF-750 (**1** and **2**, irreversible inhibitors)). Works are in progress to further exploit the imide pharmacophore embedded into various cyclic templates for the search of new FAAH inhibitors of pharmacological interest.

EXPERIMENTAL SECTION

Chemistry. All solvents, including anhydrous solvents and reagents were purchased from Acros Organics, Alfa Aesar, Cayman chemical, Fluka, Sigma-Aldrich, or VWR and used without any further purification. [^{3}H]-AEA (60 Ci/mmol) was purchased from American Radiolabeled Chemical (St Louis, MO). UltimaGold scintillation liquid was bought from Perkin-Elmer. All reactions under dry conditions were performed under argon atmosphere in flame-dried glassware. Nuclear Magnetic Resonance (^{1}H NMR and ^{13}C NMR) spectra were recorded at 300 MHz for proton and 75 MHz for carbon (Bruker Avance 300) or 500 MHz for proton and 125 MHz for carbon (Bruker Avance 500) using deuterated chloroform, methanol, or benzene. Chemical shifts are reported in ppm relative to the signals of residual nondeuterated solvents ($CDCl_3$, 7.26 and 77.16 ppm; CD_3OD, 3.31 and 49.00 ppm; C_6D_6, 7.16 and 128.06 ppm). NMR coupling constants (J) are reported in hertz. Melting points (mp) were determined on a Büchi B-540 apparatus calibrated with caffeine, vanillin, and phenacetin. Rotations were recorded on a Perkin-Elmer 241 MC polarimeter, at 20 °C, in $CHCl_3$, except for compound **20** in CH_3OH. Concentrations are given in percentage (g/100 mL). Low resolution mass spectra were acquired using a Thermo Finnigan LCQ spectrometer in negative mode of electrospray ionization (ESI). High Resolution Mass Spectrometry (HRMS) analyses were performed at the University College London (UK). Infrared (IR) spectra were recorded using a FTIR-8400S Shimadzu apparatus. Products were analyzed as thin films deposited on a Se−Zn crystal by evaporation from CH_2Cl_2 solutions. TLC analysis was performed on Merck silica-gel $60F_{254}$ with detection under UV light, and flash chromatography was performed on silica gel (40−60 mesh) purchased from Rocc (Belgium). Purity of tested compounds was assessed by HPLC on a chiralpak IA column (4.6 mm × 250 mm, 5 μm particle size) using a hexane/isopropanol eluent (95:05), at a flow rate of 1.0 mL/min, and on a symmetry C18 column (4.6 mm × 250 mm, 5 μm particle size) using a gradient of acetonitrile/H_2O eluent (50:50 to 100:0), at a flow rate of 1.2 mL/min (purity ≥97%). For compound **9**, contamination with the C3 diastereoisomer is less than 20%.

General Procedure for *N*-Alkylation (11 and 16). To a stirred solution of the starting azetidinone (1 equiv) in tetrahydrofuran (9.2 mL/mmol) at r.t. were added tetrabutylammonium hydrogen sulfate (0.2 equiv), sodium iodide (4 equiv), potassium hydroxide (2 equiv), and the suitable alkyl bromide (4 equiv). The mixture was stirred for 15 h, the inorganic precipitate was filtered off and washed with tetrahydrofuran, and the filtrate was concentrated under vacuum. After purification by flash chromatography (cyclohexane/ethyl acetate), a colorless oil was obtained (**11** and **16**).

*1-(Pent-4-enyl)-3(S)-[1(R)-(tert-butyldimethylsilyloxy)-ethyl]-azetidin-2-one (**11**).* Yield: 76% (198 mg from 0.87 mmol of the starting material). $[\alpha]_D = -36.9$ (c = 3.0). $R_f = 0.29$ (cyclohexane/ethyl acetate: 5/2). MS (ESI): m/z 298.16 ($(M + H)^+$), 320.17 ($(M + Na)^+$). ^{1}H NMR (300 MHz, $CDCl_3$): δ = −0.06 (s, 3H), −0.05 (s, 3H), 0.74 (s, 9H), 1.05 (d, 3H, J = 6.3 Hz), 1.50 (m, 2H), 1.95 (m, 2H), 2.94−3.19 (m, 5H), 4.06 (m, 1H), 4.80−4.95 (m, 2H), 5.65 (ddt, 1H, J = 10.5 Hz, J = 17.5 Hz, J = 6.5 Hz). ^{13}C NMR (75 MHz, $CDCl_3$): δ = −5.0, −4.6, 17.8, 22.5, 25.6, 26.8, 31.0, 40.89, 40.92, 57.0, 65.0, 115.2, 137.2, 168.1. IR (cm^{-1}): υ = 2856−2953, 1747, 1641, 1472, 1404, 1252, 835. HRMS: $C_{16}H_{31}NO_2SiNa$ calculated, 320.2022; found, 320.2036.

General Procedure for Silyl Ether Deprotection (12, 17, and 22). To a stirred solution of silyl ether (1 equiv) in dry tetrahydrofuran (33 mL/mmol) at r.t. was added, dropwise, a solution of tetrabutyl ammonium fluoride in tetrahydrofuran (5 equiv). The solution was stirred for 1 h, and then acetic acid was added (2.2 equiv). The solution was stirred for an additional 15 min and then extracted three times with dichloromethane. The organic layers were combined, washed with brine and water, dried over $MgSO_4$, filtered, and concentrated under vacuum. After purification by flash chromatography (ethyl acetate/methanol), a colorless oil (**12**) or a white solid (**17** and **22**) was obtained.

*1-(Pent-4-enyl)-3(S)-[1(R)-hydroxyethyl]-azetidin-2-one (**12**).* Yield: 93% (135.5 mg from 0.79 mmol of **11**). $[\alpha]_D = -22.4$ (c = 1.1). $R_f = 0.33$ (ethyl acetate/methanol: 99/1). MS (ESI): m/z 184.20 ($(M + H)^+$), 206.15 ($(M + Na)^+$). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.20 (d, 3H, J = 6.3 Hz), 1.57 (m, 2H), 2.02 (m, 2H), 2.98−3.23 (m, 5H), 3.25 (broad s, 1H), 4.07 (m, 1H), 4.82−5.04 (m, 2H), 5.72 (ddt, 1H, J = 10.5 Hz, J = 17.2 Hz, J = 6.5 Hz). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 21.4, 26.7, 31.0, 41.1, 41.6, 56.7, 64.8, 115.4, 137.3, 168.6. IR (cm^{-1}): υ = 3402, 2928, 1717, 1641, 1418, 1238. HRMS: $C_{10}H_{18}NO_2$ calculated, 184.13375; found, 184.13297.

1-(Pent-4-enyl)-3(*S*)-[1(*R*)-(4-phenylbutanoyloxy)-ethyl]-azetidin-2-one (5). To a stirred solution of **12** (1 equiv) in dry

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dichloromethane (20 mL/mmol) at r.t. were added pyridine (2 equiv) and 4-phenylbutanoyl chloride (2 equiv) under argon atmosphere. After stirring overnight, the mixture was diluted in dichloromethane, and the excess of acyl chloride was quenched by a 10% aqueous solution of Na_2CO_3. The organic layer was washed with 3 N aqueous solution of HCl and brine, dried over $MgSO_4$, filtered, and concentrated under vacuum. After purification by flash chromatography (dichloromethane/ethyl acetate), a colorless oil was obtained. Yield: 80% (33 mg from 0.12 mmol of **12**). $[\alpha]_D = -2.1$ (c = 1.8). $R_f = 0.34$ (cyclohexane/ethyl acetate: 5/3). MS (ESI): m/z 330.18 ($(M + H)^+$), 352.15 ($(M + Na)^+$). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.35 (d, 3H, J = 6.3 Hz), 1.61 (m, 2H), 1.93 (m, 2H), 2.07 (m, 2H), 2.30 (t, 2H, J = 7.5 Hz), 2.63 (t, 2H, J = 7.6 Hz), 3.07−3.33 (m, 5H), 4.90−5.08 (m, 2H), 5.21 (m, 1H), 5.77 (ddt, 1H, J = 10.5 Hz, J = 17.2 Hz, J = 6.5 Hz), 7.12−7.31 (m, 5H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 18.7, 26.6, 26.8, 31.0, 33.9, 35.1, 41.3, 42.7, 54.5, 68.6, 115.7, 126.1, 128.5, 128.6, 137.3, 141.4, 166.7, 172.7. IR (cm^{-1}): υ = 2862−2930, 1744, 1728, 1641, 1454, 1413, 1240, 1134. HRMS: $C_{20}H_{27}NO_3Na$ calculated, 352.1889; found, 352.1900.

General Procedure for *O*-Alkylation (6 and 18). To a stirred suspension of sodium hydride (4 equiv) in dry dimethylformamide (6 mL/mmol of alcohol precursor) at 0 °C was added, dropwise, the alcohol precursor (1 equiv) in dry dimethylformamide (6 mL/mmol of alcohol precursor) under argon atmosphere. The suspension was stirred for 30 min at 0 °C, and then freshly dried potassium iodide (3 equiv) and 4-phenyl-1-butyl bromide (3 equiv) were added. The suspension was stirred for an additional 30 min and then allowed to warm up to r.t. After 4 h, the reaction was quenched, at low temperature, with an aqueous saturated solution of NH_4Cl, and the aqueous layer was extracted several times with diethyl ether. The organic layers were combined, dried over $MgSO_4$, filtered, and concentrated under vacuum. After purification by flash chromatography (cyclohexane/ethyl acetate), a colorless oil was obtained in all cases.

*1-(Pent-4-enyl)-3(S)-[1(R)-(4-phenylbutoxy)-ethyl]-azetidin-2-one (**6**).* Yield: 92% (196.1 mg from 0.68 mmol of **12**). $[\alpha]_D = -25.7$ (c = 1.1). R_f = 0.47 (cyclohexane/ethyl acetate: 5/3). MS (ESI): m/z 316.16 ($(M + H)^+$), 338.23 ($(M + Na)^+$). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.21 (d, 3H, J = 6.3 Hz), 1.51−1.72 (m, 4H), 2.07 (m, 2H), 2.61 (t, 2H, J = 7.5 Hz), 3.04−3.29 (m, 4H), 3.40 (td, 1H, J = 6.3 Hz, J = 12.5 Hz, AB system), 3.57 (td, 1H, J = 6.3 Hz, J = 12.5 Hz, AB system), 3.72 (m, 1H), 4.95−5.08 (m, 2H), 5.77 (ddt, 1H, J = 10.5 Hz, J = 17.2 Hz, J = 6.5 Hz), 7.12−7.31 (m, 5H). ^{13}C NMR (75 MHz, $CDCl_3$): δ =18.5, 26.8, 28.1, 29.7, 31.0, 35.7, 41.1, 42.1, 55.7, 68.9, 72.7, 115.4, 125.7, 128.3, 128.4, 137.4, 142.5, 168.4. IR (cm^{-1}): υ = 2860−2932, 1747, 1641, 1452, 1407, 1103. HRMS: $C_{20}H_{29}NO_2Na$ calculated, 338.2096; found, 338.2107.

*1-(4-Methoxybenzyl)-3(S)-[1(R)-(4-phenylbutoxy)-ethyl]-azetidin-2-one (**18**).* Yield: 91% (156 mg from 0.46 mmol of **17**). $[\alpha]_D = 8.0$ (c = 1.9). R_f = 0.23 (cyclohexane/ethyl acetate: 1/1). MS (ESI): m/z 368.24 ($(M + H)^+$), 390.29 ($(M + Na)^+$). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.20 (d, 3H, J = 6.2 Hz), 1.48−1.77 (m, 4H), 2.62 (t, 2H, J = 7.4 Hz), 3.04−3.24 (m, 3H), 3.39 (td, 1H, J = 6.2 Hz, J = 12.4 Hz, AB system), 3.56 (td, 1H, J = 6.2 Hz, J = 12.4 Hz, AB system), 3.75 (m, 1H), 3.79 (s, 3H), 4.21 (d, 1H, J = 14.9 Hz, AB system), 4.39 (d, 1H, J = 14.9 Hz, AB system), 6.85 (d, 2H, J = 8.6 Hz), 7.12−7.31 (m, 7H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 18.5, 28.2, 29.9, 35.9, 42.1, 45.1, 55.3, 56.0, 69.0, 72.7, 114.1, 125.8, 128.4, 128.5, 129.5, 142.6, 159.3, 168.5. IR (cm^{-1}): υ = 2860−2932, 1747, 1610, 1512, 1452, 1402, 1246. HRMS: $C_{23}H_{29}NO_3$Na calculated, 390.2045; found, 390.2056.

General Procedure for the Reduction of Azetidin-2-one (7 and 19). To a stirred suspension of aluminum chloride (3 equiv) in dry diethyl ether (12 mL/mmol of azetidin-2-one) at 0 °C was added lithium aluminum hydride (3 equiv) under argon atmosphere. The suspension was stirred for 10 min, then refluxed for 30 min, and finally, the azetidin-2-one (1 equiv) was added dropwise in dry diethyl ether (6 mL/mmol of azetidin-2-one). After 4 h, the reaction mixture was cooled, and water was added. The aqueous layer was extracted with dichloromethane. The organic layers were combined, dried over $MgSO_4$, filtered, and concentrated under vacuum. After purification by flash chromatography (dichloromethane/methanol), a colorless oil was obtained in all cases.

*1-(Pent-4-enyl)-3(S)-[1(R)-(4-phenylbutoxy)-ethyl]-azetidine (**7**).* Yield: 99% (148 mg from 0.50 mmol of **6**). $[\alpha]_D = -26.0$ (c = 3.0). R_f = 0.21 (dichloromethane/methanol: 9/1). MS (ESI): m/z 302.17 ($(M + H)^+$). ^{1}H NMR (500 MHz, $CDCl_3$, 25 °C): δ = 0.97 (d, 3H, J = 6.3 Hz), 1.51−1.72 (m, 4H), 2.04 (m, 2H), 2.59 (m, 2H), 2.90 (m, 3H), 3.29 (m, 1H), 3.41 (broad signal, 1H), 3.60 (m, 2H), 3.70 (broad signal, 1H), 4.16 (broad signal, 2H), 4.80−4.95 (m, 2H), 5.64 (ddt, 1H, J = 10.5 Hz, J = 17.2 Hz, J = 6.5 Hz), 7.05−7.30 (m, 5H). ^{13}C NMR (125 MHz, $CDCl_3$, 25 °C): δ = 16.4, 23.3, 28.2, 29.5, 30.3, 34.8, 35.6, 53.6 (broad signal), 54.1, 54.5, 68.5, 71.8 (broad signal), 116.5, 125.8, 128.3, 128.3, 136.0, 142.1. IR (cm^{-1}): υ = 2854−2930, 1641, 1452, 1377, 1157, 1088. HRMS: $C_{20}H_{32}NO$ calculated, 302.2484; found, 302.2469.

*1-(4-Methoxybenzyl)-3(S)-[1(R)-(4-phenylbutoxy)-ethyl]-azetidine (**19**).* Yield: 73% (64 mg from 0.25 mmol of **18**). $[\alpha]_D = -19.8$ (c = 1.0). R_f = 0.21 (dichloromethane/methanol: 94/6). MS (ESI): m/z 354.13 ($(M + H)^+$), 390.29 ($(M + Na)^+$). ^{1}H NMR (500 MHz, $CDCl_3$, 25 °C): δ = 0.90 (d, 3H, J = 6.2 Hz), 1.54−1.80 (m, 4H), 2.56 (t, 2H, J = 7.2 Hz), 2.75 (m, 1H), 3.24 (td, 1H, J = 6.2 Hz, J = 12.4 Hz, AB system), 3.34 (m, 1H), 3.47 (m, 1H), 3.52−3.62 (m, 2H), 3.67 (s, 3H), 3.84−3.98 (m, 4H), 6.76 (d, 2H, J = 8.6 Hz), 7.06−7.20 (m, 5H), 7.26 (d, 2H, J = 8.6 Hz). ^{13}C NMR (125 MHz, $CDCl_3$, 25 °C): δ = 16.5, 28.4, 29.8, 34.8, 35.7, 53.0, 53.7, 55.3, 57.8, 68.6, 72.6, 114.6, 122.2, 125.9, 128.3, 128.40, 128.43, 131.4, 133.7, 142.2, 160.4. IR (cm^{-1}): υ = 2841−2928, 1612, 1516, 1454, 1375, 1252, 1180, 1088, 1030. HRMS: $C_{23}H_{32}NO_2$ calculated, 354.2433; found, 354.2424.

3(*S*)-[1(*R*)-(4-Phenylbutoxy)-ethyl]-azetidine (20). To a stirred solution of **19** (1 equiv) in acetonitrile (23 mL/mmol of **19**) was added dropwise a solution of cerium ammonium nitrate (4 equiv) in water (2 mL/mmol of CAN). The solution was stirred at 70 °C during 1 h, and then water was added (13 mL/mmol of **19**). The aqueous layer was extracted three times with ethyl acetate. The organic layers were combined, dried over $MgSO_4$, filtered, and concentrated under vacuum. After purification by flash chromatography (dichloromethane/methanol), a colorless oil was obtained. Yield: 86% (31 mg from 0.15 mmol of **19**). $[\alpha]_D = -21.6$ (c = 3.1). R_f = 0.20 (dichloromethane/methanol: 94/6). MS (ESI): m/z 234.19 ($(M + H)^+$). ^{1}H NMR (500 MHz, MeOD): δ = 1.05 (d, 3H, J = 6.2 Hz), 1.58−1.77 (m, 4H), 2.64 (m, 2H), 2.90 (m, 1H), 3.40 (td, 1H, J = 6.3 Hz, J = 12.6 Hz, AB system), 3.56 (m, 1H), 3.65 (td, 1H, J = 6.5 Hz, J = 12.9 Hz, AB system), 3.95 (dd, 1H, J = 7.2 Hz, J = 10.6 Hz), 4.01−4.09 (m, 3H), 7.08−7.27 (m, 5H). ^{13}C NMR (125 MHz, MeOD, 25 °C): δ = 16.4, 29.3, 30.6, 36.7, 38.9, 69.8, 74.9, 126.7, 129.3, 129.4, 143.7 (two carbons are not visible because they are masked by the deuterated methanol signal). ^{13}C NMR (125 MHz, C_6D_6, 25 °C): δ = 16.5, 28.5, 30.0, 36.2, 38.0, 49.5, 49.8, 69.0, 75.2, 126.1, 128.7, 129.0, 143.0 (all carbons are visible). IR (cm^{-1}): υ = 2859−2932, 1643, 1339, 1254. HRMS: $C_{15}H_{24}NO$ calculated, 234.1858; found, 234.1863.

1-(Pent-4-enoyl)-3(*S*)-[1(*R*)-(4-phenylbutoxy)-ethyl]-azetidine (8). To a stirred solution of 4-pentenoic acid (1.5 equiv) in dimethylformamide (12 mL/mmol) was added *N*,*N*-diisopropylethylamine (3 equiv) and PyBOP (1.5 equiv) under argon atmosphere. The solution was stirred for 10 min, then a solution of compound **20** in dimethylformamide (12 mL/mmol) was added, and the mixture was stirred overnight. The reaction mixture was diluted with water and diethyl ether, and the aqueous phase was extracted three times with diethyl ether. The organic layers were combined, washed twice with an aqueous solution of 3 N HCl, dried over $MgSO_4$, filtered, and concentrated under vacuum. After purification by flash chromatography (cyclohexane/ethyl acetate), a colorless oil was obtained. Yield: 54% (23 mg from 0.13 mmol of **20**). R_f = 0.18 (cyclohexane/ethyl acetate: 1/1). MS (ESI): m/z 316.20 ($(M + H)^+$),

338.13 ((M + Na)$^+$). ^{1}H NMR (500 MHz, $CDCl_3$, 25 °C): δ = 1.08 (d, 3H, J = 6.1 Hz), 1.54−1.76 (m, 4H), 2.15 (m, 2H), 2.36 (m, 2H), 2.57 (m, 1H), 2.63 (m, 2H), 3.31 (td, 1H, J = 6.4 Hz, J = 12.8 Hz, AB system), 3.46 (m, 1H), 3.60 (td, 1H, J = 6.4 Hz, J = 12.5 Hz, AB system), 3.75 (broad signal, 1H), 3.92 (broad signal, 1H), 4.05 (broad signal, 2H), 4.96−5.14 (m, 2H), 5.84 (ddt, 1H, J = 10.5 Hz, J = 17.2 Hz, J = 6.5 Hz), 7.14−7.34 (m, 5H). ^{13}C NMR (125 MHz, $CDCl_3$, 25 °C): δ = 16.6, 28.2, 29.0, 29.80, 29.83, 30.7, 34.4, 35.8, 50.7 (broad signal, 2C), 68.9, 76.4, 115.4, 125.9, 128.4, 128.5, 137.4, 142.5, 172.6. IR (cm^{-1}): υ = 2858−2926, 1628, 1454, 1373, 1335, 1113, 1088. HRMS: $C_{20}H_{29}NO_2Na$ calculated, 338.2096; found, 338.2087.

1-(4-Methoxyphenyl)-3(*S*)-[1(*R*)-(4-phenylbutoxy)-ethyl]-azetidin-2-one (23). To a stirred suspension of sodium hydride (1.1 equiv) in dry dimethylformamide (6 mL/mmol of alcohol precursor) at 0 °C was added, dropwise, the alcohol precursor (1 equiv) in dry dimethylformamide (6 mL/mmol of alcohol precursor) under argon atmosphere. The suspension was stirred for 30 min at 0 °C, and then freshly dried potassium iodide (3 equiv) and 4-phenyl-1-butyl bromide (3 equiv) were added. The suspension was stirred for an additional 30 min and then allowed to warm up to r.t. After 4 h, the reaction was quenched, at low temperature, with an aqueous saturated solution of NH_4Cl, and the aqueous layer was extracted several times with diethyl ether. The organic layers were combined, dried over $MgSO_4$, filtered, and concentrated under vacuum. After purification by flash chromatography (cyclohexane/ethyl acetate), a colorless oil was obtained. Yield: 37% (85 mg from 0.64 mmol of **22**). R_f = 0.50 (cyclohexane/ethyl acetate: 1/1). MS (ESI): m/z 354.12 ((M + H)$^+$), 376.29 ((M + Na)$^+$). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.28 (d, 3H, J = 6.3 Hz), 1.48−1.72 (m, 4H), 2.57 (t, 2H, J = 7.5 Hz), 3.29 (m, 1H), 3.41 (m, 1H), 3.49−3.64 (m, 3H), 3.77 (s, 3H), 3.81 (m, 1H), 6.86 (d, 2H, J = 9.0 Hz), 7.04−7.36 (m, 7H). Chemical shifts of the minor C-3 diastereoisomer are given in parentheses. ^{13}C NMR (125 MHz, $CDCl_3$): δ = 18.7 (16.6), 28.0 (28.1), 29.7 (29.6), 35.68 (35.73), 41.7 (40.5), 55.2 (53.7), 55.5, 69.0 (68.9), 72.9 (72.2), 114.4, 117.5, 125.7 (125.7), 128.3, 128.4, 132.23 (132.17), 142.5, 156.0, 164.9 (164.7). IR (cm^{-1}): υ = 2860−2930, 1738, 1512, 1454, 1246. HRMS: $C_{22}H_{28}NO_3$ calculated, 354.20692; found, 354.20643.

3(*S*)-[1(*R*)-(4-Phenylbutoxy)-ethyl]-azetidin-2-one (24). To a stirred solution of **23** (1 equiv) in acetonitrile (23 mL/mmol of **23**) at −15 °C was added dropwise a solution of cerium ammonium nitrate (4 equiv) in water (2 mL/mmol of CAN). The solution was stirred at low temperature for 15 min, and then water was added (13 mL/mmol of **23**). The aqueous layer was extracted three times with ethyl acetate. The organic layers were combined, dried over $MgSO_4$, filtered, and concentrated under vacuum. After purification by flash chromatography (cyclohexane/ethyl acetate), a colorless oil was obtained. Yield: 80% (47 mg from 0.24 mmol of **23**). R_f = 0.23 (cyclohexane/ethyl acetate: 1/1). MS (ESI): m/z 354.12 ((M + H)$^+$), 376.29 ((M + Na)$^+$). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.26 (d, 3H, J = 6.3 Hz), 1.52−1.75 (m, 4H), 2.62 (d, 2H, J = 7.0 Hz), 3.19−3.51 (m, 4H), 3.52−3.62 (m, 1H), 3.66−3−80 (m, 1H), 5.74 (broad s, 1H), 7.08−7.33 (m, 5H). Chemical shifts for the minor C-3 diastereoisomer are given in parentheses. ^{13}C NMR (125 MHz, $CDCl_3$): δ = 18.5 (16.5), 28.1 (28.2), 29.8 (29.7), 35.8, 39.0 (37.8), 58.0 (56.5), 68.9, 72.9 (72.0), 125.8, 128.4, 128.5, 142.6, 169.5. IR (cm^{-1}): υ = 3309, 2853−2935, 1726, 1452, 1101. HRMS: $C_{15}H_{21}NO_2Na$ calculated, 270.1470; found, 270.1467.

1-(Pent-4-enoyl)-3(*S*)-[1(*R*)-(4-phenylbutoxy)-ethyl]-azetidin-2-one (9). To a stirred solution of **24** (1 equiv) in dry dichloromethane (8.6 mL/mmol) at r.t. were added pyridine (2 equiv) and 4-pentenoyl chloride (2 equiv) under argon atmosphere. The reaction mixture was refluxed for 24 h then diluted in dichloromethane, and the excess of acyl chloride was quenched by 10% aqueous solution of Na_2CO_3. The organic layer was washed with 3 N aqueous solution of HCl and brine, dried over $MgSO_4$, filtered, and concentrated under vacuum. After purification by flash chromatography (cyclohexane/ethyl acetate), a colorless oil was obtained. Yield: 47% (30 mg from 0.19 mmol of **24**). R_f = 0.23 (cyclohexane/ethyl acetate: 7/1). MS (ESI): m/z 330.25 ((M + H)$^+$), 352.23 ((M + Na)$^+$). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.22 (d, 3H, J = 6.3 Hz), [1.31 (d, 3H, J = 6.1 Hz)], 1.53−1.77 (m, 4H), 2.40 (m, 2H), 2.60 (t, 2H, J = 7.3 Hz), 2.71−2.82 (m, 2H), 3.25 (m, 1H), 3.33 (m, 1H), 3.55−3.64 (m, 3H), 3.81 (m, 1H), 4.97−5.11 (m, 2H), 5.82 (m, 1H), 7.13−7.30 (m, 5H). Chemical shifts of the minor C-3 diastereoisomer are given in parentheses. ^{13}C NMR (125 MHz, $CDCl_3$): δ = 18.0 (17.1), 28.01 (2C) (28.04), 29.6 (29.5), 35.7, 35.8, 39.5 (39.4), 55.0 (54.0), 69.0 (69.1), 71.9 (72.1), 115.7, 125.8, 128.36, 128.44, 136.6, 142.4, 166.3 (166.0), 170.4. IR (cm^{-1}): υ = 2856−2980, 1786, 1697, 1452, 1387, 1312. HRMS: $C_{20}H_{27}NO_3Na$ calculated, 352.1889; found, 352.1880.

Enzymes Preparation. *h*FAAH was prepared in our laboratory as previously described.[43] Mouse liver homogenates were prepared by homogenizing a mouse liver in Tris buffer (pH 7.4, 4 mL) before centrifuging it at 800*g* (15 min). The supernatant was recovered and aliquots stored until use. Aliquots of *h*FAAH or liver homogenate were boiled (45 min, in a water bath) and used as control to account for the chemical hydrolysis of our compounds of interest (blank).

IC_{50} Determination Using *h*FAAH. Tubes containing the enzyme[43] (6 μg in 10 mM Tris-HCl, 1 mM EDTA, and 0.1% (w/v) BSA, pH 7.4, 165 μL), test compounds in DMSO or DMSO alone for controls (10 μL), and [^{3}H]-AEA (50,000 dpm, 2 μM final concentration, 25 μL) were incubated at 37 °C for 10 min. Reactions were stopped by rapidly placing the tubes in ice and adding 400 μL of ice-cold chloroform/methanol (1:1, v/v) followed by vigorous mixing. Phases were separated by centrifugation at 850*g*, and aliquots (200 μL) of the upper methanol/buffer phase were counted for radioactivity by liquid scintillation counting. In all experiments, tubes containing only buffer were used as the control for chemical hydrolysis (blank), and this value was systematically subtracted. Using these conditions, URB-597 inhibits *h*FAAH with an IC_{50} value of 40 nM.

Incubations for the HPLC/MS Analyses. Test compounds (**4**, **9**, or anandamide) at 2.10^{-4} M or their vehicle alone for controls (acetonitrile 10 μL) were incubated in TE buffer (10 mM Tris-HCl, 1 mM EDTA, and 0.1% (w/v) BSA, pH 7.4, 190 μL) for 90 min at 37 °C in the presence of *h*FAAH (90 μg/tube) or liver homogenate (22.5 μg/tube). Reactions were stopped by rapidly placing the tubes in ice and adding 200 μL of ice-cold acetonitrile at which point an internal standard (10 μL of 2.10^{-4} M solution in acetonitrile) was added followed by vigorous mixing. Compound **25** and *N*-palmitoylethanolamine were used as internal standards for the experiments with **4** and **9** and anandamide, respectively. Proteins were further precipitated by centrifugation at 10000*g*, and aliquots (300 μL) of the supernatants were concentrated under reduced pressure. The residues were recovered in 30 μL of acetonitrile (**4** and **9**) or chloroform/methanol (1:1) (anandamide) for HPLC/MS analysis. In all experiments, tubes containing compounds and denatured enzymes (either *h*FAAH or liver homogenate) were used as a control for chemical hydrolysis in buffer (blank). When needed, PF-750 or URB-597 (1.10^{-3} M, 10 μL in acetonitrile) was used as FAAH inhibitors and preincubated for 5 min with the enzyme preparation (180 μL) before adding the test compounds.

HPLC/MS Analyses of the Incubation Medium. The residues obtained following *h*FAAH or liver incubation were analyzed by HPLC/MS using an LTQ Orbitrap mass spectrometer (ThermoFischer Scientific) coupled to an Accela HPLC system (ThermoFischer Scientific). Separation of the hydrolytic products was performed on a C-18 Supelguard precolumn and a Supelcosil LC-18 column (3 μM, 4 × 150 mm) (Sigma-Aldrich). Chromatographic conditions (0.5 mL/min) were as follows: (i, for **4** and **9**) from A (methanol/H_2O/acetic acid; 60:40:0.1) to B (methanol/acetic acid; 100:0.1) in 15 min, followed by a 5 min plateau with B and 8 min re-equilibration with A; and (ii, for anandamide) from A (methanol/H_2O/acetic acid; 75:25:0.1) to B

(methanol/acetic acid; 100:0.1) in 15 min, followed by a 5 min plateau with B and 8 min re-equilibration with A, using a flow rate of 0.5 mL/min. The MS analyses were performed in the positive mode with an APCI ionization source. The capillary and APCI vaporizer temperatures were set at 250 and 400 °C, respectively.

■ ASSOCIATED CONTENT

Supporting Information. Experimental procedures and spectroscopic details of compounds **15**, **16**, **17**, **21**, and **22**; discussion about the rearrangement which resulted in **15**; ^{1}H and ^{13}C NMR spectra of azetidines **7**, **8**, and **20**; and complementary figures of HPLC/MS analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

SAR, structure−activity relationship; LC, liquid chromatography; MS, mass spectrometry; *h*FAAH, human fatty acid amide hydrolase; CB, cannabinoid; GPCR, G-protein coupled receptor; CB_1, cannabinoid receptor subtype-1; CB_2, cannabinoid receptor subtype-2; AEA, anandamide; 2-AG, 2-arachidonoylglycerol; CNS, central nervous system; MALDI, matrix-assisted laser desorption/ionization; TBDMS, *tert*-butyldimethylsilyl; THF, tetrahydrofurane; TBAF, tetra-*n*-butylammonium fluoride; DCM, dichloromethane; DMF, dimethylformamide; ACN, acetonitrile; PMB, paramethoxybenzyl; PMP, paramethoxyphenyl; PyBOP, benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate; CAN, ceric ammonium nitrate; DIEA, *N*-*N*, diisopropylethylamine; DMSO, dimethyl sulfoxide; PEA, *N*-palmitoylethanolamine; AUC, area under curve

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