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Synthesis and pharmacological studies of novel β-lactamic inhibitors of human Fatty Acid Amide Hydrolase (hFAAH): Evidence of a reversible and competitive mode of inhibition

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Parce que l'une des plus belles choses au monde est de s'apercevoir combien est grand le nombre de personnes impliquées dans nos projets, nos passions, nos vies; combien sans elles, la route aurait été longue et terrassante;
combien notre propre évolution est intimement liée à elles …

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***

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Puis vient la recherche !
Quel parcours du combattant ! Les « ça ne marche pas ! », les « %£µ@*$$$*@& » après avoir brisé son tube RMN avec 10 mg de précieux produit final à l'intérieur, les « j'en ai marre de cette thèse », les « j'en peux plus de rédiger », les « I did it ! »… Tous ces grands moments qui composent une thèse ne seraient pas marqués dans mon esprit si je ne les avais pas partagés avec toute une équipe de joyeux collègues ! Merci à tous ceux qui ont rendu tous ces moments inoubliables, tant en chimie qu'en pharmaco, à LLN city ou à Wolu avec tous ceux qui sont passés dans les labos CHOM-ORGJ ou CMFA mais aussi tous ceux que j'ai pu croiser dans le Lavoisier ou le Van Helmont. Merci également aux deux étudiants qui m'ont accompagnée durant ce projet, Joséphine et Guillaume.

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… (soupirs) …

« Challenge accepted » !

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Résumé

C'est dans un contexte médicinal que la recherche sur les effets du cannabis a évolué vers la découverte puis la compréhension du système endocannabinoïde. La plupart des effets, bénéfiques ou psychotropes, engendrés par la consommation de cannabis a été attribuée à l'activation de deux récepteurs, appelés récepteurs cannabinoïdes CB_1 et CB_2, par une molécule liposoluble le tétrahydrocannabinol (THC). La mise en évidence de ces deux récepteurs, jusqu'alors orphelins, a été le point de départ concernant l'hypothèse qu'un système cannabinoïde endogène ou « endocannabinoïde » puisse réguler le même genre d'effets (*e. g.* modulation de l'inflammation, perception de la douleur, stimulation de l'appétit). Plusieurs années se sont écoulées avant d'isoler et de démontrer que deux dérivés de l'acide arachidonique biosynthétisés dans l'organisme, l'arachidonoyléthanolamide (anandamide) et l'arachidonylglycérol (2-AG), jouaient effectivement ce rôle. En effet, ces deux neurotransmetteurs produits « à la demande » activent tous les deux les récepteurs cannabinoïdes entraînant ainsi divers effets physiologiques similaires à ceux du THC, sans toutefois engendrer les effets psychotropes. Cette dernière propriété signe l'originalité et les avantages du système endocannabinoïde *versus* cannabinoïde.

Afin d'exploiter le système endocannabinoïde à des fins thérapeutiques (anti-inflammatoire, anti- douleur), il faut cependant contrer le système de régulation des ligands endogènes mettant en jeu plusieurs enzymes de dégradation. La Fatty Acid Amide Hydrolase (FAAH) et la Monoacylglycerol lipase (MAGL) sont les deux enzymes responsables de la dégradation de l'anandamide et du 2-AG respectivement, limitant ainsi leur action. A l'heure actuelle, de nombreux inhibiteurs de ces deux enzymes ont été décrits comme actifs *in vitro* et *in vivo*, augmentant ainsi localement la concentration d'endocannabinoïdes et l'intensité des effets mesurés.

C'est dans un contexte très compétitif que se situe notre projet de thèse, à savoir la découverte de nouveaux inhibiteurs de la FAAH humaine. Connaissant la structure de la FAAH et sachant qu'elle appartient à la grande famille des protéases à sérine, nous avons utilisé le savoir-faire du laboratoire dans le domaine des β-lactames (antibiotiques) pour adapter un précurseur de carbapénèmes (inhibiteur de DD-peptidases bactériennes) à notre objectif. Pour ce faire, nous avons tout d'abord synthétisé un panel de composés lipophiles que nous avons testés *in vitro* sur la FAAH et la MAGL humaine. Cette première étape nous a permis de dégager une structure « lead » active sélectivement sur la FAAH vis-à-vis de la MAGL. Après avoir montré pour la première fois que des β-lactames pouvaient être également de bons inhibiteurs de la FAAH humaine, nous avons optimisé cette structure et réalisé une étude relation structure-activité (RSA) jusqu'à obtenir une inhibition nanomolaire (IC_{50} = 5,3 nM).

Par la suite, le mode d'action de nos β-lactames a été étudié par des méthodes originales développées dans cette thèse. Nous avons pu ainsi mettre en évidence une inhibition compétitive réversible, mécanisme tout à fait unique dans la famille des inhibiteurs d'hydrolases à sérine de type β-lactame.

Ce manuscrit est présenté sous la forme d'un recueil composé d'une revue et de trois articles publiés dans des revues scientifiques.

Abstract

Research on cannabis effects has evolved in a medicinal context towards the discovery and the understanding of the endocannabinoid system. Most of the effects, beneficial or psychotropic, produced by the cannabis consumption have been ascribed to the activation of two receptors, called cannabinoid receptors CB1 and CB2, by a liposoluble molecule the tetrahydrocannabinol (THC). Displaying the existence of these two receptors has accelerated the research about an eventual endogenous cannabinoid or "endocannabinoid" system which might regulate similar effects (*e. g.* inflammation modulation, nociception, appetite stimulation). Later, two derivatives of arachidonic acid, the arachidonoylethanolamide (anandamide) and the arachidonylglycerol (2-AG), were isolated and shown to play this role. Indeed, these two neurotransmitters produced "on demand" both activate the cannabinoid receptors which induce various similar physiological effects to those produced by THC, without inducing the psychotropic ones. This last property signs the originality and the advantages of the endocannabinoid versus cannabinoid system.

However, to take advantage of endocannabinoid system for therapeutic use (against inflammation or pain), the regulation system of the endogenous ligands, which is composed of degradation enzymes, must be counteract. Fatty Acid Amide Hydrolase (FAAH) and Monoacylglycerol lipase (MAGL) are both responsible of the degradation of anandamide and 2-AG respectively, limiting their action. To date, a lot of inhibitors of these two enzymes have been described as active in vitro and in vivo, locally increasing anandamide concentration and intensity of measured effects.

Our project takes place in a highly competitive context where the discovery of new inhibitors of FAAH constitutes a real challenge. Knowing FAAH structure and that it belongs to the great family of serine proteases, we used the know-how of our laboratory in the β-lactam field (antibiotics) to adapt a carbapenem precursor (DD-peptidase inhibitors) to our aim. Thus, we first synthesized a panel of lipophilic compounds which were tested in vitro against human FAAH and MAGL. This first step allowed us to identify a lead structure which is selectively active against FAAH versus MAGL. As we showed for the first time that β-lactams can be good to excellent inhibitors of human FAAH, we optimized this structure and realized a structure activity relationship study (SAR) until we culminated with a nanomolar inhibition (IC_{50} = 5.3 nM).

Then, the mode of action of our compounds was studied by original methods which were developed during this thesis. Thanks to that, we were able to show that our inhibitors act in a competitive and reversible manner, which is a unique mechanism in the β-lactam family of serine hydrolases inhibitors.

This manuscript is presented as a compilation of a review and three articles published in scientific journals.

Abbreviations, functions of the compounds

ABHD6: α/β-hydrolase-6

ABHD12: α/β-hydrolase-12

ACN: acetonitrile

AEA: anandamide

2-AG: 2-arachidonoylglycerol

2-AGE: noladin ether

AIDS: acquired immune deficiency syndrome

cAMP: cyclic adenosine monophosphate

CAN: ceric ammonium nitrate

CB: cannabinoid

CBD: cannabidiol

Cbz: carbobenzyloxy

CMVp: cytomegalovirus protease

CNS: central nervous system

COX-2: cyclo-oxygenase-2

DCC: dicyclohexylcarbodiimide

DCM: dichloromethane

DMAP: *N-N'*-dimethylaminopyridine

DMF: dimethylformamide

DMSO: dimethylsulfoxide

eCB: endocannabinoid

equiv: equivalent

FAAH: fatty acid amide hydrolase

GPCR: G protein-coupled receptor

GPR55: G protein-coupled receptor 55

GPR119: G protein-coupled receptor 119

HLE: human leukocyte elastase

HMDS (LiHMDS): hexamethyl disilazane

HRMS: high resolution mass spectrometry

HTS: high throughput screening

Hz: hertz

IC_{50}: half maximal inhibitory concentration

IR: infrared spectroscopy

LC/MS: liquid chromatrography-mass spectrometry

LOX: lipo-oxygenases

MAGL: monoacyl glycerol lipase

MAPK: mitogen-activated protein kinases

mp: melting point

MS: mass spectrometry

NAAA: *N*-acylethanolamine-selective acid amidase

NADA: *N*-arachidonyldopamine

NAEs: *N*-acylethanolamines

NMR: nuclear magnetic resonance

OAc: acetate

OEA: oleoylethanolamide

OL-135: reversible and selective α-keto-oxazole-type inhibitor of FAAH

PBP: penicillin binding protein

PEA: palmitoylethanolamide

PF-750: irreversible and selective urea-type inhibitor of FAAH

PF-04457845: irreversible and selective urea-type inhibitor of FAAH

PMB: paramethoxybenzyl

PPAR: peroxisome-proliferator activated receptor

PPE: porcine pancreatic elastase

PSA: prostate specific antigen, or polar surface area

PyBOP: benzotriazolyloxy-tris(pyrrolidino)-phosphonium hexafluorophosphate

rt: room temperature

SAR: structure activity relationship

TBAF: tetrabutylammonium fluoride

TBDMS: tert-butyldimethylsilyl

THF: tetrahydrofurane

$Ti(OtBu)_4$: titanium tetrabutyloxide

Δ^9-THC: Δ^9-tetrahydrocannabinol

TRPV1: transient receptor potential cation channel subfamily V member 1

URB-597: irreversible and selective carbamate-type inhibitor of FAAH

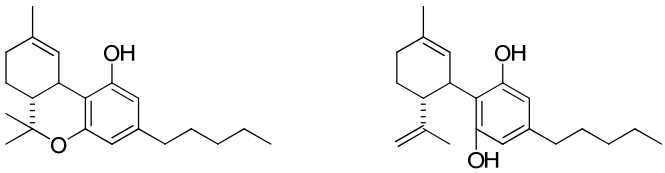
Chapter 1

I On endocannabinoid system considered as one of the promising fields in pain treatment and various CNS disorders.

I.1 The endocannabinoid system and its exogenous modulators: discovery of anandamide, the first endocannabinoid

I.1.1 From cannabinoids…

Cannabis has been consumed and has been considered not only as a recreational drug but also and especially as a medicinal plant for its ancestral use. Before the Christian era, essentially Indian, Chinese, Assyrian and Persian civilizations mentioned the use and the consumption of *Cannabis Sativa* in their cultures, religions or medicines. Beneficial effects such as the psychoactive ones have already been highlighted by these ancient populations. Besides, they used these properties in their everyday life: as analgesic for headache or toothache, anti-inflammatory for rheumatic pain, antispasmodic for colic and diarrhea, appetite stimulant or tranquillizer for anxiety or hysteria; the spiritual side of the plant was turned to account in religious use (Buddhism or probably Shamanism) to facilitate meditation.[1; 2] Progressively, the medical use of cannabis was more described and expanded to the rest of the world until the beginning of the twentieth century. After the structural elucidation of the main psychoactive compounds contained in the plant, Δ^9-tetrahydrocannabinol (Δ^9-THC, Figure 1)[3] and cannabidiol (CBD, Figure 1), the medical application of cannabinoids was really considered and became an attractive field in CNS drug discovery.[4]



Δ^9-THC CBD

Figure 1. Main pharmacologically active components of *Cannabis Sativa*

Cannabis extracts and synthetic analogues of Δ^9-THC became common medicines prescribed in various therapeutic conditions: i) as analgesic in neuropathic pain which is induced by multiple sclerosis neuropathy (mixture of Δ^9-THC and CBD, Sativex®)[5]; ii) as antiemetic used to treat side-effects of chemotherapy (Nabilone, Cesamet®)[6]; iii) as appetite stimulator for AIDS patients (Dronabinol, Marinol®).[7] However, cannabis and its derivatives were - and are still - considered as illicit drugs, which justify the control of their consumption in a lot of countries, and to date, several opinions diverge about the safety related to their induced psychoactive effects.[8] This is why the growing interest in cannabinoid medicines drove the research towards the endogenous cannabinoid system.

I.1.2 … to endocannabinoids

Additionally to safety issues, it was really unavoidable to discover whether an endogenous cannabinoid system exists. About thirty years separate the structural elucidation of the first cannabinoids (cannabidiol, 1963 and Δ^9-tetrahydrocannabinol, 1964)[3] and the discovery of the first elements of the endocannabinoid system. Indeed, in 1990, the first cannabinoid receptor (called cannabinoid because of binding with Δ^9-THC) was cloned and named CB_1 receptor,[9] and, in 1992, a second receptor, CB_2,[10] and their first endogenous ligand, called anandamide,[11] were isolated. These discoveries drove to the evidence of the existence of an endocannabinoid system and then, to an endogenous capacity of the human body to produce and regulate some identical beneficial effects due to cannabis consumption. Since then, the researches on endocannabinoid system were dramatically accelerated.

I.2 The endocannabinoid system: regulation and pharmacology.

This system has been largely studied and is now well described; the wide diversity of endogenous ligands binding the two cannabinoid receptors, CB_1 and CB_2, was established using the most studied and principal ones, anandamide (AEA) and 2-arachidonoylglycerol (2-AG). In the same time, lots of studies were achieved to understand the biosynthesis, the regulation and the degradation of these ligands which drove to the description of various enzymes. Thereby, the acquired knowledge considerably evolved and the endocannabinoid system became more complex, spreading to other "endocannabinoid-like" entities which will be briefly described at the end of this section (I.2.4).

I.2.1 Cannabinoid receptors

Although they are both activated by cannabinoids and endocannabinoids, the two cannabinoid receptors, CB_1 and CB_2, diverge in their localization and function. Both belong to the GPCR (G protein-coupled receptor) superfamily and enhance cascade pathways such as inhibition of adenylyl cyclase, activation of mitogen-activated protein kinases (MAPK), and only in the case of CB_1, regulation of certain K^+ and Ca^{2+} channels.[12]

I.2.1.1 CB_1 receptors

CB_1 receptors are highly expressed in various brain regions such as cortex, hippocampus, amygdala, basal ganglia and cerebellum. Such distribution explains well all the behavioural effects due to their activation by the cannabinoids (like cannabis consumption) or endocannabinoids. Indeed, CB_1 receptors are closely connected with cognitive effects, such as memory or reversal learning,[13] but also with control of motor function,[14] addiction processes, food intake, analgesia or central nervous system diseases such as depression, anxiety or schizophrenia.[15; 16] In addition to CNS, they are also expressed in the peripheral nervous system. Psychoactive effects of cannabinoids and synthetics agonists are mainly assigned to CB_1 activation, probably due to its high expression within the brain.

I.2.1.2 CB_2 receptors

In contrast to CB_1 receptors, CB_2 ones were said, for a long time, to play a predominant role in the peripheral nervous system. They are indeed mainly located in immune cells at the periphery such as macrophages, lymphocytes, T-cells and monocytes.[17] Besides, they are proposed to play a key role in inflammatory processes. However, they are also localized in a minor level into the brain, where they are supposed to be involved, like CB_1 receptors, in the proliferation, differentiation and survival of neuronal cells.[18; 19]

I.2.2 Endogenous ligands

Establishing a list of all molecules considered as endocannabinoids is a complex task because of the multitude of cross-talking pathways involved in the endocannabinoid system.[20] Indeed, some bioactive lipids, possessing close structural features, exhibit cannabimimetic responses without activating CB receptors, and moreover, they are also regulated by the same enzymes (it will be discussed later in 1.2.3 section about enzymatic pathways). Therefore, only the so-called endogenous molecules responsible of the activation of cannabinoid receptors are discussed hereafter. All these ligands are derived from an arachidonic acid chain with a polar head.

To date, five bioactive lipids are considered to be the main endocannabinoids (Figure 2 and Table 1), including the two most studied ones, anandamide (AEA) and 2-

arachidonoylglycerol (2-AG). They were found to bind and activate all cannabinoid receptors, enhancing physiological cannabimimetic effects in the mouse "tetrad": hypothermia, antinociception, inhibition of spontaneous activity and mobility.

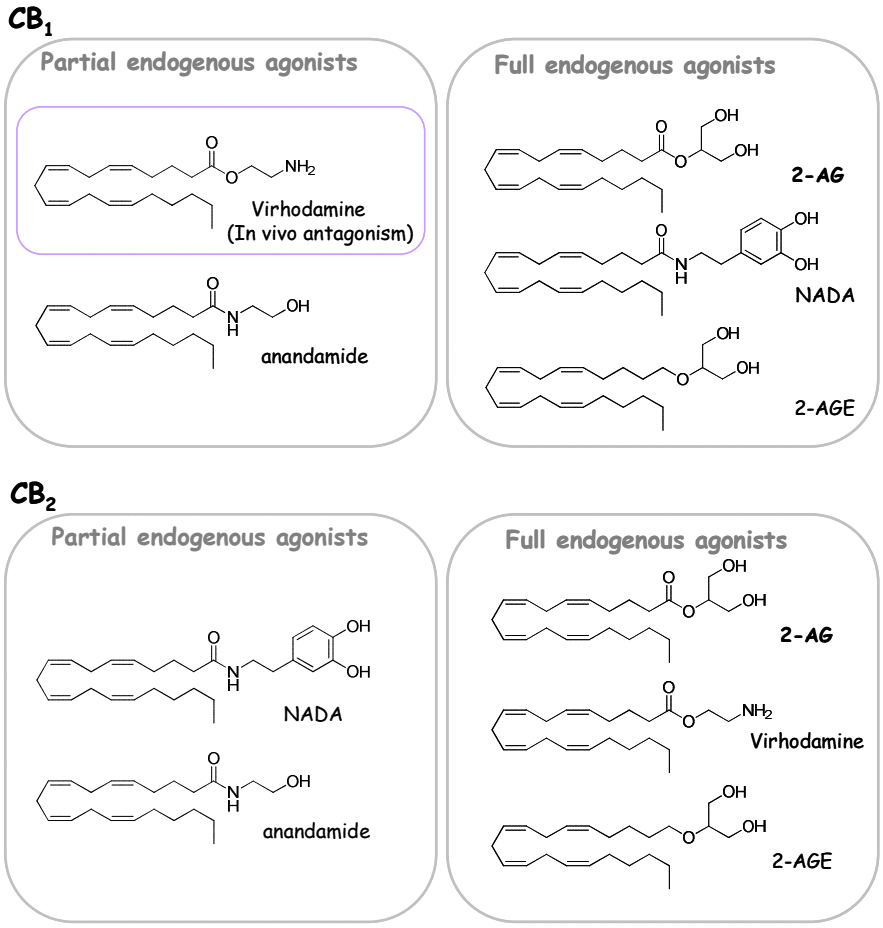


Figure 2. Functionality of the most studied endocannabinoids with regard to the two cannabinoid receptors, CB_1 and CB_2.

Table. 1 binding affinities published by the respective authors of the five most studied endocannabinoids.

Endogenous ligand	Affinity for CB_1 (nM)	Affinity for CB_2 (nM)
Anandamide (AEA)	61-89[21]	260-371[22]
2-arachidonoylglycerol (2-AG)	58.3[23]	120-145[22]
Noladinether (2-AGE)	21.2[26]	480[27]
Virhodamine	1906[25]	1401[25]
N-arachidonoyldopamine (NADA)	250[24]	nd (40* more)[24]

However, they do not interact nor activate receptors to the same extent. Thus, AEA was found to be a partial agonist of CB_1[21] and CB_2[22] while 2-AG is a full agonist of both receptors.[22; 23]

N-Arachidonyldopamine (NADA) is a full agonist of CB_1 while it acts as a partial one at CB_2.[24] Virhodamine has a partial efficacy on CB_1 but is a full agonist of CB_2.[25] Note that virhodamine was found to have an antagonist activity towards CB_1 *in vivo*. Finally, noladin ether (2-AGE) was assessed to be a full agonist of both CB_1[26] and CB_2[27]. All the binding affinities for the two cannabinoid receptors are summarized in Table 1 while Figure 2 illustrates the functional difference between them.

Importantly, there is a common feature to all the endocannabinoids. These lipids are biosynthesized "on demand", *i. e.* they are not stored near their site of action. Therefore, they are produced from arachidonic acid in the synapses and released outside the cell to interact with their receptors. Their activities are rapidly interrupted by a regulation via degrading-enzymes which will be described in section I.2.3.

I.2.3 Enzymatic pathways

I.2.3.1 Overview of endocannabinoids regulation

As endocannabinoids are produced "on demand" and rapidly hydrolyzed, the related enzymatic system holds a major place in the running of the endocannabinoids set. Considering the different endogenous ligands of CB receptors, it is easy to imagine the multiple biochemical pathways involved in their regulation. First of all, we can observe that the *N*-acylethanolamines (NAEs, *i. e.* AEA, Palmitoylethanolamide (PEA) and oleoylethanolamide (OEA)) biosynthesis pathways are distinct from those of 2-AG, while the biosynthesis of the other endocannabinoids still remains unexplored. To date, three pathways of NAEs biosynthesis have been elucidated, involving five different enzymes.[28; 29] Observations may suggest a tissue localisation specificity, NAEs selectivity and cross-regulations between the pathways. Concerning 2-AG, two pathways, involving four enzymes, have been proposed to lead to its biosynthesis.[28; 29] After the synthesis step, endocannabinoids are released out of the cell to interact with CB receptors or other targets. Although endocannabinoids are described to be "lipidic" enough to pass the membrane by diffusion, they are also suspected to cross it thanks to a carrier which facilitates the transport not only from inside to outside the cell but also inversely.[30; 31] Indeed, endocannabinoids are degraded in the intracellular environment and they must pass again the membrane to be hydrolyzed by the degrading-enzymes which terminate the endocannabinoid signalling. In addition to enzymes responsible of endocannabinoids degradation, which will be more detailed in the

next sections, some other enzymes could be mentioned. Indeed, for instance, cyclo-oxygenase-2 (*i. e.* COX-2) oxygenates anandamide into prostaglandine-ethanolamides (*i. e.* prostamides)[32] and lipo-oxygenases (LOX) drive to oxygenated products. Similar oxygenations are also described concerning 2-AG.

I.2.3.2 Endocannabinoid-degrading enzymes

In this section, we will briefly approach the notion and the consequences of such degradation. More details about structures, functions and pharmacology of each principal enzyme (FAAH, MAGL and NAAA) will be given in section II.

The study of the endocannabinoid-degrading enzymes was particularly investigated.[33] Initially, Fatty Acid Amide Hydrolase (FAAH) and Monoacylglycerol Lipase (MAGL), which regulate and degrade mainly AEA and 2-AG respectively (Figure 3), were the prominent centres of interest, but additional endocannabinoid-degrading enzymes were shown to also play key roles in the eCB system. Thus, a second FAAH-based mechanism was recently described for essentially regulating oleamide, an endocannabinoid-like.[34] Although this enzyme shares only 20 % homology with FAAH, it was called FAAH-2. Two serine hydrolases, α/β-hydrolase-6 (ABHD6) and α/β-hydrolase-12 (ABHD12), were also recently discovered and described as complementary 2-AG-degrading enzymes in the brain.[35] Interestingly, MAGL and ABHDs are localized in different subcellular divisions suggesting distinct roles, at different moments, in the 2-AG pharmacology. In addition, another enzyme, called *N*-acylethanolamine-hydrolyzing acid amidase (NAAA), was found to regulate the levels of NAEs and principally PEA, which exerts eCB-like effects via distinct pathways from CB receptors.[36] This diversity of enzymes involved in the regulation and degradation of all the endogenous ligands drove to the hypothesis that distinct roles should result from their actions. Consequently, it appears interesting to target them selectively to induce selective physiological and pharmacological effects. FAAH and MAGL are now well described and, to date, their 3D structures are elucidated.[37-39] In this thesis work we will focus on the FAAH activity, especially about its inhibition, in order to improve endogenous anandamide level.

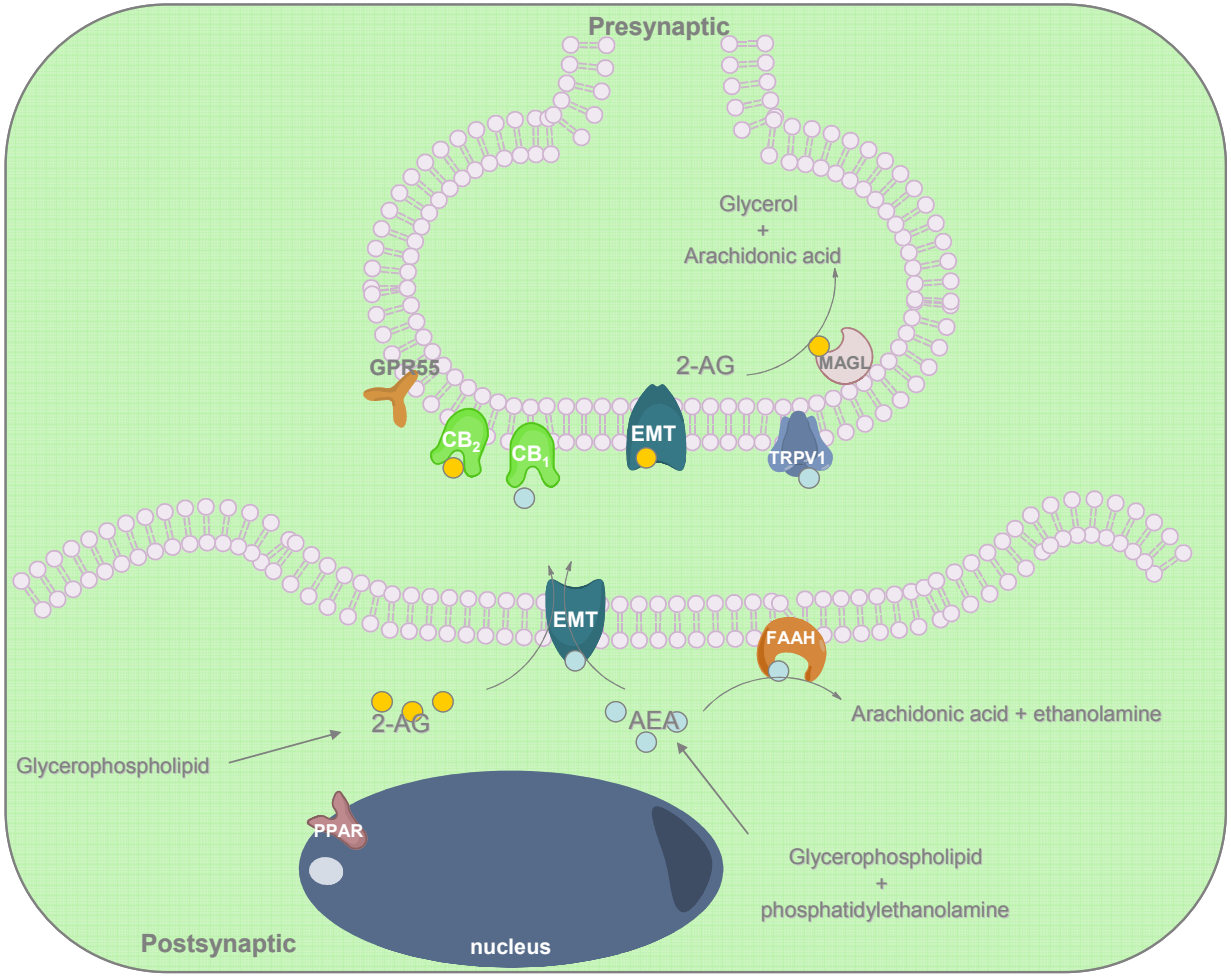


Figure. 3 The endocannabinoid system at synapses. AEA, anandamide; 2-AG, 2-arachidonoylethanolamine; CB, cannabinoid receptors; EMT, endocannabinoid membrane transporter; FAAH, fatty acid amide hydrolase; MAGL, Monoacylglycerol lipase; PPAR, peroxisom-proliferator activated receptor; TRPV1, transient receptor potential cation channel subfamily V member 1; localisation of CB_2 and GPR55 receptors is not established at synapses, here they are represented at presynaptic position.

I.2.4 Additional targets or "endocannabinoid-like system"

Anti-inflammatory and anti-nociceptive properties are also mediated by other systems distinct from the endocannabinoid one. Other pathways meet characteristics of the endocannabinoid system: (i) receptors which drive to quite identical physiological effects; (ii) ligands which do not bind CB receptors but lead to close effects and are degraded by endocannabinoid enzymes; (iii) new enzymes found to degrade "endocannabinoid-like" compounds. All these discoveries made the endocannabinoid system becomes more and more complex and quite ambiguous (Figure 3). Thus, peroxisome-proliferator activated receptors (PPAR), vanilloid receptors (TRPV1) and two orphan receptors, GPR55 and GPR119, have been shown to interact with endocannabinoids but also with NAEs which do not bind to CB receptors. Palmitoylethanolamide (PEA), oleoylethanolamide (OEA), oleamide and *N*-acyltaurines have similar properties than endocannabinoids but bind PPAR or TRPV1 receptors. However, they are degraded by FAAH or NAAA, except oleamide which is hydrolyzed by FAAH-2. (for a complete review see Alexander, 2007[20]).

I.3 Conclusion

These recent findings and knowledge about cannabinoids and endocannabinoids allowed the understanding of all the mechanisms implied in the endocannabinoid system in a large manner (*i. e.* endocannabinoid and endocannabinoid-like systems). Considering all the positive effects, its use as therapeutic is increasingly explored. Three kinds of approaches are taking advantage of CB_1 and/or CB_2 activation beneficial effects. First, a large number of synthetic agonists have been described, which allow to enhance the effects resulting directly from receptor activation.[40; 41] However, most of the time, psychoactive effects, due to CB_1 activation are produced and also, in some cases, a lack of selectivity is observed. The recent, but quite less developed, inquiry of allosteric modulators of cannabinoid receptors offers the second strategy, increasing the effects of the endogenous ligands without displaying the characteristic side-effects.[42] Another good alternative to prevent the cannabinoid "tetrad" (hypomotility, hypothermia, analgesia and catalepsia) has been to imagine inhibitors of endocannabinoid-degrading enzymes.[43; 44] During this thesis, we followed this third strategy on FAAH using the β-lactam template, widely known for being a good structural motif to inhibit serine proteases.

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Chapter 2

II Focus on "inhibitors of the endocannabinoid-degrading enzymes, or how to increase endocannabinoid's activity by preventing their hydrolysis"

***

During our thesis, we were invited to review the importance of endocannabinoid-degrading enzymes inhibitors in the patent literature. This chapter consists in an adaptation of the review published in recent patents on CNS drug discovery. Herein, we detailed features of principal enzymes of the system (FAAH, MAGL and NAAA) and their main inhibitors described and published in patent literature. We also briefly presented those from classical literature. In addition, we documented the clinical trials concerning some FAAH inhibitors.

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Inhibitors of the endocannabinoid-degrading enzymes, or how to increase endocannabinoid's activity by preventing their hydrolysis

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Endocannabinoids are lipid transmitters binding and activating the cannabinoid receptors. Both cannabinoid receptors and endocannabinoids, such as 2-arachidonoylglycerol and anandamide, have been shown to control numerous physiological and pathological processes, including in the central nervous system. Thus regulating endocannabinoid levels *in-vivo* represents an interesting therapeutic perspective in several CNS-related diseases. To date four enzymes - Fatty Acid Amide Hydrolase (FAAH), *N*-Acylethanolamine-hydrolyzing Acid Amidase (NAAA), Monoacylglycerol Lipase (MAGL), α/β-Hydrolase Domain 6 (ABHD6) – were shown to control endocannabinoid levels in tissues or in intact cells. While the searches for NAAA and ABHD6 inhibitors are still in their beginning, a growing number of selective and potent inhibitors are now available to inhibit FAAH and MAGL activities. Here, based on the literature and patent literature, we review the compounds of the different chemical families that have been developed to inhibit these enzymes, with a special emphasis on FAAH and MAGL inhibitors.

Adapted from a published review, in recent patents on CNS drug discovery, **2012**, 7 (1), 49-70.

II.1 Introduction

The effects of cannabinoids (from natural, synthetic and endogenous origin) are mainly mediated by two G protein-coupled receptors, the cannabinoid receptors CB_1 and CB_2. These two receptors are activated by endogenous bioactive lipids named endocannabinoids, which are produced in an activity-dependent manner (*i.e.* following cell stimulation) from phospholipid precursors present in the cell membranes. So far, two types of endocannabinoids, based on an arachidonic acid moiety, have been fully characterized. Indeed, *N*-arachidonoylethanolamine (anandamide, AEA) is a member of the large family of *N*-acylethanolamines, whereas 2-arachidonoylglycerol (2-AG) is an acylglycerol. The activity of these lipid mediators at the cannabinoid receptors is terminated essentially following their hydrolysis by several lipases.[1] Initially, Fatty Acid Amide Hydrolase (FAAH)[2] and Monoacylglycerol Lipase (MAGL),[3-4] were described as the main enzymes regulating the activity of AEA and 2-AG, respectively. More recently, additional endocannabinoid-degrading enzymes were also shown to have key roles in the endocannabinoid system. Thus, a second FAAH-related mechanism was recently described for essentially regulating oleamide.[5] Although it shares only 20 % homology with FAAH, this novel enzyme was called FAAH-2. Two serine hydrolases, α/β-hydrolase 6 (ABHD6) and α/β-hydrolase 12 (ABHD12),[6] were also recently discovered and described as complementary 2-AG-degrading enzymes in the brain[7]. Interestingly, MAGL and ABHD6 and 12 are present in different subcellular locations suggesting distinct roles in controlling 2-AG activities. In addition, another enzyme, called *N*-Acylethanolamine-hydrolyzing Acid Amidase (NAAA), was found to regulate the levels of *N*-acylethanolamines.[8]

Because the activation of the cannabinoid receptors results in multiple beneficial effects, numerous CB_1 and CB_2 agonists are being described since the characterization of Δ^9-THC structure and of its activity at cannabinoid receptors. However direct and constant activation of the receptors resulting from this strategy presents several drawbacks, including receptor desensitization, and numerous CNS-related side effects for the CB_1 receptor agonists. Conversely, increasing selectively the levels of an endocannabinoid is expected to result in a subset of the effects induced by the agonist but with more limited side effects.[9] Therefore there is a strong rationale for the preparation of potent and selective inhibitors of endocannabinoid degradation.

Thus, following a brief summary of the enzymes' characteristics, we will review the different classes of inhibitors described in the patent literature. The main focus will be on the compounds able to inhibit FAAH and MAGL since those are the primary enzymes controlling the endocannabinoid levels. However, we will also briefly describe the novel inhibitors developed to block the activity of NAAA.

II.2 FATTY ACID AMIDE HYDROLASE

II.2.1 Pharmacology of FAAH inhibition or why inhibiting FAAH activity?

FAAH is a membrane-bound serine hydrolase which belongs to a distinct class of enzymes characterized by the amidase signature (AS). It possesses an atypical catalytic triad consisting in Ser-Ser-Lys (instead of the classical Ser-His-Asp) which confers to FAAH the ability to hydrolyse amide bonds of various endogenous bioactive lipids.[10-11]

Essentially known for being the main anandamide-degrading enzyme[12], FAAH also hydrolyses and thus regulates the endogenous levels of other bioactive lipids (**figure 1**). Indeed, several *N*-acylethanolamines (NAEs) - including *N*-palmitoylethanolamine (PEA), which does not activate CB receptors but induces anti-inflammatory responses via the PPAR receptors, and the satiating agent *N*-oleoylethanolamine (OEA) - also undergo a FAAH-dependent catabolism.[13-14] Furthermore, the levels of other classes of amide-derived lipids, like the *N*-acyl taurines (NATs), which activate transient receptor potential (TRP) ions channels,[15] and fatty acid primary amides (FAPAs) such as the sleep-inducing lipid oleamide,[12, 16-17] are also regulated by FAAH. Interestingly, while the levels of these mediators are increased upon FAAH inhibition the levels of two other bioactive lipids, *N*-arachidonoyldopamine (a TRPV1 agonist)[18] and *N*-arachidonoylglycine (the putative GPR18 receptor endogenous agonist)[19], are decreased following FAAH inhibition.

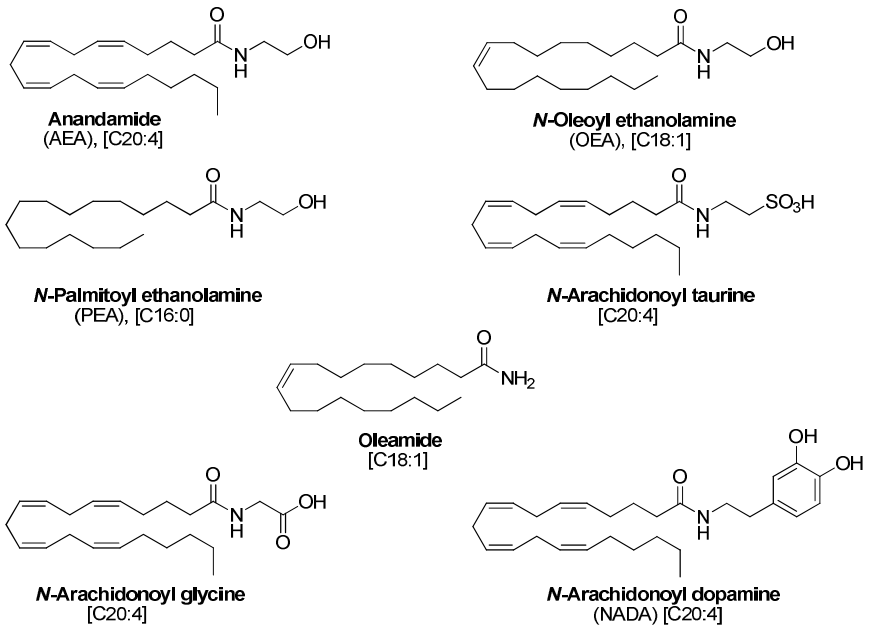


Figure 1. Endogenous bioactive lipids regulated by FAAH

Considering all its different substrates, FAAH inhibition will result in numerous effects, with several of them not mediated by anandamide or by cannabinoid receptors. These non-CB1 and non-CB2 effects can be mediated by G protein-coupled receptors (e.g. GPR18, GPR119), ion channels (e.g. TRPV1) or nuclear receptors (e.g. PPAR)[20-21]. To date, only the consequences of increasing the levels of the *N*-acylethanolamines are relatively well characterized both in cells and *in-vivo*. Anandamide was shown to be involved in number of physiological processes[22-25] including appetite regulation[26], pain[27-28] and inflammation, but also various CNS and psychiatric disorders[29], with anxiety and depressive disorders being the most studied. Concerning other substrates, oleamide was shown to be involved in the sleep induction,[30-31] PEA is widely described as analgesic and anti-inflammatory molecule[27-28, 32] and OEA either as satiating factor or also as analgesic[33]. OEA regulates feeding and body weight through activation of the nuclear receptor PPAR-alpha.[34] Today, FAAH inhibition is generally considered as a sound therapeutic strategy in the treatment of pain and inflammation[20, 35-39] as well as anxiety and depression[40-42].

II.2.2 FAAH, structure and function

Soon after the discovery of FAAH, X-ray crystallographic studies were performed to further understand its mode of action, but also to improve the research and the development of its

inhibitors. Thus the rat isoform (*r*FAAH) was crystallised in presence of methylarachidonoylfluorophosphonate (MAFP), an irreversible and non selective inhibitor, and the structure was solved with a 2.8 Å resolution.[43] This three dimensional structure revealed the presence of several domains implicated in distinct functions. *i*) A large domain composed by hydrophobic and basic residues covers the active site and allows the enzyme to anchor to the membrane. *ii*) Near this hydrophobic plateau, lies a channel responsible for the entry of the substrate. Commonly called the membrane access channel (MAC), this cavity allows a direct access for the lipid substrate from the membrane to the active site. *iii*) Close to the active site, a hydrophobic cavity is present. This acyl-chain binding pocket (ABP) interacts with the side-chain of the substrate. *iv*) Finally, the cytosolic port (CP) was found to interact with the polar head of the substrate and is connected with the cytosol. Moreover, the active site is able to accommodate a water molecule to hydrolyse the acyl-enzyme complex. As a result, the hydrophobic moiety and the hydrophilic one are released towards the MAC and CP, respectively. (For complete reviews, see [44-45]) Further helping the drug development of FAAH inhibitors, an engineered "humanised" rat FAAH (*h/r*FAAH)[46] was produced and successively co-crystallised with three compounds representative of the major FAAH inhibitors classes.[47-50] This constitutes an interesting tool, as a large number of FAAH inhibitors present differences in activity depending on the origin of the enzyme, that is mouse FAAH (mFAAH) or human FAAH (hFAAH). An alternative strategy was to develop an homology model of *h*FAAH based on the reported X-ray structure of *r*FAAH.[51] This model was validated by docking the selective inhibitor PF-750 resulting in similar interactions to those found in the co-crystal structure of PF-750 into *h/r*FAAH.

A large number of FAAH inhibitors have been described over the years, starting from natural substrates analogues to well-adapted traditional types of serine hydrolase inhibitors.[52-55] Indeed, a wide variety of electrophilic functions has been used to target the enzyme's active site, generating large sets of structure-activity relationships aiming at improving, not only the potency, but also the selectivity of the inhibitors. Among the numerous templates described in the patent literature (**figure 2**), three main chemical families have been extensively studied. Below, we will review them in their order of development, starting from the α-keto heterocycles,[56] then the carbamate-based inhibitors[57] and finally, the urea-derived inhibitors[58].

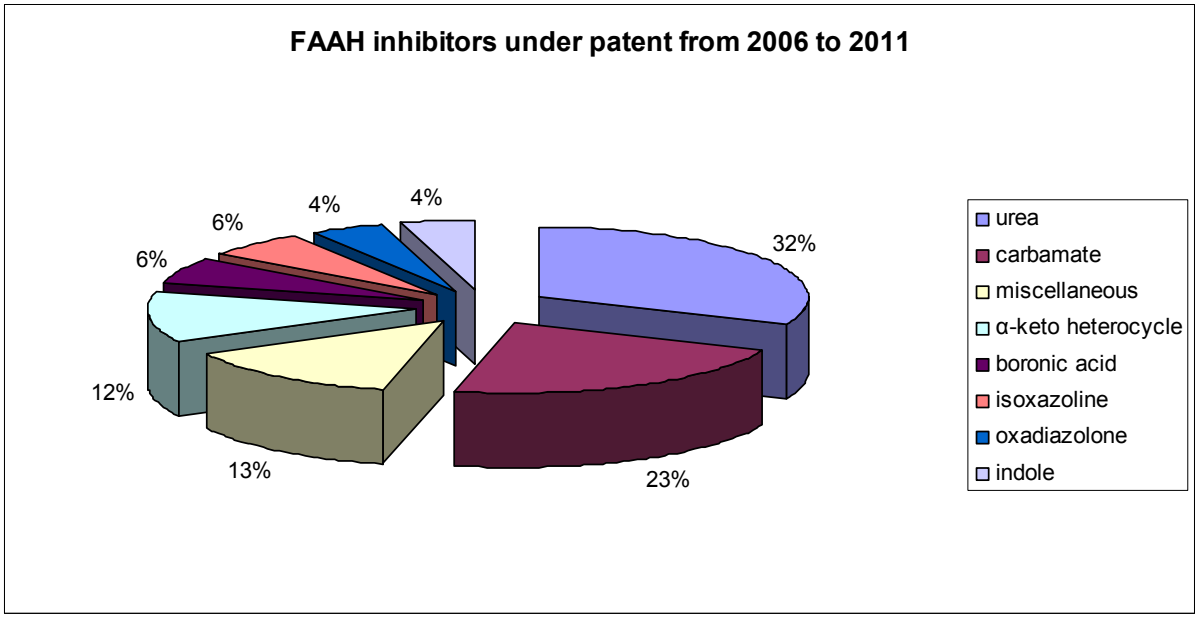


Figure 2. Pie-chart, based on the chemical family, of the FAAH inhibitors families found in the patent literature (2006-2011).

II.2.3 FAAH inhibitors

II.2.3.1 α-keto heterocycle-based FAAH inhibitors

Activated ketones were disclosed very early in the development of FAAH inhibitors. Disclosed for inhibiting serine proteases,[59-60] a first series was described by Dale Boger's group, who generated a series of arachidonoyl- and oleoyl-based α-keto heterocycles[56]. The inhibition is based on the attack by FAAH's active serine on the electrophilic carbonyl of the inhibitor. The resulting reversible tetrahedral intermediate was recently observed in OL-135 – *h/r*FAAH co-crystals.[48] After studying a large range of heterocycles, α-keto oxazoles and α-keto oxazolopyridines were identified as the most efficacious moieties. Therefore, Boger's group refined its inhibitors and reported more potent heterocyclic inhibitors exemplified with OL-135 (**figure 3**, **1**, IC_{50} = 2.1 nM and > 100 µM on *m*FAAH and on mMAGL, respectively), a potent and highly selective pharmacological tool commonly used and based on the pyridyl oxazole template.

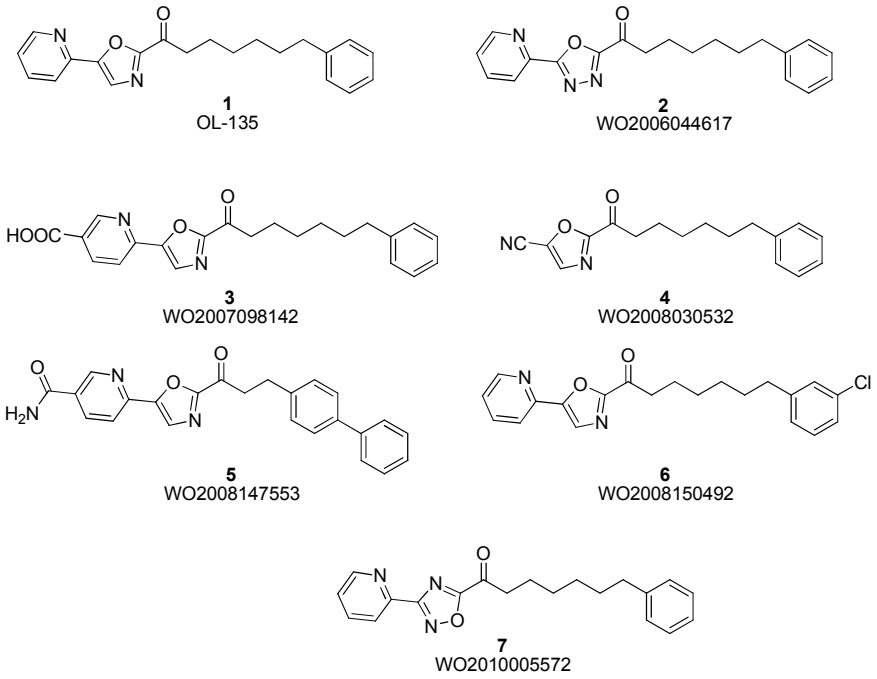
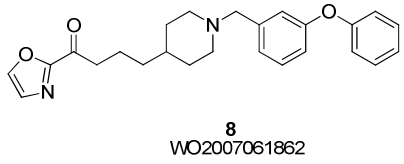


Figure 3. FAAH inhibitors based on α-keto heterocycle templates described by Boger *et al.*

From this study, an optimal C6 linker length with a phenyl end-group was chosen to replace the fatty acid chain. Beside the acyl chain, the authors also explored the impact of the heterocycle nature. Thereby, analogues were synthesised, based on 2-keto-1,3,4-oxadiazole scaffold with a subnanomolar activity[61] (**2**, **figure 3**, K_i = 290 pM on *r*FAAH) and more recently, 2-keto-1,2,4-oxadiazole scaffold[62] (**7**, K_i = 920 pM and 340 pM on *h*FAAH and *r*FAAH, respectively). Similarly to the C6 alkylphenyl chain, the H-bond acceptor pyridine group appeared to be responsible for the higher potency. Boger's group also investigated the substitution of either the pyridine or oxazole ring.[63-64] Again highly potent inhibitors were obtained featuring subnanomolar activities (**figure 3**, **3**, K_i = 200 pM and 2 nM on *h*FAAH and *r*FAAH, respectively; **4**, K_i = 900 pM on *h*FAAH). Focusing on the lipophilic portion that binds the ACB pocket, a series of bioisosteres was synthesized. For instance, equal activity to the C6 alkylphenyl chain was found for the C2 alkylbiphenyl chain, resulting in the inhibitor **5** [65] (**figure 3**, K_i = 400 pM and 500 pM, on *h*FAAH and *r*FAAH, respectively). Finally, the same authors also studied the substitution of the phenyl ring at the end of the acyl side-chain, resulting in inhibitors such as **6** [66] (**figure 3**, K_i = 400 pM on *r*FAAH).

Meanwhile, another study on α-keto heterocycles was undertaken by the researchers of Janssen Pharmaceuticals. Based on the structure of OL-135, (**1**, **figure 3**) including the oxazole ring, they oriented their efforts toward the insertion of a piperidinyl scaffold which allows for a wide diversity of substitutions. They published two patents containing SAR

studies, and exemplified here with compounds **8**[67] (**figure 4**, 400 pM and 4.7 nM on *h*FAAH and *r*FAAH, respectively) and **9**[68] (**figure 4**, Ki = 2 nM and 2 nM on *h*FAAH and *r*FAAH, respectively).



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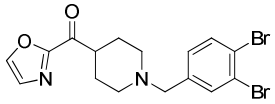

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Figure 4. FAAH inhibitors based on α-keto heterocycle templates described by Janssen Pharmaceutica.

II.2.3.2 Carbamate-based FAAH inhibitors

Carbamate-based FAAH inhibitors were inspired by the structures of previously reported inhibitors of serine hydrolases. This function is usually used to inhibit serine proteases in an irreversible manner. Indeed, the tetrahedral intermediate evolves towards a stable acyl-enzyme complex. This mechanism of action was put forth, first by MS analyses[69] and then by X-ray structures[50], in studies involving URB-597 (or KDS-4103, **10**, **figure 5**) the lead compound of this class of inhibitors[70-71]. URB-597 is largely used as pharmacological tool, both *in-vitro* and *in-vivo* (IC_{50} = 4.6 nM on *r*FAAH). In addition, analogues of **10** were synthesized in order to increase their stability towards oxidative metabolism. Indeed, hydroxylation of the C4 position was observed following *in-vivo* administration of **10**. Thereby, this position was blocked by adding various substituents, like the gem-dimethyl found in **11**[72] (**figure 5**), allowing a significant reduction of anandamide hydrolysis (50 % of control, at 30 nM).

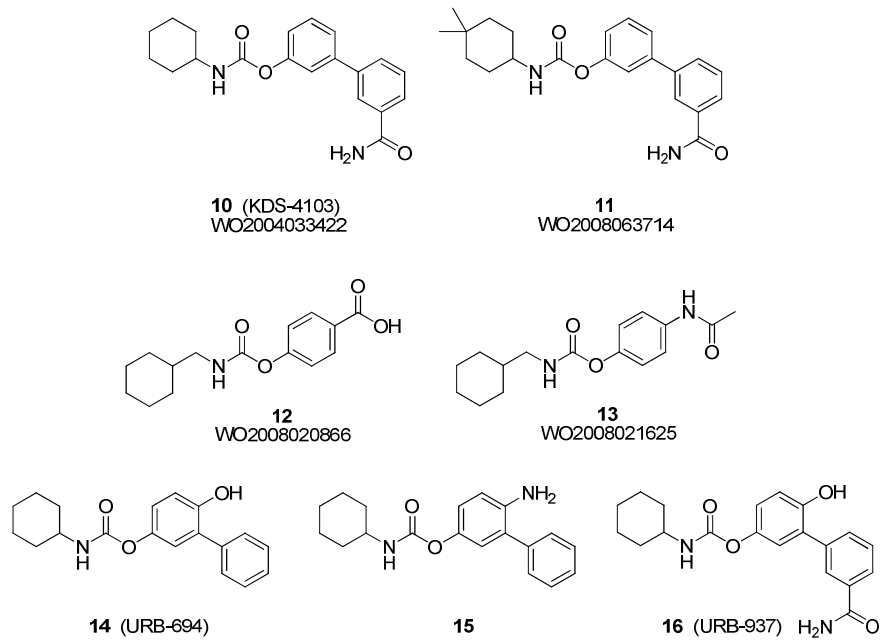

Figure 5. Carbamate-type FAAH inhibitors developed by Piomelli *et al.* and Kadmus Pharmaceuticals

Additional series of analogues were also synthesized, including compound **12**[73] (**figure 5**, $IC_{50} < 0.1$ μM). This compound is interesting as it exhibits a good oral bioavailability (C_{max} in plasma 2-fold higher compared to **10**) and a low CNS uptake (C_{max} in brain 10-fold lower compared to **10**) which may thus allow to target selectively FAAH in the periphery while not affecting FAAH activity in the CNS. This feature may be useful to treat pain and inflammation disorders by acting at peripheral sites, without inducing potential CNS (side)effects. Another carbamate derivative, compound **13**[74] (**figure 5**, $IC_{50} < 0.1$ μM), also possesses a good oral bioavailability and its administration results in enhanced OEA and PEA levels in blood (7.55 and 8.85 ng/mL compared to 4.18 and 4.17 ng/mL for OEA and PEA, respectively). The same authors described some other inhibitors but neither their potency nor *in-vivo* activity were disclosed.[75-76] Piomelli and co-workers also developed new KDS-4103-based inhibitors with the aim to reduce its activity towards liver carboxylesterases. It is indeed known that **10** (URB-597, **figure 5**), while being quite selective, has several off-targets[77-78], including carboxylesterases, which could prevent its further development. Thus, URB-694 (**14**, **figure 5**, $IC_{50} = 30.0$ nM) and the aniline analogue **15** (**figure 5**, $IC_{50} = 27.2$ nM)[79], both possessing electron-donating substituents on the phenyl ring that reduce the electrophilicity of the carbonyl, were described. This decreased electrophilicity resulted in more selective compounds that retained their good activity against FAAH *in-vitro* and *in-vivo*.[80] Therefore, novel URB-694-based inhibitors could soon be developed with improved

selectivity for FAAH. Note that recently, a first URB-694 derivative, URB-937, (**16**, **figure 5**, IC_{50} = 26.8 nM) was disclosed to selectively inhibit FAAH in the periphery.[81]

In addition, *O*-phenylcarbamates were also described by Astellas (**17, figure 6**, IC_{50} = 12 nM) and Myllymaeki and co-workers (**18**, **figure 6**, IC_{50} = 240 pM, *r*FAAH).[82-83]

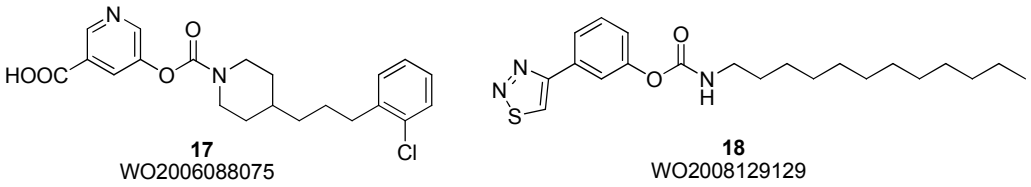

Figure 6. Carbamate-type FAAH inhibitors described by Astellas Pharma and Saario *et al.*

Due to their properties towards FAAH, carbamate-based inhibitors were also largely investigated by Sanofi-Aventis. Numerous series were described, based on various *O*- and *N*-substituents including alkyl, piperazinyl, azetidinyl or thioazolyl, as illustrated in **figure 7**.[84-91] These inhibitors were all described for having an analgesic activity and their inhibition potencies against *m*FAAH are summarized in **figure 8**.

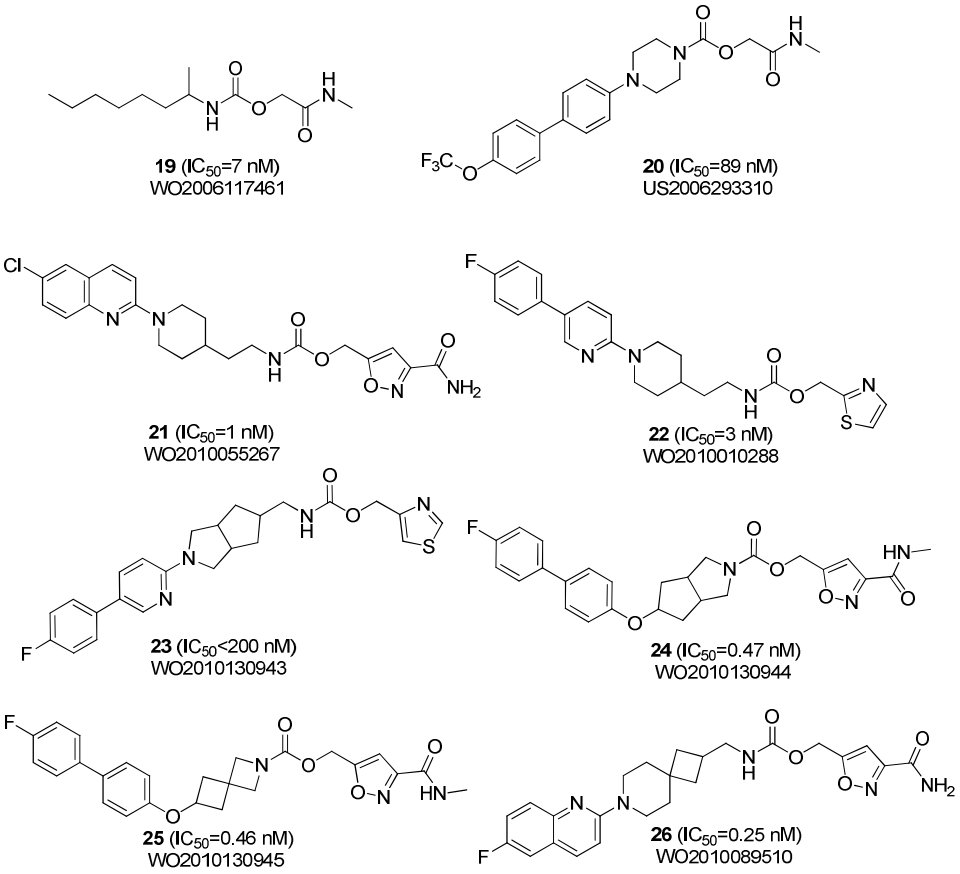

Figure 7. Carbamate-type FAAH inhibitors described by Sanofi-Aventis

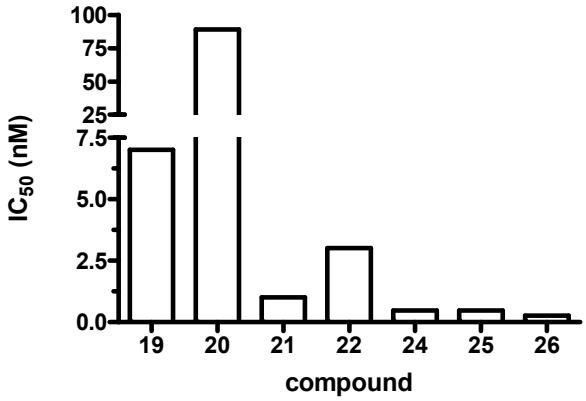

Figure 8. IC_{50} values of the carbamate-based inhibitors from Sanofi-Aventis depicted in figure 7

Two other carbamate-based families were developed at Sigma-Tau Pharmaceuticals: one is based on an enol carbamate template[92] (**27** and **28**, **figure 9**, IC_{50} and K_i both < 10 nM, *m*FAAH) and the other is based on an oxime carbamate[93] (**29**, **figure 9**, IC_{50} and K_i both < 10 nM, *m*FAAH). These compounds are described as selective for FAAH over various cannabinoid-related targets (< 60 % versus CB_1, CB_2, TRPV1, NAPE-PLD, AMT, DAGL, MAGL, at a concentration equal to 1000-fold their IC_{50} against FAAH).

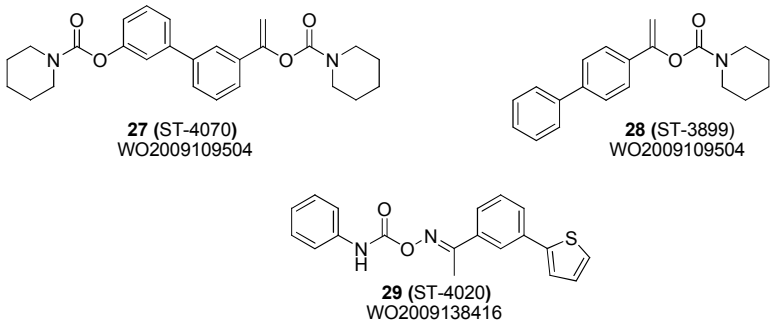

Figure 9. Carbamate-type FAAH inhibitors described by Sigma-Tau Pharmaceuticals

Interestingly, **27-29** inhibit FAAH in a reversible manner which is quite unexpected for carbamate-type inhibitors. *In-vivo*, compound **28** exhibits analgesic activity and reduces anxiety without affecting locomotor activity, whereas **29** was able to reduce anxiety as well as the hyperalgesia in a model of neuropathic pain.

II.2.3.3 Urea-based FAAH inhibitors

Due to its high resistance towards chemical and biological hydrolysis, the urea function is usually not considered as a pharmacophore to inhibit serine hydrolases. However, it was

shown that adding a good leaving group, such as an aniline function, transforms urea into a more reactive moiety, which could then function as enzyme inhibitor. Urea-based FAAH inhibitors originated from high-throughput screening studies of industrial chemical libraries. Both Janssen Pharmaceuticals and Takeda companies described compounds based on a piperazinyl urea moiety (**30** and **31**, **figure 12**). Compound **30** exhibits an IC_{50} value of 16 or 50 nM depending on the source of enzyme (human and rat, respectively)[94], while for **31** 100 % of enzyme (*r*FAAH) inhibition was obtained at 1 µM.[95]

However, the development of urea-based FAAH inhibitors really started with the discovery at Pfizer of PF-622 and PF-750 (**32** and **33**, **figure 10**, k_{inact}/K_i = 621 $M^{-1}.s^{-1}$ and 791 $M^{-1}.s^{-1}$ on *h*FAAH, respectively).[46, 58]

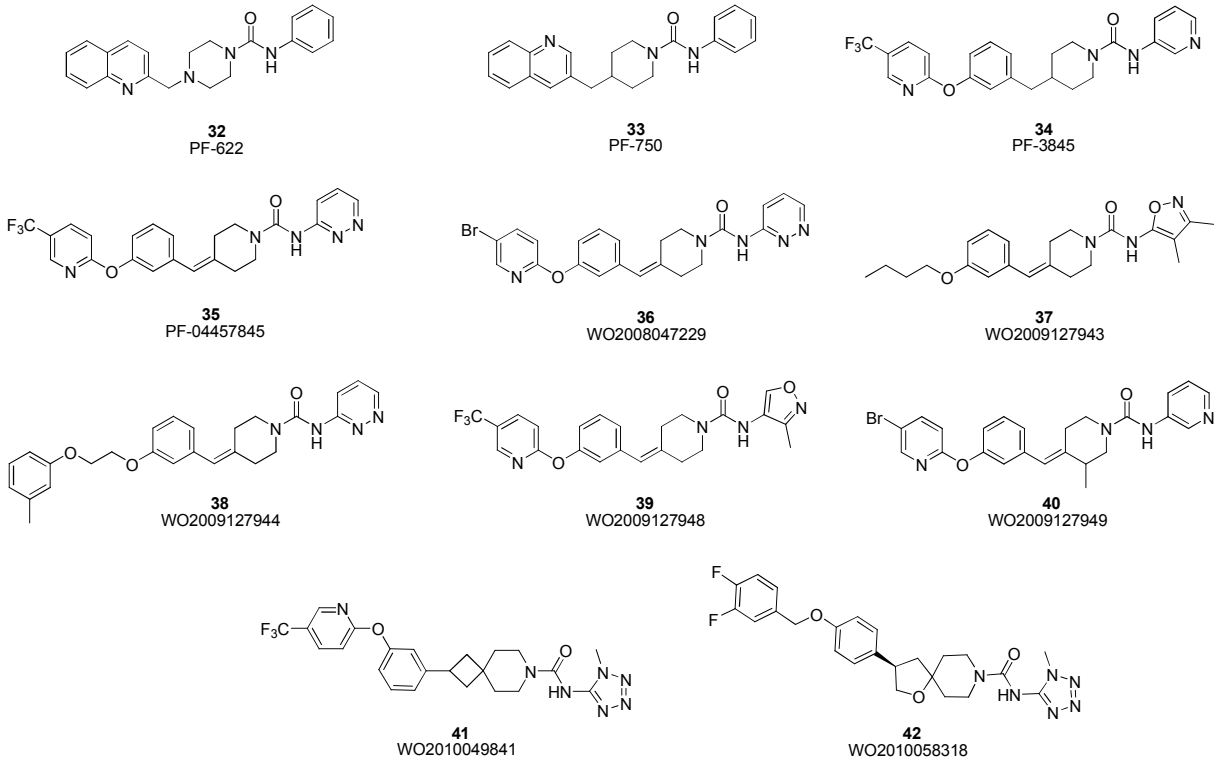

Figure 10. Evolution of Pfizer's piperidinyl urea-type FAAH inhibitors

Indeed, after a high-throughput screening study to improve either drug-like pharmacokinetic properties and/or selectivity, Pfizer published a new kind of mechanistic class of inhibitors, based on the piperidinyl urea scaffold, showing a combination of potency and excellent selectivity.[58] Of note, the development of this class of inhibitors at Pfizer beneficiated from the *h/r*FAAH three dimensional structure.[46] Thus, the stable acyl-enzyme complex was identified by MALDI-MS analyses and then by X-ray structures with *h/r*FAAH, which confirmed the addition to the active serine and the irreversible mechanism. These studies led

to a new chemical family obtained by replacing, not only the quinoline group of **32** and **33** with a biaryl ether group, but also the aniline leaving group with a 3-aminopyridine one. These changes resulted in the inhibitor PF-3845 (**34**, **figure 10**) which possesses much higher activity (k_{inact}/K_i = 14,310 $M^1.s^{-1}$)[47]. Pfizer further described in several patents the synthesis and pharmacological evaluations of numerous urea-based inhibitors (e.g. compounds **35-40**, **figure 10**).[96-100] One line of research was to design more rigid compounds using a methylenepiperidine scaffold while incorporating a polar moiety to improve the pharmacokinetic parameters. This led to the clinical candidate PF-04457845 (**35**, **figure 10**, k_{inact}/K_i = 40,300 $M^{-1}.s^{-1}$) which contains a pyridazinyl moiety instead of the 3-aminopyridine one. This inhibitor exhibits a high selectivity for FAAH, excellent potency and good pharmacokinetic profile. The same company also prepared a new series of rigid piperidines where the methylene group was replaced with a C4-spirocycle (**41** and **42**, **figure 10**).[101-102] The later inhibitors possess, similarly to the inhibitors containing a methylene unit, a high potency against FAAH activity.

It is difficult to directly compare the inhibitory activity of Pfizer's ureas with the other types of inhibitors since their potency is expressed in the literature as k_{inact}/K_i values instead of the IC_{50} values traditionally reported. However, the use of k_{inact}/K_i values appears more suited than IC_{50} values when studying irreversible inhibitors. Note that in the same assay, the well-known URB-597 (**10**, KDS-4103, **figure 5**) shows a k_{inact}/K_i value of 1,590 $M^{-1}.s^{-1}$ (**figure 11**). Based on all the reported assays, piperidinyl ureas appeared to be more selective and efficacious than KDS-4103.[47]

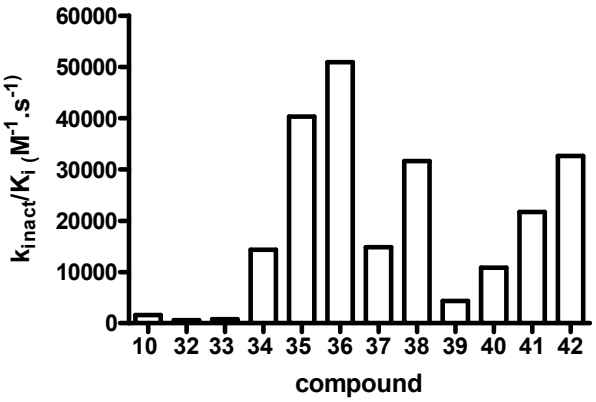

Figure 11. Potency of Pfizer's ureas compared to the carbamate-based inhibitor KDS-4103 (**10**, **figure 5**). Note that a highly potent inhibitor is characterized by a high k_{inact}/K_i value (and a low IC_{50} value).

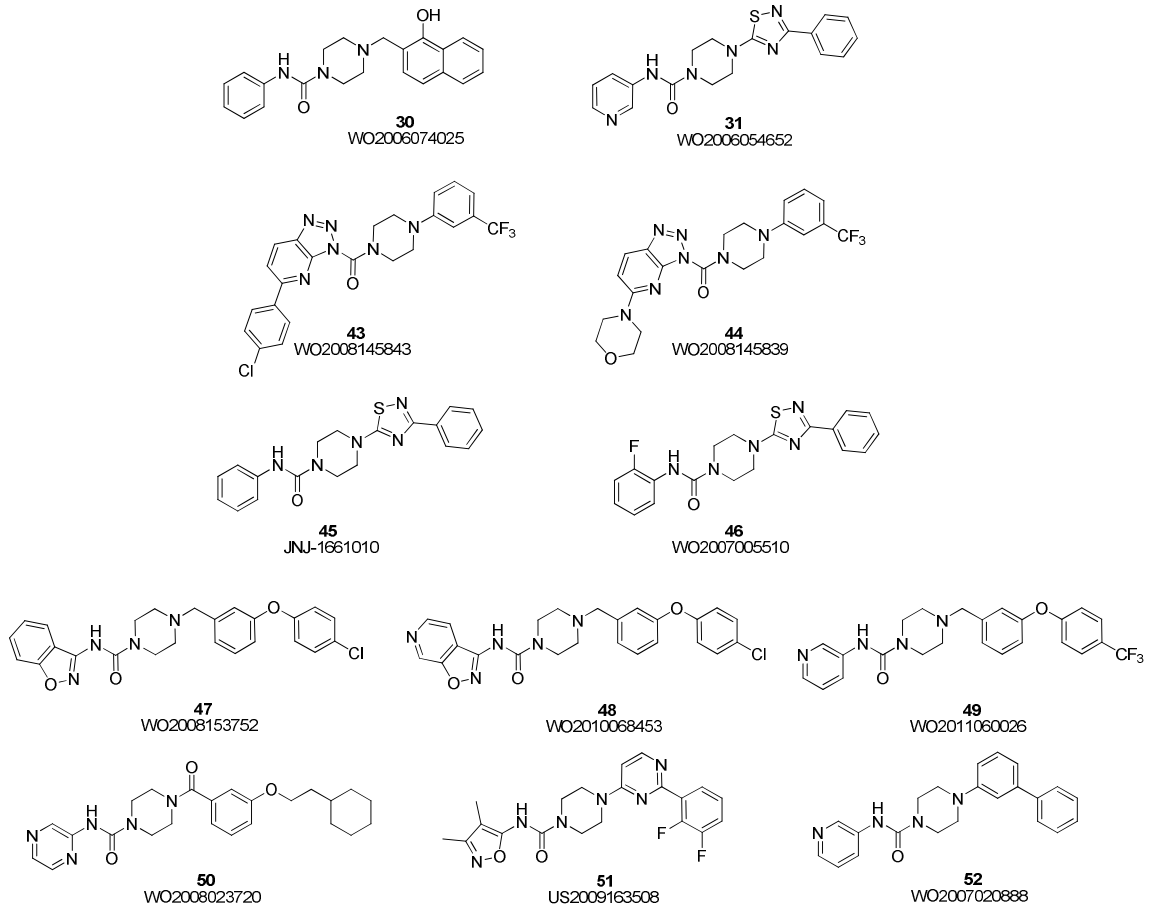

Figure 12. Urea-type FAAH inhibitors

While other urea-derivatives have been investigated by several pharmaceutical companies, piperazinyl urea remains the most common template. *i*) Sanofi-Aventis described two compounds exhibiting dual inhibition against both *m*FAAH and *m*MAGL (**43** and **44**, **figure 12**).[103-104] *ii*) Janssen Pharmaceuticals reported two additional series of compounds (**45-49, figure 12** and **53, figure 13**). Compound **46**[105] (**figure 12**, IC_{50} = 19 nM and 6 nM on *h*FAAH and *r*FAAH) is derived directly from JNJ-1661010 (**45**, **figure 12**) structure, whereas compounds **47-49**[106] contained a biaryl ether motif. When administered i*n-vivo,* **47** (**figure 12**, IC_{50} = 8 nM and 10 nM on *h*FAAH and *r*FAAH, 20 mg/kg, po) showed analgesic effects in a model of mechanical allodynia. Its activity was improved by replacing the benzisoxazole moiety with an isoxazolopyridine resulting in compound **48** described as a subnanomolar inhibitor.[107] Inspired from the structure of **48**, **49** (**figure 12**, IC_{50} = 1 nM) conserves a biaryl ether moiety, but a pyridine replaces the isoxazolopyridine motif.[108] *iii*) Other piperazinyl urea-based FAAH inhibitors were shown to possess *in-vivo* efficacy. For instance, Astella published a series of inhibitors, illustrated with **50** (**figure 12**, IC_{50} = 160 pM, *r*FAAH), which seem to be useful in the context of overactive bladder,[109] and Takeda

published two families of compounds based on an isoxazole or pyridine moiety, exemplified here with **51,** which exhibited analgesic effect at 10 mg/kg,[110] and **52**, which was proposed to treat sleep disorders[111]. *iv*) More recently, azetidinyl ureas were described as FAAH inhibitors. Vernalis described compound **53** (**figure 13**) which exhibits an IC_{50} value of 3 nM on *h*FAAH.[112] Janssen Pharmaceuticals investigated also azetidinyl ureas with compound **54** (**figure 13**), which presents an IC_{50} value of 1 nM both on *h*FAAH and *r*FAAH,[113] and the recently published rigid spirocyclic compound **55** (**figure 13**, 10 nM and 20 nM on *h*FAAH and *r*FAAH, respectively)[114].

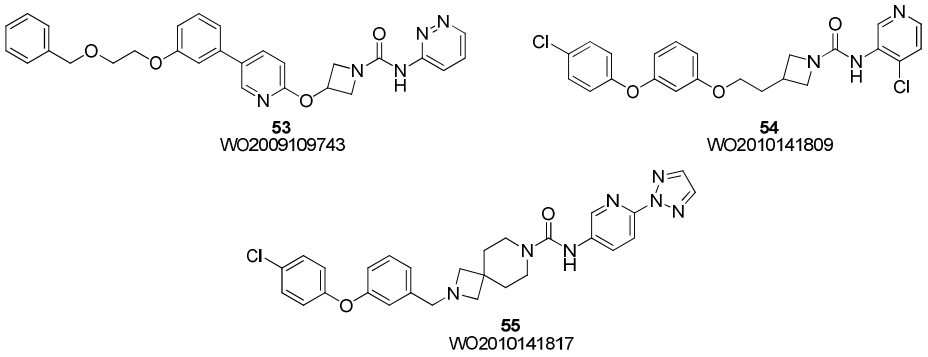

Figure 13. Urea-type FAAH inhibitors from Vernalis and Janssen Pharmaceuticals

II.2.3.4 Boronic acid-based FAAH inhibitors

Recently, a new kind of FAAH inhibitors using a boronic acid as the electrophilic function was reported. This function has been already described for inhibiting serine proteases in a reversible manner.[115] Indeed, boron's ability to go up from a trigonal planar geometry to a tetrahedral geometry allows boronic acids to form a transient and reversible tetrahedral intermediate with the nucleophilic serine. Both Infinity Pharmaceuticals (**56**, **figure 14**)[116] and Minkkila and co-workers (**57**, **figure 14**)[117] published in 2008, the first arylboronic acids described as FAAH inhibitors. These two inhibitors exhibited nanomolar activities ($K_i \leq$ 10 nM for **56** and IC_{50} = 9.1 nM for **57**) and a reversible inhibition of the enzyme. In the case of compound **56**, kinetic data consolidated the hypothesis of a reversible inhibition. Furthermore, supplementary investigations were undertaken to unravel the interactions between the inhibitor and the enzyme. Both the molecular modelling studies and mutagenesis studies of *r*FAAH demonstrated that the phenyl moiety of these inhibitors interacts with the enzyme's hydrophobic channel.

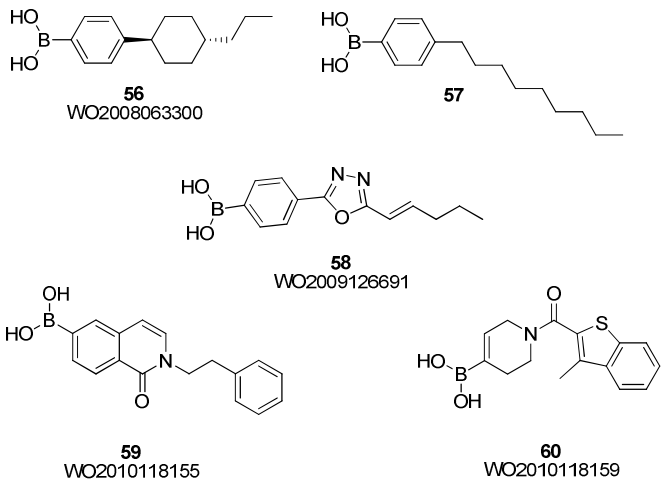

Figure 14. Boronic acids developed as FAAH inhibitors

Infinity Pharmaceuticals further covered this area with three patents in 2009 and 2010. Thus, they developed several series of FAAH inhibitors based on various substituted arylboronic acids, exemplified with compounds **58**[118] and **59**[119] (**figure 14**, K_i ≤10 nM), and also based on a tetrahydropyridine boronic acid[120] (*i.e* compound **60**, **figure 14**, K_i ≤10 nM).

II.2.3.5 FAAH inhibitors of miscellaneous structures

Whereas initially, carbamate and activated ketone-based inhibitors were mainly described, a wider diversity of templates has been explored since 2006. Some indole and pyrrole derivatives were investigated by Ironwood Pharmaceuticals (Microbia) which published several patents from 2006 to 2011.[121-125] Therein, different series of compounds were disclosed that were, not only FAAH inhibitors, but that also interact with one or several targets involved in inflammation and pain (e.g. COX-1 or COX-2). For example, compounds **61**[122], **62**[123] and **63**[125] (**figure 15**) were found to inhibit *h*FAAH at submicromolar concentrations.

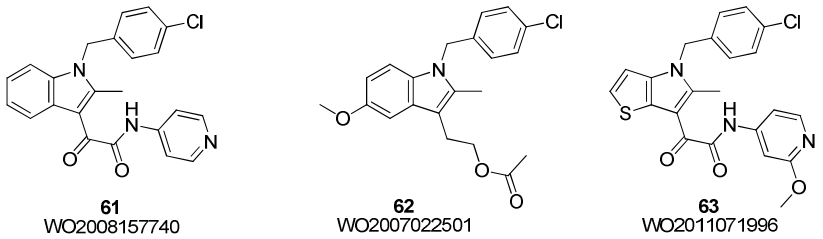

Figure 15. 2-Methylindole-based inhibitors of FAAH

Bial (Portela & C^{o}) reported several series of oxadiazolones (**64**, **65** and **66**, **figure 16**) as FAAH inhibitors being selective for peripheral over CNS located FAAH.[126-128]

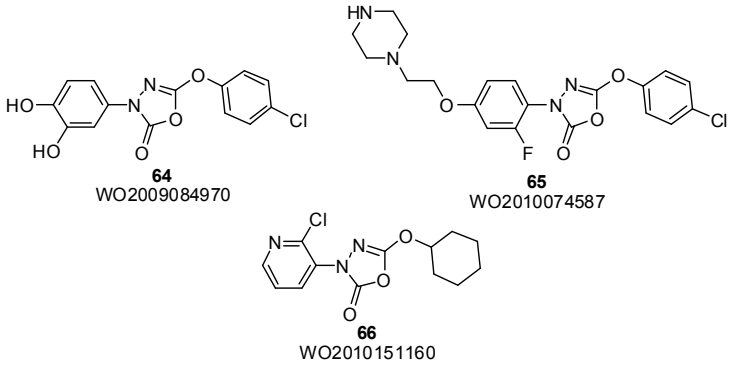

Figure 16. Oxadiazolone-type FAAH inhibitors

Indeed, following administration of the inhibitors to mice (30 mg/kg, p.o.) the residual activity in liver was found to be very low (5 %, 6 % and 29 % compared to the control, for **64**, **65** and **66** respectively) whereas it was almost completely conserved in brain (83 %, 86 % and 84 % compared to the control, for **64**, **65** and **66** respectively).

Merck designed various heterocycle-based FAAH inhibitors like imidazole, pyrazole or oxazole cycles. Thus, two series of imidazole derivatives were described which inhibit *h*FAAH with nanomolar and subnanomolar activities (**figure 17**, compounds **67** and **68**, IC_{50} values of 6.3 nM and 0.2 nM, respectively).[129-130] In addition, a pyrazole series was published, illustrated with **69** (**figure 17**, IC_{50} value of 0.47 nM)[131] and also an oxazole one, represented by **70** (**figure 17**)[132]. The inventors described the later inhibitor as having good cell permeability (IC_{50} = 5 and 20 nM, in cell lysate and in whole cell, respectively).

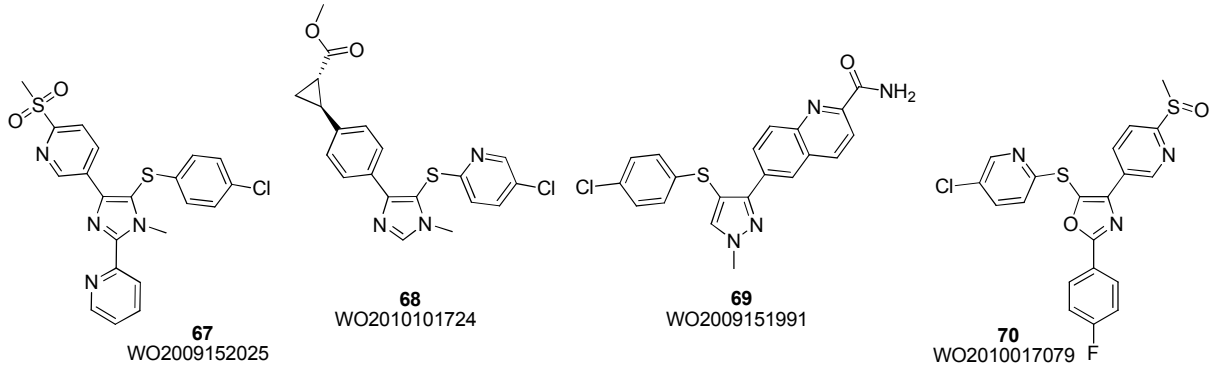

Figure 17. Central heterocyle-based FAAH inhibitors from Merck.

Renovis published several lipophilic and polycyclic compounds as FAAH modulators. For instance, compounds **71**[133] and **72**[134] exhibited nanomolar IC_{50} values (**figure 18**, IC_{50} < 100 nM and IC_{50} = 1.2 nM for **71** and **72**, respectively) while compound **73** (**figure 18**)[135] showed more than 75 % of FAAH inhibition at 1 µM compared to the control.

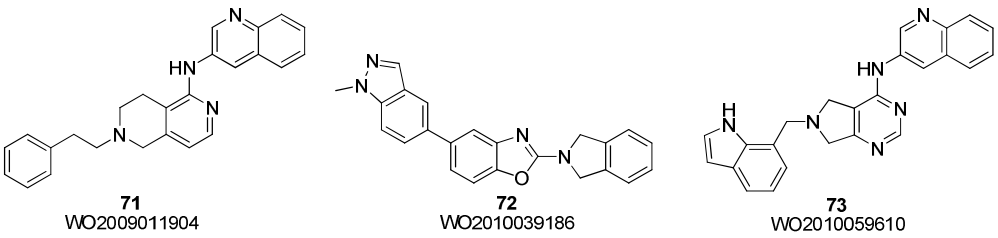

Figure 18. FAAH inhibitors from Renovis

Janssen Pharmaceuticals developed also a family of inhibitors based on a pyrimidine moiety with a C6 aryl group and a C4 amine function. Compounds **74** [136] and **75** (**figure 19**)[137] featured nanomolar activities (**figure 19**, IC_{50} = 1 and 3 nM for **74** and IC_{50} = 7 and 240 nM for **75**, against *h*FAAH and *r*FAAH, respectively).

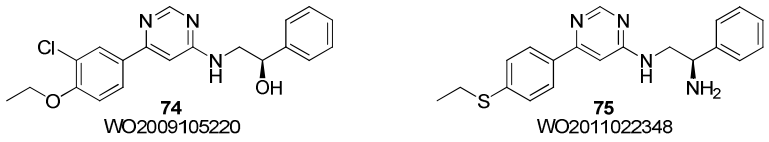

Figure 19. Pyrimidine-based FAAH inhibitors from Janssen Pharmaceuticals

Very recently, Infinity Pharmaceuticals identified the isoxazoline heterocycle as a new template for FAAH inhibition. Four series of compounds were presented based on this new scaffold. The representative compounds **76**, **77**, **78** and **79** (**figure 20**) were reported to have $K_i \leq 100$ nM and an irreversible mode of *h*FAAH inhibition.[138-140] Indeed, the authors reported evidence for a covalent FAAH inhibition *via* kinetic data, on one hand, and rapid dilution experiments, on the other hand, confirming the irreversible or slowly reversible inhibition. This mechanism of action was explained by the nucleophilic addition of the active Ser-241 on the isoxazoline C=N function followed by the elimination of the leaving group, i.e. Br or ArO substituent at C3.

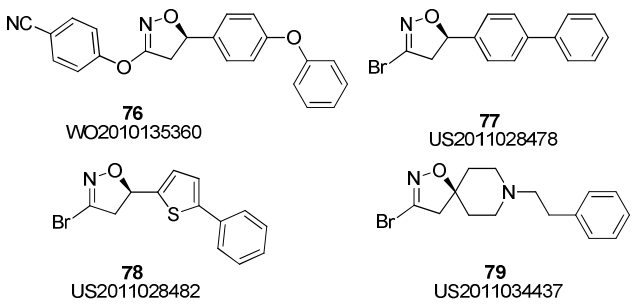

Figure 20. Isoxazoline-based FAAH inhibitors described by Infinity Pharmaceuticals

II.2.3.6 Other structures not covered by the patents

Since 2006, number of inhibitors have been published in the literature but are not covered by the patents. We have summarized here the main families. A family of 2-thioxoimidazolidin-4-ones was described to inhibit FAAH in a reversible and competitive manner (**80**, **figure 21,** pI_{50} = 5.86)[141]. Then, benzothiazole-based inhibitors were reported to reversibly inhibit FAAH with nanomolar activity (**81**, **figure 21**, IC_{50} = 1.7 nM)[142]. During the same year, two distinct series of paracetamol[143] and ibuprofen[144] analogues were disclosed to block FAAH activity with good to moderate potency (**82** and **83**, **figure 21**, IC_{50} = 100 nM and pI_{50} = 5.86, respectively). A unique series of 1-indol-1-yl-propan-2-ones was also described for a dual inhibition towards FAAH and cytosolic phospholipase $A_2\alpha$ (**84**, **figure 21**, IC_{50} = 47 nM and 2.2 µM against FAAH and $cPLA_2\alpha$, respectively).[145] In 2009, *β*-lactam-based inhibitors were disclosed to inhibit FAAH in a reversible manner without being processed by the nucleophilic serine (**85**, **figure 21**, IC_{50} = 8 nM).[51, 146] Additionally, the first potent non-covalent and competitive inhibitors of FAAH were disclosed (**86**, **figure 21**, IC_{50} = 36nM).[147]

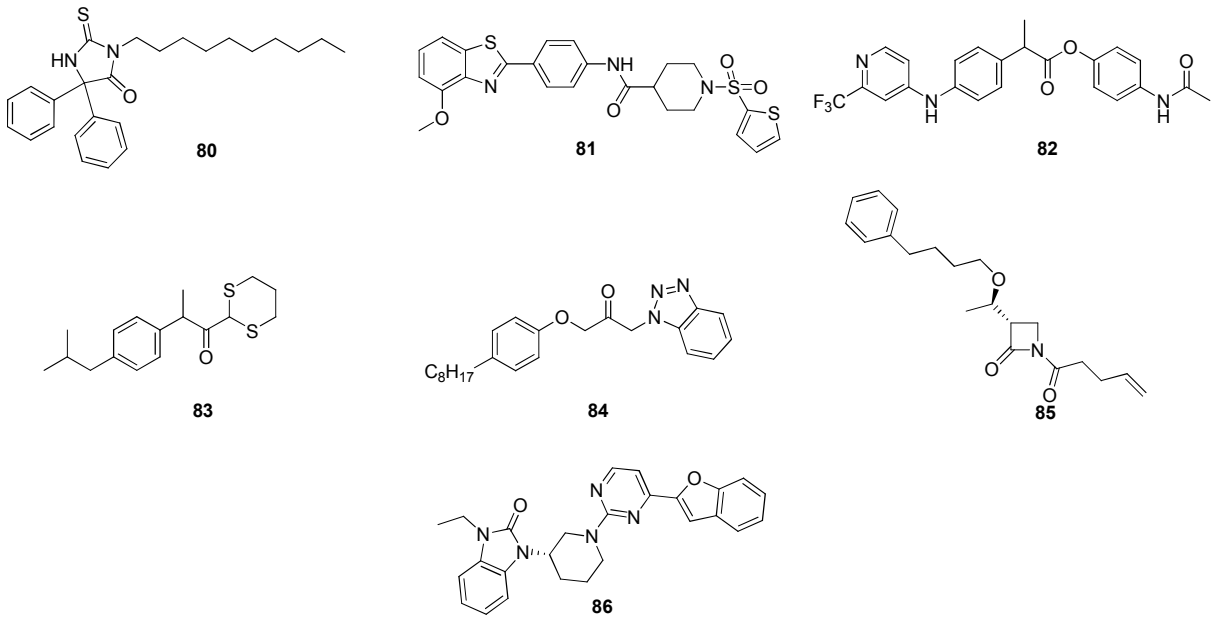

figure 21. FAAH inhibitors not covered by the patents

II.2.4 Current clinical trials involving FAAH inhibitors

Based on the preclinical studies reported so far, the most promising therapeutic applications for FAAH inhibitors are to be found in the treatment of pain and mood, and sleep disorders.

Recently, Pfizer undertook a phase II clinical trial with PF-04457845 (**35, figure 10**) to evaluate its efficacy, safety and tolerability in knee osteoarthritis (NCT00981357). Another small scale clinical trial (NCT01092845) aimed at studying the effect of **35** on sleep. Indeed, a positive effect on sleep would represent a proof-of-concept for the CNS efficacy of the compound, and more largely of increasing AEA levels, in humans.

In addition, phase II clinical trials were also undertaken to evaluate SSR-411298, a FAAH inhibitor developed by Sanofi-Aventis, for treatment of major depressive disorders in the elderly patients (NCT00822744). To date, neither the inhibitor structure nor results were reported concerning these investigations. Note however that although the development of SSR-411298 in this indication has been abandoned, other indications (e. g. pain, NCT01439919) are being investigated.

Infinity Pharmaceuticals is developing IPI-940 (no structure available) in order to treat various types of pain. Phase I resulted in positive data, IPI-940 is presented as a well-tolerated compound with good pharmacokinetic, pharmacodynamic and safety properties. Purdue Pharmaceutical Products is expected to initiate Phase II studies with this compound. Finally, Vernalis has its own FAAH inhibitor, V158866 (IC_{50} = 24 nM) entering Phase I clinical trials.

We are at the early stages of the clinical development of FAAH inhibitors; the results of the first Phase II trials are eagerly awaited to determine whether FAAH inhibition will prove to be a viable drug target.

II.3 MONOACYLGLYCEROL LIPASE

II.3.1 MAGL, structure and mechanism of action

Although the existence of a monoacylglyceride hydrolase in the adipose tissues was reported decades ago [148-149], MAGL became more actively investigated after its role in controlling 2-AG (**figure 22**) levels was demonstrated.[3-4]

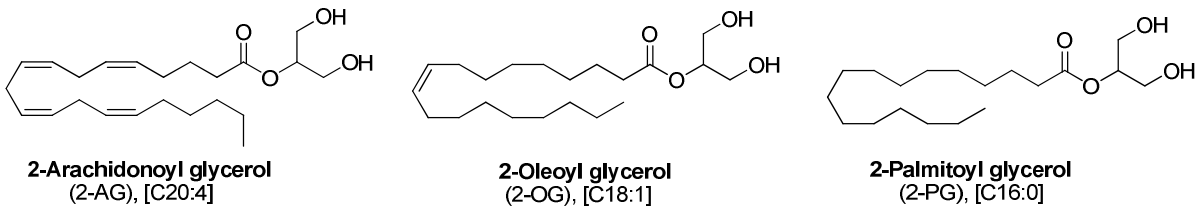

Figure 22. Known endogenous substrates of MAGL

Molecular cloning allowed determining the catalytic triad of the enzyme and its classification as a member of the α/β hydrolase family.[150] MAGL activity is governed by the classical Ser-His-Asp catalytic triad of the serine hydrolases. Additionally, four cysteine residues were shown to interact with some enzyme inhibitors [151-153]. Thus for instance, *N*-arachidonoylmaleimide, disulfiram and octhilinone were developed as MAGL inhibitors targeting those cysteine residues.[151, 153-154]

Very recently, the *h*MAGL's three dimensional structure was independently elucidated by two research teams, with a resolution of 2.2 Å[155] and 2.7 Å[156] respectively. The publication by Sanofi-Aventis described also a co-crystal between MAGL and SAR-629, one of their own MAGL inhibitors (**87**, **figure 23**). *In silico* modelling of the tetrahedral intermediate between 2-AG and the active serine,[155-156] as well as the X-ray structure of MAGL-SAR-629 co-crystal[156] allowed important structural features to be established. i) At the surface of the enzyme, a large highly hydrophobic cavity which leads to the active site is present. This channel, made of several hydrophobic residues, appears to be suitable for interacting with the lipophilic chain of the substrate and seems to govern substrate specificity. ii) a lid (or cap) is present at the entrance of the channel. This lid is suggested to allow MAGL to interact with the cell membrane, thus helping in recruiting its lipophilic substrates from the membrane. Note that MAGL is found in both soluble and particulate fraction suggesting that the interaction between the lid and the membrane is reversible. iii) Closer to the active site a hydrophilic pocket is present and appears to be able to accommodate the substrate's glycerol moiety. This pocket, named "alcohol-binding pocket"[155] or "exit-hole"[156] by the two groups, contains three residues Ala51 (Ala61 in Sanofi's paper), His121 (His131) and Tyr194 (Tyr204), important for substrate recognition, and thus potential residues to be targeted by novel inhibitors. iv) Two non-catalytic cysteines, Cys201 (Cys211) and Cys242 (Cys252), which are supposed to be targeted by Michael-acceptor inhibitors, are in the vicinity of the catalytic site. Cys242 (Cys252) lies very close to the active Ser, deeply buried in the catalytic pocket, and Cys201 (Cys211) is farther from the active serine but remains accessible to inhibitors from the active site. On the contrary, Cys208 (Cys218) is described as pointing toward the outside of the enzyme. It is expected that these crystal structures will aid the development of novel MAGL inhibitors.

II.3.2 Pharmacology of 2-AG or why inhibiting MAGL hydrolase activity?

2-AG is present at high levels in the brain, where it exerts an important role in controlling neurotransmitters release, and is also present in the periphery throughout the organism. (for a review see [157]) Indeed, beside its role as transmitter, 2-AG is an intermediate in lipid metabolism and, very likely, only a limited fraction of the 2-AG available acts as lipid mediator. Among the proposed roles for 2-AG, it was demonstrated to be involved in various processes like neuroprotection[158-160], appetite [161], cognitive and affective behaviours, or nociception[162] and inflammation, resulting from CNS and peripheral system locations, respectively. (for a complete review, see [157]) Furthermore, several studies demonstrated the involvement of 2-AG in controlling cell proliferation and invasion, suggesting that MAGL inhibitors could be relevant in cancer treatment.[163-164] Preclinical studies suggested that MAGL inhibition could represent an interesting strategy for treating pain[20, 36, 165-167], inflammation[37, 166, 168], vomiting, nausea[169] and anxiety [40].
Investigations for MAGL inhibitors are more recent that those on FAAH, resulting in a limited number of inhibitors. (see [54, 170]) Below we will review the available patents describing MAGL inhibitors.

II.3.3 MAGL inhibitors

II.3.3.1 Urea-based MAGL inhibitors

Sanofi-Aventis was the first pharmaceutical company which published MAGL inhibitors. Urea-based compounds bearing piperazinyl and triazole or triazolopyridine moieties as substituents were designed and developed.

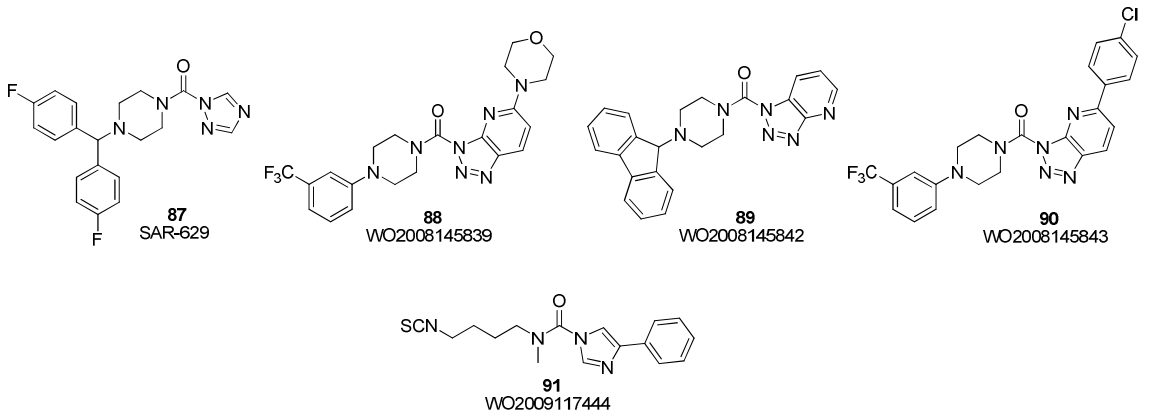

Figure 23. Urea-type MAGL inhibitors

Compounds **88**[104], **89**[171] and **90**[103] (**figure 23**) were found to inhibit *m*MAGL at nanomolar concentrations (IC_{50} values of 4 nM, 4 nM and 2 nM for **88**, **89** and **90**, respectively) and to exhibit either selectivity for MAGL over FAAH, or dual nanomolar inhibition of both enzymes. Makriyannis' group at Northearsten University also disclosed several urea-based MAGL inhibitors such as **91**[172] (IC_{50} = 42 nM against *h*MAGL). Note that, due to its isothiocyanate function, this compound could be useful as a covalent probe to explore MAGL properties, and notably the cysteine residues.

II.3.3.2 Carbamate-based MAGL inhibitors

Piomelli's group reported the ability of a carbamate derivative, URB-602 (**92**, **figure 24**), to inhibit MAGL (IC_{50} value of 28 µM)[173]. However, this compound lacks selectivity since it inhibits FAAH with a similar potency[174-175].

Much more recently, by screening its own library of carbamates, Cravatt's group found piperidinyl and piperazinyl carbamates which were able to inhibit MAGL without affecting FAAH activity.[176] The authors demonstrated that an increased steric hindrance improved the selectivity toward MAGL. This work resulted in the design of a selective and potent MAGL inhibitor with the synthesis of compound JZL-184 (**93**, **figure 24**).[177-178] Indeed, with the incorporation of two oxygen atoms in the 3 and 4 positions of the phenyl rings, they obtained an excellent selectivity in the range of 400-fold (IC_{50} values of 10 nM and 4690 nM for MAGL and FAAH, respectively). In addition, inspired by the selective piperazinyl urea-based FAAH inhibitor **32** (PF-622, **figure 10**) and by **93**, Cravatt et al. developed a series of compounds, such as **94** (JZL-195, **figure 24**), which inhibited FAAH and MAGL with a similar potency without affecting other serine hydrolases (IC_{50} values of 13 and 19 nM for FAAH and MAGL, respectively).[9]

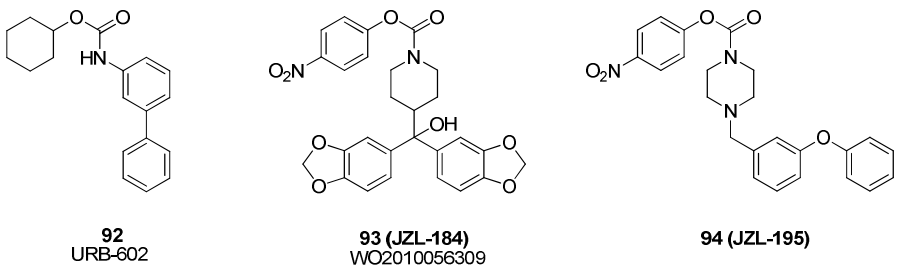

Figure 24. Carbamate-type MAGL inhibitors

Today, compound **93** is extensively used as a reference pharmacological tool to study the effects of MAGL inhibition. Several publications report the uses of **93** for increasing 2-AG levels and the resulting effects, for instance, in cancer pathogenesis[164], neuropathic pain[36], anxiety[40] and colon inflammation[168].

II.3.3.3 MAGL inhibitors of miscellaneous structure

MAGL inhibitors based on an activated ketone were described by Makriyannis' group at Northeastern University. The α-keto oxadiazole derivatives, such as **97**,[179] are quite active against MAGL, although they remain more active against FAAH (**figure 25**, IC_{50} = 71 nM and K_i = 17 nM against *h*FAAH).

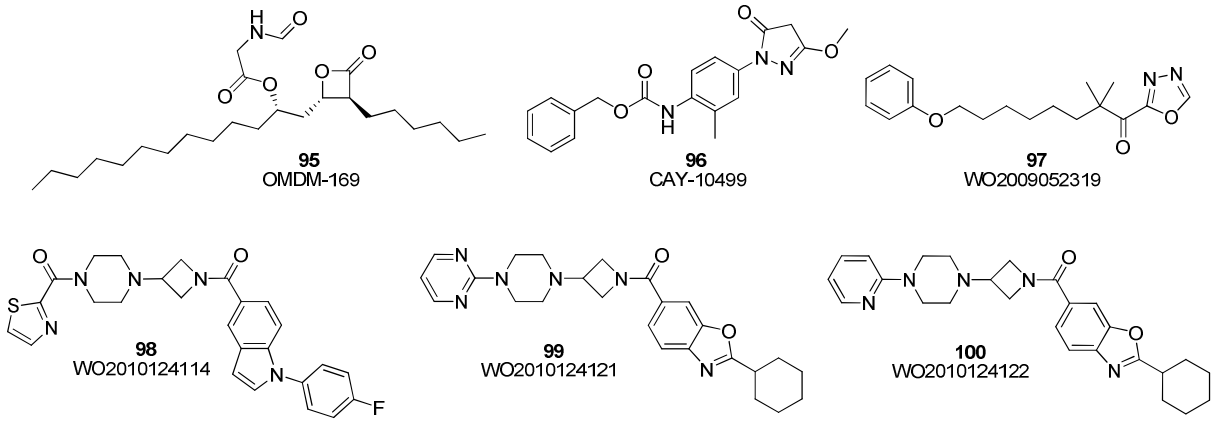

Figure 25. MAGL inhibitors of various structures

Janssen Pharmaceuticals published a series of three patents describing MAGL inhibitors based on an amide function. Each patent is illustrated with a lead compound (**98**-**100, figure 25)** that inhibit MAGL with an IC_{50} value of 10.4 nM, 10 nM and 50 nM, respectively) which was tested in various *in-vitro* and/or *in-vivo* pharmacological evaluations.[180-182] Thus for instance, **98** was able to increase 2-AG levels in an *ex-vivo* preparation of rat brain. *In-vivo*, compound **98** (30 mg/kg, po) completely prevented the CFA-induced heat hypersensitivity and partially the CFA-induced pressure hypersensitivity. Compound **99** was similarly tested in various experimental models to assess its anti-nociceptive properties. The group also published crystal structures of different MAGL mutants and co-crystallised forms with compounds **99** and **100** with resolutions of 1.35 and 2.3 Å, respectively.[183-184]

II.3.3.4 Other recent structures not covered by patents

A *β*-lactone inspired of the serine hydrolase inhibitor, tetrahydrolipstatin, was designed by Di Marzo's group. OMDM169 (**95**, **figure 25**) inhibits *h*MAGL with an IC_{50} value of 0.89 µM in a competitive manner. However, the authors also disclosed that compound **95** also inhibits *r*FAAH with an IC_{50} value of 3.0 µM.[165] Also of interest, is the finding that the 5-methoxy-1,3,4-oxadiazol-2(3H)-one moiety (**96**, **figure 25**) is also able to inhibit MAGL activity, thus offering an additional template for the development of inhibitors of the enzyme.[185-186]

II.4 N-ACYLETHANOLAMINE ACID AMIDASE

II.4.1 NAAA, structure and mechanism

Like FAAH, the *N*-Acylethanolamine-hydrolyzing Acid Amidase (NAAA) is also able to cleave amide bonds of saturated and unsaturated NAEs.[8] NAAA is thought to exert almost all of its hydrolytic activity towards PEA (**figure 2**) since other NAEs are hydrolysed at much lower rates[186]. Moreover, it is notable that NAAA does not hydrolyse 2-AG. Although NAAA, like FAAH, exerts its activity towards NAEs, there is no sequence homology between these two enzymes, and whereas the optimum pH for FAAH activity is around 9, NAAA's activity is the highest at pH 5.[188-189]. This is actually consistent with the subcellular localisation of NAAA in the lysosomes.[189-190]. Moreover, NAAA shares high sequence homology with the human acid ceramidase family, and its mode of action and structural features are closer to those of this hydrolase family than to FAAH.[191] For instance, similarly to what is found for the choloylglycine hydrolase superfamily[192], and more precisely for the acid ceramidase family, the precursor form of NAAA is auto-catalytically cleaved into two subunits, α and β, at acidic pH. Then, this cleavage leads to the appearance of the unmasked *N*-terminal nucleophilic residue responsible for the catalytic activity of NAAA.[193]. Wang *et al.* also identified Cys126 as the *N*-terminal residue and Cys126/Arg142/Asp154 as the residues constituting the catalytic triad of the human NAAA. As a consequence, the strategy for NAAA targeting is mainly based on the cysteine hydrolase activity of the enzyme, contrasting to the strategies used to target the serine hydrolases of the endocannabinoid system (i.e. FAAH, MAGL, and ABHD6). Because the discovery and initial characterisation of NAAA are quite recent, only a very limited number of studies have been published to date.

II.4.2 Pharmacology of PEA or why inhibiting NAAA hydrolase activity?

Several studies suggested that the role of NAAA is to regulate NAEs levels in macrophages and peripheral tissues.[194] As PEA is NAAA's primary substrate, its inhibition appears to be a relevant alternative to FAAH inhibition in the induction of anti-inflammatory[195], analgesic[27-28] and neuroprotective effects[196]. Indeed, these effects can be mediated by PEA through receptors that are distinct from cannabinoid receptors (e.g. PPARα)[197].

II.4.3 NAAA inhibitors

II.4.3.1 Substrate-like NAAA inhibitors

The initial studies on NAAA inhibitors consisted in the synthesis of substrate analogues. Thus esters (**101** and **106**, IC_{50} values of 19 and 10 µM respectively, **figure 26**), retroesters (**102**, IC_{50} value of 53.8 µM, **figure 26**), amides and retroamides (**103**, **104** and **105**, IC_{50} values of 31.8, 4.5 and 8.3 µM respectively, **figure 26**) of palmitic acid were developed.[198-201]

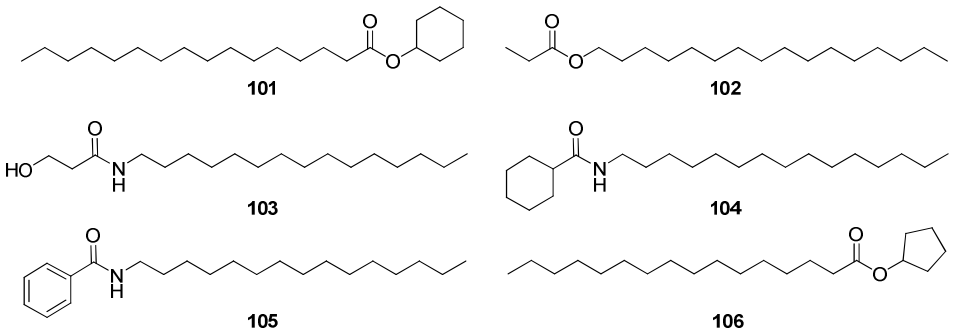

Figure 26. Substrate-based NAAA inhibitors

Compounds **101**, **102** and **103** were tested against *r*FAAH and *r*NAAA (solubilised from the 12000*g pellet of rat lung homogenates) and were found to be selective at 100 µM for NAAA versus FAAH (84, 71 and 77 % of NAAA inhibition versus 36, 0 and 8 % of FAAH inhibition, for compounds **101**, **102** and **103** respectively).[197-198] Similarly, compounds **104** and **105** do not inhibit FAAH at 100 µM. The inhibition mechanism of **104** was further investigated and was found to act by a reversible and non-competitive mechanism. This compound was also shown to inhibit NAAA in intact macrophages and in macrophage homogenates.[200]

More recently, another study based on PEA analogues has been published. The authors used recombinant *r*NAAA expressed in HEK cells to test novel series of NAAA inhibitors. Among

the assayed compounds, **106** (**figure 26**) was found to be a selective and competitive NAAA inhibitor.[201]

II.4.3.2 β-lactone-based inhibitors

To date, and to our knowledge, the only inhibitors known for inhibiting NAAA with a sub-micromolar activity are based on the *β*-lactone template.

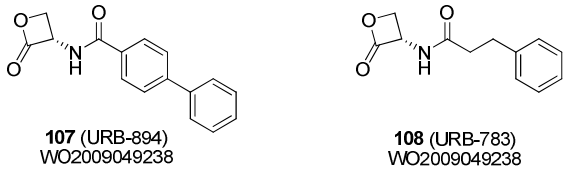

Figure 27. *β*-lactone-type NAAA inhibitors

Compounds **107** and **108** (**figure 27**)[202] were found to inhibit *r*NAAA (recombinant HEK-NAAA cells) in a non-competitive and reversible manner with IC_{50} values of 115 and 420 nM, respectively. As no crystal structure of NAAA is available, the authors built a model of NAAA catalytic site based on its high homology with conjugated bile acid hydrolase (CBAH). This model, which was validated by the docking of the tetrahedral intermediate between PEA and Cys131 (*r*NAAA), is to date, the only tool available for designing new inhibitors of NAAA.[203] Compound **107**[204] as well as compound **108**[202] exhibited anti-inflammatory effects in various inflammation models where URB-597 (KDS-4103 or **10**, **figure 5**) had no effect, suggesting that NAAA is solely implicated.

II.5 CURRENT AND FUTURE DEVELOPMENTS

When looking at the variety of compounds described here, it is safe to say that we now have the tools to fully explore the consequences of FAAH and MAGL selective inhibition. The early thinking was that using an inhibitor would allow to increase local levels of endocannabinoids due to their on-demand production. However, it appears that the administration of a FAAH or MAGL inhibitor results in increased endocannabinoid levels throughout the body (see for instance [205-206].). Although this results in a situation not that different from agonist administration, advantages of inhibiting the endocannabinoid hydrolysing enzymes still exist. First, by selectively inhibiting FAAH or MAGL only a subset

of the effects obtained following agonists administration are observed. Thus when looking at the cannabinoid tetrad of effects,[23] - *i.e.* antinociception, catalepsy, hypolocomotion, hypothermia - all the effects are present following CB_1 agonist administration, but only antinociception is induced upon FAAH inhibition. Another interesting point is that neither selective FAAH inhibition nor selective MAGL inhibition induce a cataleptic behaviour in mice. However, upon blockade of both enzymes catalepsy is present, as it is following CB_1 agonist administration (**Table 1**, and see [9]).

Table 1.

	FAAH inhibition	MAGL inhibition	FAAH/MAGL dual inhibition
Antinociception	+	+	+++
Catalepsy	-	-	++
Hypolocomotion	-	+	+
Hypothermia	-	-	-

Based on the published studies, it appears that FAAH inhibition generates less CNS-related side effects compared to MAGL inhibition. Thus for instance MAGL, but not FAAH, inhibition reduces locomotion. Another difference between MAGL and FAAH inactivation is the adaptations in CB_1 signalling observed following MAGL, but not FAAH, complete and chronic inhibition.[207-208] These adaptations, resulting in functional antagonism of the endocannabinoid system, provoke a lower analgesic effect upon MAGL chronic inhibition compared to FAAH inhibition, even though acute MAGL inhibition induces similar effects than acute FAAH inhibition.[208] Based on these studies, it has been suggested, but has not been demonstrated yet, that partial blockade of MAGL could preserve its analgesic potential also during chronic administration.

It is also worth noting that FAAH inhibition seems to be safe although a large number of bioactive lipids, besides NAEs, are hydrolysed by the enzyme. For instance, chronic inhibition of FAAH increases NAE levels, but also *N*-acyltaurines which are transient receptor potential channels agonists.[206] Conversely, brain levels of the GPR18 receptor endogenous agonist *N*-arachidonoylglycine are decreased following FAAH inhibition.[19] The question whether MAGL inhibition results in the exclusive modulation of monoacylglycerols (and corresponding fatty acids)[178, 205] remains open. Of great interest is the recent demonstration that MAGL-produced arachidonic acid is further metabolized in prostaglandins. Thus inhibition of MAGL results in increased levels of 2-AG, but also in

decreased prostaglandins levels, further supporting MAGL as an interesting anti-inflammatory target.[209]

Because NAEs (*e.g.* anandamide and *N*-palmitoylethanolamine) and 2-AG levels are profoundly affected throughout the body by FAAH and MAGL, respectively, inhibition, one could question the interest in pursuing inhibitors of the additional endocannabinoid hydrolysing enzymes (NAAA, ABHD6, ABHD12). One argument in favour of these enzymes can be found in the localisation of specific enzymes at the tissue and cell level. This results in enzymes controlling pools of signalling mediator. Thus, although MAGL controls 85 % of 2-AG hydrolysis in whole brain homogenates, ABHD6 selective inhibition[210-212] in intact neurons and in brain slices results in increased 2-AG levels and 2-AG induced-synaptic plasticity, respectively.[7] Note that it was also recently demonstrated that carboxylesterase-1 (CES-1) participates in the control of 2-AG metabolisms in macrophages.[213] When looking at NAAA, its inhibition in intact macrophages reduces AEA degradation to a similar, if not higher, extent than FAAH inhibition.[203, 214] These two examples underscore the potential of targeting NAAA, ABHD6, and perhaps ABHD12, to more precisely fine-tune endocannabinoid levels in a subset of cells inside a tissue. Thus, in addition to advancing the development of FAAH and MAGL inhibitors from bench to bedside, efforts aiming at inhibiting the other endocannabinoid-hydrolyzing enzymes should bring exciting new developments in the endocannabinoid field.

Conflict of interest:

The authors do not have any conflict of interest.

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Chapter 3

III Is serine hydrolase inhibition based on the use of β-lactam small ring adapted for inhibiting Fatty Acid Amine Hydrolase (FAAH)?

III.1 The long story of β-lactams

III.1.1 antibacterial activity of natural and remarkable β-lactams

Who does not know the legendary story of penicillin G discovery? Everyone has heard at least once the serendipitous observation of Alexander Fleming about *Penicillium* fungi, in 1928. Consequently, we have to thank Fleming for a famous negligence and his brilliant interpretation, but also Howard Florey, Ernst Chain and Norman Heatley for their contributions to purification, large scale production and use of penicillin in human medicine. Besides, in 1945, three of them received the prestigious Nobel Prize for that great discovery which revolutionized treatments of infectious diseases. From that finding, researches in this field had considerably grown and a large number of natural compounds, such as Cephalosporin C from *Cephalosporium Acremonium*, Cephamycins, Thienamycin, Clavulanic acid from various *Streptomyces* strains and Nocardicin from *Nocardia Uniformis,* were isolated and found to be, as Penicillin G, β-lactam antibiotics (Figure 1).

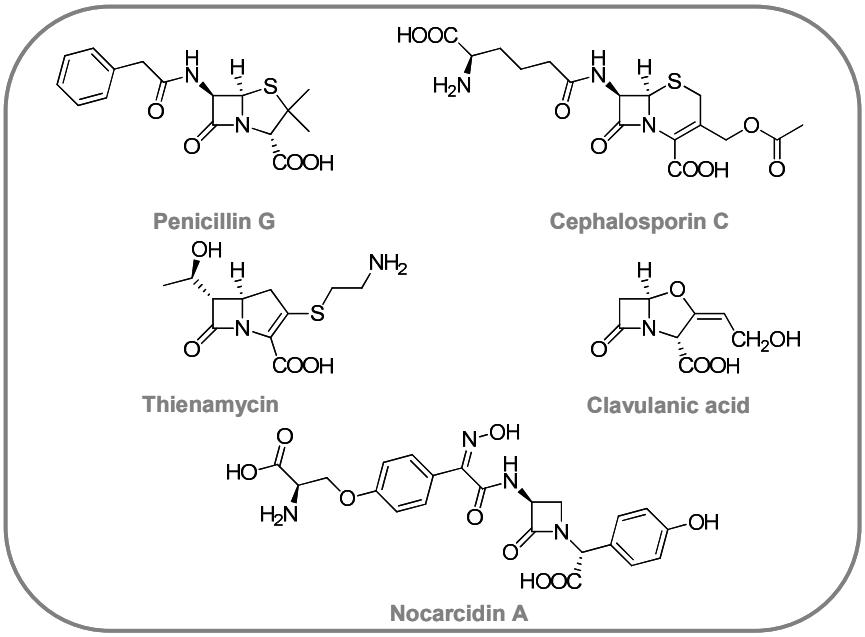

Figure 1. First natural β-lactam antibiotics isolated

β-Lactam antibiotics were classified according to the nature of the ring fused to the four-membered ring. Thus, penicillins (*i. e.* penams, oxapenams, carbapenams, penems and carbapenems) feature a five-membered ring, while cephalosporins (*i. e.* cephems, carbacephems and oxacephems) feature a six-membered ring. Monocyclic β-lactams constitute the monobactam family (Figure 2).

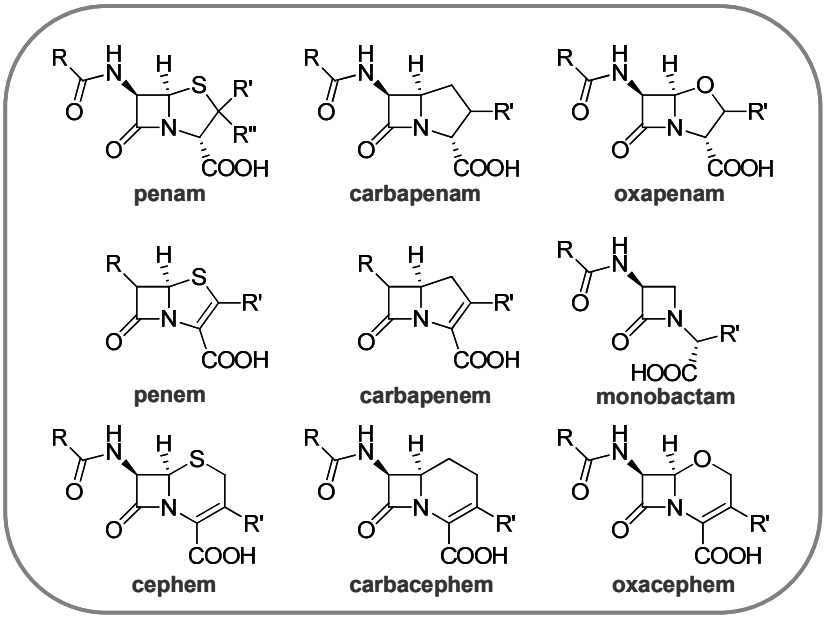

Figure 2. Classification of usual β-lactams families

The "magic ring", *i. e.* β-lactam core, is able to interact with "elements" of bacteria to inhibit their proliferation until their death. The mechanism of inhibition and the target-enzymes of penicillins and cephalosporins have been disclosed over the last thirty years.

III.1.2 Mode of action of β-lactam antibiotics

Bacteria are prokaryotic monocellular organisms delimited by a membrane consisting in a capsule layer, a cell wall and an internal membrane which protect the cytoplasmic elements inside the cell (figure 3).

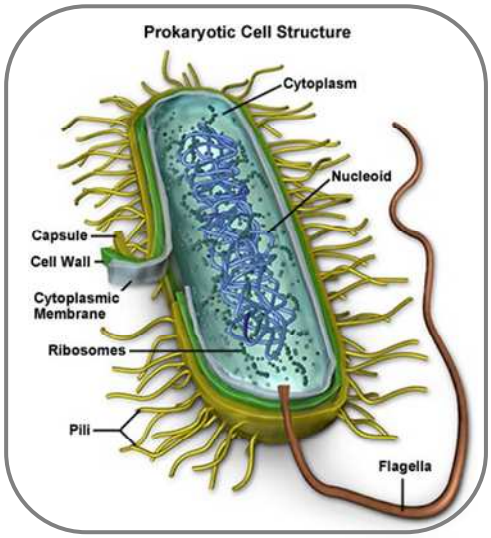

Figure 3. Prokaryote structure

The cell wall is a unique structure, involving *D*-aminoacids, without equivalent in eukaryotic organisms. The so-called "cell wall" was demonstrated to be the "weak point" of bacteria exposed to β-lactam antibiotics. The wall is composed of a peptidoglycan layer whose role is to prevent osmotic pressure variations inside the cell and to preserve cellular shape and structure. Its composition varies depending on the nature of bacteria. Bacteria belonging to Gram positive subfamily feature large peptidoglycan layer while those belonging to Gram negative subfamily have a thinner one with an additional layer of lipopolysaccharides and phospholipids (Figure 4). Thus, without such protective coat, bacterial cells lose their stability and permeability until dying.[1]

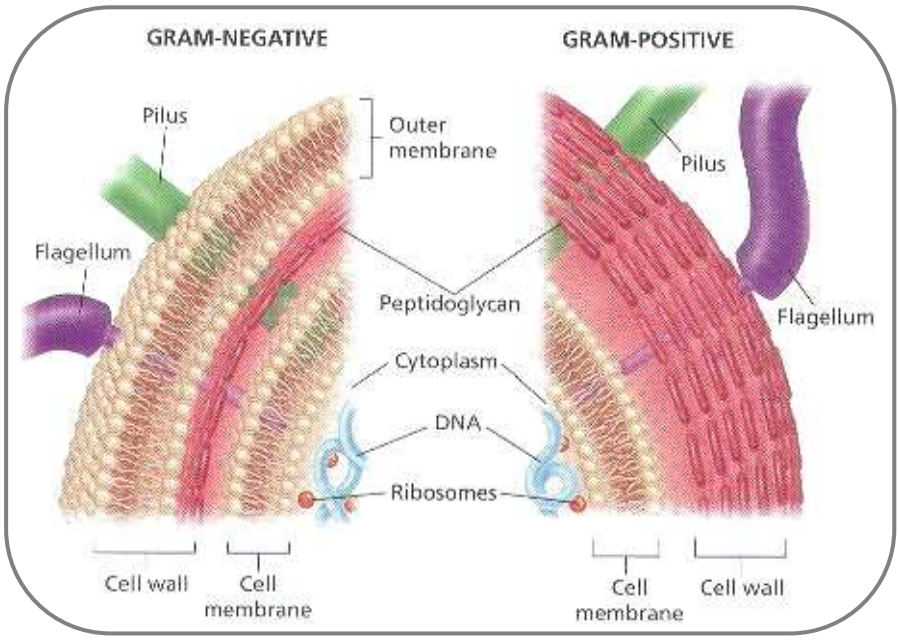

Figure 4. Composition of bacteria cell wall.

The peptidoglycans are biosynthesized and regulated by several enzymes in the bacterial cells. Among them, the targets of the β-lactam antibiotics are the enzymes which catalyze the final peptide cross-linking steps in peptidoglycan synthesis (Figure 6).[2] They are named Penicillin Binding Proteins (PBPs, also called *DD*-peptidases) because they were found to have affinity for and to bind to penicillin. By blocking constitutive enzymes, essential for achieving the rigidity of the wall, the β-lactams exert their remarkable antibacterial effects.

Among all the PBPs inhibited by β-lactams, the *DD*-peptidase activity, which could be a *DD*-transpeptidase, *DD*-carboxypeptidase or a *DD*-endopeptidase activity, was found to be their common feature. In fact, the PB domain is relatively conserved between the various PBPs and

their catalytic site involves an active serine, within a chymotrypsin-like catalytic triad, responsible of the peptidase activity.[3] In the case of PBPs, and more generally of a lot of serine proteases, three amino acids are involved in the hydrolytic processing, namely serine, histidine and aspartic acid (Ser-His-Asp).[4,5] The concerted action between these three amino acids is depicted at the figure 5.

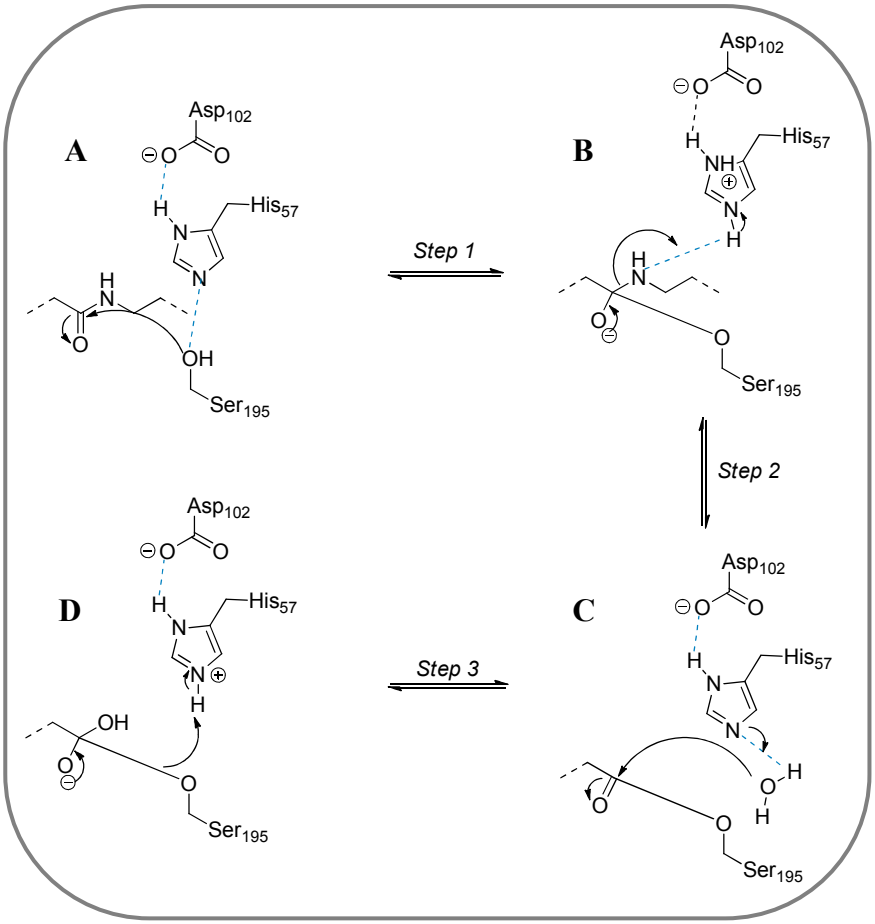

Figure 5. Proteolytic mechanism used by chymotrypsin and chymotrypsin-like enzymes. **A.** Substrate is attacked by the serine; **B.** first tetrahedral intermediate and loss of leaving group; **C.** Deacylation step by water molecule; **D.** releasing of free catalytic triad and carboxylic acid product from substrate.

An important role is played by hydrogen bonds which are the "driving force" of the catalysis. A first tetrahedral intermediate (**B**, Figure 5) is formed by the nucleophilic attack of serine on the substrate carbonyl (Step 1), which is assisted by the base role of histidine (**A**, Figure 5). Hydrogen bond between aspartate and positively-charged histidine allows its stabilisation. Then, the expulsion of the leaving group occurs (Step 2), supported with charged histidine which then reacts as an acid entity. That expulsion conducts to the formation of the "acyl-enzyme" intermediate which is hydrolyzed thanks to a water molecule (Step 3), still assisted

by histidine (**C**, Figure 5). Finally, the second tetrahedral intermediate collapses leading to the release of the initial catalytic triad and the hydrolyzed substrate (**D**, Figure 5).[47]

The natural substrate of PBPs is a peptide strand of peptidoglycan terminated by two *D*-Ala units. The transpeptidation reaction catalyzed by PBPs uses the ε-amino group of a lysine residue (of another strand of peptidoglycan) as nucleophile to cleave the amide bond and release one *D*-Ala unit (Figure 6).

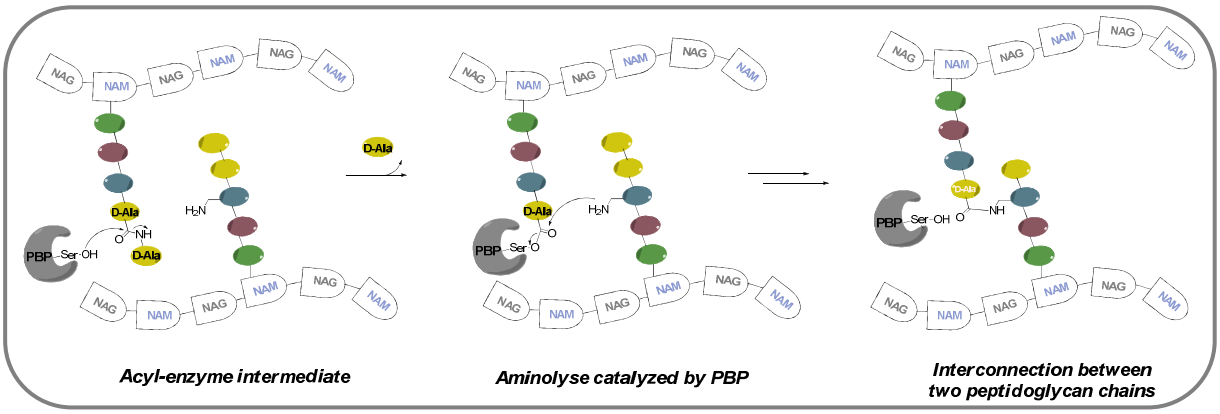

Figure 6. Peptide bond formation between two pentapeptidic chains from two peptidoglycan strands, catalyzed by *DD*-peptidase. Polymers of alternating N-acetylglucosamine (NAG) and N-acetylmuranic acid (NAM) units, which constitute peptidoglycan strands, are interconnected by the action of *DD*-peptidase. Hydrolysis of the motif *D*-Ala-*D*-Ala leads to the formation of an acyl-enzyme intermediate. This complex is aminolyzed by the terminal amine function of a basic aminoacid residue from the pentapeptide driving to the binding of the two peptidoglycan chains.

Due to the structural similarity between penicillins and X-*D*-Ala-*D*-Ala peptides (Figure 7), the PBPs recognize β-lactams as their substrates and form the first "acyl-enzyme" intermediate by active serine nucleophilic attack on the β-lactam carbonyl (C_2) (Equivalent of Step 1, Figure 5), followed by C_2-N_1 bond cleavage (Equivalent of Step 2, Figure 5). Two parameters were determined to explain the recognition and processing of β-lactam motifs by *DD*-peptidases: the Cohen distance (c)[8] – *i.e.* lactam O-atom to carboxylate C-atom distance – and the Woodward height (h) – *i.e.* height of pyramid formed by the N-atom of the β-lactam and the three adjacent C-atoms at the base – appeared to be correlated with the good activity

of β-lactams.[9] The "acyl-enzyme" intermediate (Figure 8a) is extraordinarily stable because the "leaving-group" (N_1) remains in fact linked to the substrate via the C_3-C_4 bridge of the four-membered ring. In few cases, acyl-enzyme intermediates (between purified PBPs and antibiotics) have been crystallized and X-ray data collected, confirming the mode of action described above.[10]

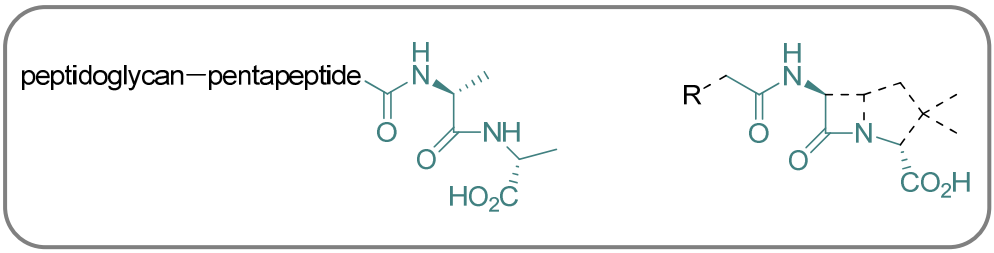

Figure 7. Similarity between *D*-Ala-*D*-Ala motif and β-lactam antibiotics

III.1.3 β-lactams and serine hydrolase inhibition mechanism

In the case of β-lactam antibiotics, because the hydrolysis of the acyl-enzyme (penicilloyl-PPB) intermediate is a very slow process, this kind of inhibition is said to be irreversible (Figure 8a). Kinetic studies have confirmed this mechanism and the stability of the inhibition. With more elaborated antibiotics (*i. e.* modified side-chains on the β-lactam ring), another possibility is the so-called "suicide inhibition". It consists in a second nucleophilic attack, after serine attack, from a nucleophile present either in the cavity of the enzyme (Figure 8b)[11] or released by the ring opening of the inhibitor, which converts it into a new reactive species (Figure 8c)[12]. In both cases, a very stable "acyl-enzyme" intermediate is obtained and an irreversible inhibition occurs.[7] Here also, crystallographic data have confirmed the suicide-type mechanisms.[13]

In addition, to counteract the antibacterial activity of β-lactam antibiotics, bacteria have developped defense enzymes which are named β-lactamases.[14] These are serine-enzymes working as *DD*-peptidases but with different kinetic parameters. β-Lactamases recognize β-lactam antibiotics to form acyl-enzyme intermediates which are rapidly hydrolyzed, leading to inactive antibiotics. Inhibitors of β-lactamases are synthetic β-lactam derivatives designed to produce an irreversible suicide-type inhibition.

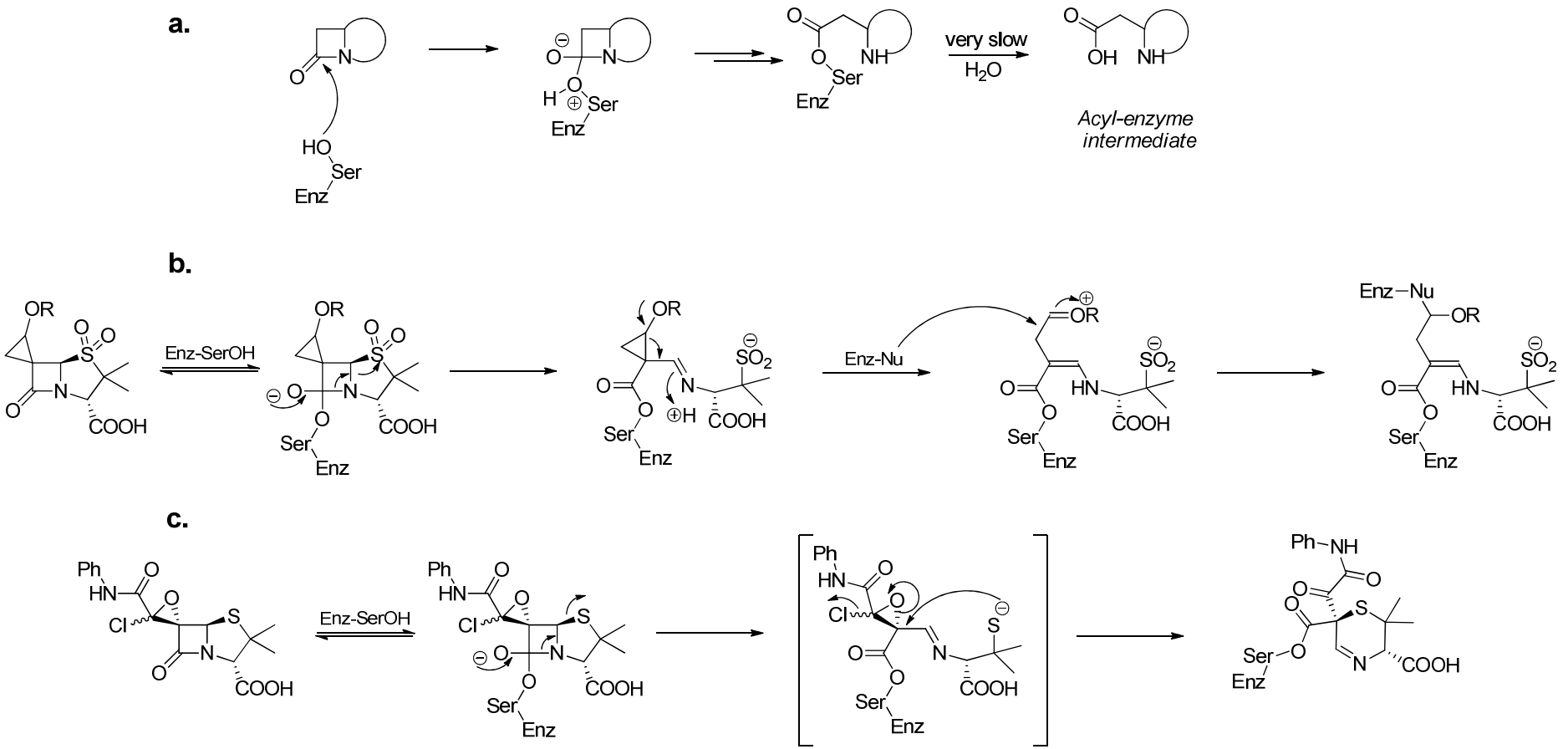

Figure 8. a) general reactivity of β-lactam ring with a serine hydrolase, formation of the tetrahedral intermediate and then the acyl-enzyme intermediate. Its hydrolysis is a so slow step that inhibition is said to be irreversible. **b)** Suicide-type inhibition with a nucleophilic residue inside the active site[11] and **c)** with an intramolecular nucleophile appearing after the serine attack[12].

Anti-β-lactamase compounds are actually given to the patients in combination with β-lactam antibiotics in view of protecting them against enzymatic hydrolysis. Both past and recent[15] stories of β-lactam antibiotics (penicillin-like) illustrate well the interest of the β-lactam core as a template for the inhibition of serine hydrolases in general. A lot of examples are now described and published in the literature. Thus, human leukocyte elastase (HLE) and porcine pancreatic elastase (PPE),[16] prostate specific antigen (PSA),[17] cathepsin G, human cytomegalovirus protease (hCMV),[18] thrombin[19] and many others were found to be effectively inhibited by diverse and varied β-lactams.

III.2 Objectives and strategy

As previously said, FAAH is responsible of the degradation of anandamide, an endocannabinoid which binds to cannabinoid receptors CB_1 and CB_2. Although FAAH does not share the usual catalytic triad Ser-His-Asp, it belongs to the large family of serine hydrolases. Strategies, early developed in the literature, to inhibit FAAH were based on traditional serine hydrolase inhibitors (*e. g.* fluorophosphonates, activated ketones, and later carbamates and ureas). Surprisingly, β-lactams were not considered before the works of our laboratory. This is why, we imagined to design new inhibitors based on the β-lactam scaffold to target human FAAH.

Preliminary works were carried out with potential inhibitors selected from a library of compounds initially prepared to inhibit various (bacterial) serine proteases. Allan Urbach found that some compounds showed activities towards human FAAH. The hit compounds **1, 2** and **3** (Figure 9) inhibited FAAH with moderate activities (IC_{50} values of 21.9, 4.5 and 0.098 µM, respectively). Interestingly, they are selective of FAAH versus MAGL (IC_{50} values of 817, 657 and 23.3 µM, respectively).[20]

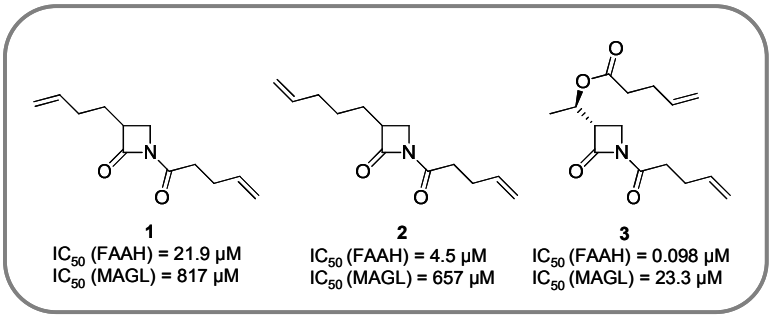

Figure 9. Preliminary hit compounds from Allan Urbach thesis (2006)[21]

Inspired by compound **3** structure, our approach is based on the synthesis of novel molecules from the commercially available (3*R*,4*R*)-4-acetoxy-3-[(*R*)-1-(tert-butyldimethylsilyloxy)ethyl]azetidin-2-one **4** (Figure 10), a chiron used in the preparation of carbapenem antibiotics. Our aim is to design lipophilic inhibitors which will be able to fit the catalytic pocket of human FAAH and therefore to improve the affinity and activity against FAAH while conserving the selectivity versus MAGL.

This thesis is a complete medicinal chemistry project, including the design, synthesis and pharmacological evaluation of new kinds of inhibitors. The organic chemistry evolved in function of the pharmacological results, following an iterative process.
We did not use high throughput screening (HTS) but dedicated small libraries of easily accessible β-lactams. Thanks to the starting material **4**, we will be able to independently functionalize the *N*- and *O*- positions (Figure 10). Moreover, purified human FAAH and human MAGL, available at the Louvain Drug Research Institute (LDRI) allow the elaboration of all sorts of testing, to assess the level of activity and the mode of inhibition.
A human FAAH model will be built based on homology modelling with rat FAAH, allowing to inform us how our inhibitors are placed in the catalytic site and what the interactions are between compounds and amino acid residues. In addition, the enzymatic processing of the novel β-lactams will be studied in order to confirm or not their classical irreversible mode of action. Here again, organic chemistry will undeniably complement pharmacology. As the crystallized form and X-ray data of an engineered human/rat FAAH became available during the thesis, a lot of structural features could be understood.

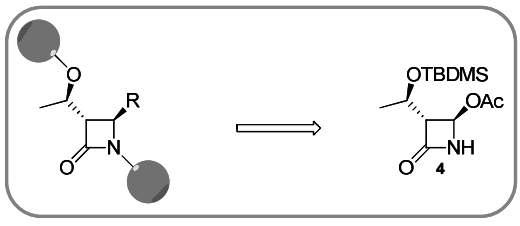

Figure 10. Synthesis from a carbapenem chiron

III.3 Thesis Outline

During our thesis, the project progressed as fast as questioning came. The opportunity to work in chemistry and pharmacology laboratories drove continuously the researches towards

original answers to our questions. Accordingly, all the results were published in a linear and chronological manner following our reflection. Thus, the manuscript is presented as a compilation of one review and three full articles published (or submitted).
The first chapter is a general introduction of the endocannabinoid system dealing with the presentation of its principal actors and main features. The second chapter is adapted from our review which was published in Recent patents in CNS drug discovery and more deeply presents the degradation-enzymes implied in endocannabinoid system and all the inhibitors described until today, especially in patent literature. This leads us to the third chapter where a brief story of the use of β-lactams in medicinal chemistry is depicted and our project to inhibit FAAH with β-lactams is introduced.

The chapters 4, 5 and 6 consist in the chronological progress of our researches published or submitted in the journal of medicinal chemistry.

In the first full-paper (chapter 4), we prepared two novel families from compound **4** (Figure 10). These compounds were obtained by a three-step synthesis route. Among the thirty synthesized azetidinones, a lead compound (IC_{50} = 5.3 nM, **19b**, chapter 4) was described to be a potent and selective inhibitor of FAAH versus MAGL. In addition, exploratory mechanistic studies revealed a surprising reversible mode of action.

Following that observation of unusual reversible mode of inhibition, deeper mechanistic studies were performed (chapter 5) using at a time organic synthesis (SAR studies), pharmacological studies but also LC/MS analysis in order to establish whether or not our compounds are substrates of FAAH. All the experiments drove to the evidence of a non-hydrolytic processing and highlighted the importance of the exocyclic carbonyl function, fixed on the β-lactam nitrogen atom, for the FAAH inhibition. The β-lactam ring itself is not the pharmacophore!

Finally, we incorporated heteroatoms in our lead structure (referenced as **19b** in chapter 4, **4** in chapter 5 and **4a** in chapter 6) in order to modulate the logP value and the polar surface area (PSA) on the one hand, which results in a modification of the solubility and permeability of our inhibitors. These two parameters vary in an inverse manner and provide information to predict the permeation of drugs through the different membranes (Blood-Brain Barrier (BBB) or peripheric barriers) and therefore their bioavailability. On the other hand, we checked

whether the presence of heteroatoms could improve the activity and/or change the mechanism of inhibition in the case of two compounds which present a good leaving group.

At the end of the manuscript, all the results and the perspectives are discussed to conclude on the innovation we have brought in the world of endocannabinoid-degrading enzymes, and what we could still do.

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Chapter 4

IV β-Lactams derived from a carbapenem chiron are selective inhibitors of human Fatty acid amide hydrolase versus human Monoacylglycerol lipase

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The first part of our work consisted in the synthesis of two novel β-lactam families based on a previous work in our laboratory. They were both tested as FAAH and MAGL inhibitors and, consequently, first elements of SAR were identified. In addition, preliminary experiments, to elucidate the mechanism and the kinetic of inhibition, were performed.
Finally, docking studies were undertaken to illustrate the reversible mode of inhibition.

Dr. Catherine Michaux performed the modelling and docking experiments.
Dr. Allan Urbach inspired our work by its PhD project, compounds **19e** and **19f** were already synthesized in this thesis.
Dr. Geoffray Labar purified hFAAH and co-performed kinetic studies with M. Feledziak.
Pr. Giulio Muccioli gave advices for pharmacological testing.

All these results were published in the Journal of Medicinal Chemistry, in 2009.

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β-lactams derived from a carbapenem chiron are selective inhibitors of human Fatty acid amide hydrolase versus human Monoacyl glycerol lipase

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A library of thirty β-lactams has been prepared from (3*R*,4*R*)-3-[(*R*)-1'-(tertiobutyldimethylsilyloxy)-ethyl]-4-acetoxy-2-azetidinone, and the corresponding de-acetoxy derivative, by sequential N- and O-functionalizations with various ω-alkenoyl and ω-arylalkanoyl chains. All compounds were selective inhibitors of hFAAH versus hMGL, and IC_{50} values in the nanomolar range (5-14 nM) were recorded for the best representatives. From time-dependant preincubation and rapid dilution studies, and from docking analyses in a homology model of the target enzyme, a reversible mechanism of inhibition of hFAAH is proposed.

Adapted from a published article, in journal of medicinal chemistry, **2009**, 52 (22), 7054-7068.

IV.1 Introduction

The 2-azetidinone template (β-lactam) has been widely described as a lead structure for the inhibition of serine hydrolases such as human leukocyte elastase (HLE)[1], prostate specific antigen (PSA)[2], thrombin[3], human cytomegalovirus[4], and mainly D,D-peptidases and β-lactamases, the bacterial target-enzymes of penicillin-type drugs used in antibiotherapy.[5] Generally, enzyme inhibition results from the interaction between the 2-azetidinone carbonyl and the active serine of the catalytic triad Ser-His-Asp. This interaction creates a covalent bond, *via* a tetrahedral intermediate, leading to a relatively stable acyl-enzyme complex, and therefore to the inhibition of the enzyme. Slow hydrolysis of the acyl-enzyme complex can regenerate the active enzyme, but in the case of so-called "suicide-substrates",[6] the inhibition is irreversible because the acyl-enzyme structure is no more sensitive towards hydrolysis. Surprisingly, the β-lactam motif has never been considered for fatty acid amide hydrolase (FAAH) inhibition, until our preliminary study which disclosed 3-alkenyl-2-azetidinones as micromolar inhibitors[7]. Like the above mentioned enzymes, FAAH is a serine hydrolase but a member of a distinct class from the chymotrypsin family. Indeed, the active site differs from traditional enzymes by the replacement of Ser-His-Asp catalytic triad with Ser-Ser-Lys triad which constitutes the so-called amidase signature (AS).[8, 9] Recently, a second AS enzyme has been discovered and termed FAAH-2;[10] regarding the original FAAH (also named FAAH-1), this enzyme shares only 20 % sequence identity, but the same amide hydrolyzing activity using a Ser-Ser-Lys triad. FAAH exerts its activity on substrates possessing an amide bond, especially endogenous fatty acid amides (FAA). The principal substrate, and the most studied, is anandamide (arachidonoylethanolamide, AEA), a partial agonist of cannabinoid receptors CB_1 and CB_2.[11] Therefore, FAAH is commonly said to belong to the endocannabinoid system which consists of different hydrolases : FAAH-1, FAAH-2, monoacylglycerol lipase (MGL)[12] and *N*-acylethanolamine-hydrolyzing acid amidase (NAAA),[13] among others.[14-17] MGL and NAAA preferentially hydrolyse 2-arachidonoylglycerol (2-AG) or 2-oleoylglycerol (2-OG) and palmitoylethanolamide respectively. FAAH hydrolyses anandamide, other endogenous fatty acid amides, but also a particular class of *N*-acylamino acids, *i. e N*-acyl taurines (NATs) which activate transient receptor potential (TRP) ions channels,[18] and oleamide,[19] a fatty acid primary amide recognized as a sleep-inducing lipid. The actual knowledge on these bioactive lipids and the role played by FAAH in the control of their levels open the door to the development of novel therapeutic agents.[20] Indeed, pharmacological investigations in animal models have shown that a large number of biological benefic effects such as appetite

stimulation, anti-inflammatory effect, sleep-induction[21], anxiety release and analgesia[22, 23] could be enhanced by controlling FAAH catabolic activity.

The search of FAAH inhibitors constitutes a domain of growing interest which has been recently reviewed.[24, 25] Potent inhibitors based on different types of electrophilic functions have been published. They are divided into two mechanistic classes : irreversible carbamates[26-31] and ureas[32, 33] inhibitors, which include the pharmacological tools **1** (URB-597)[34] and **2** (PF-622)[33], and the reversible α-keto oxazoles[35-39] inhibitors (and other heterocycles) illustrated by **3** (OL-135)[23] (Figure 1). Reaction of **1** and **2** with FAAH leads to inactive and stable acyl-enzymes. Initial proton exchange between Lys142, Ser217 and Ser241 (catalytic triad) allows the nucleophilic attack of Ser241 on the carbonyl function of the inhibitor; the resulting tetrahedral intermediate expulses the leaving group, namely the phenol moiety of **1** or the aniline group of **2**, along with proton transfer from Ser217, thus leading to Ser241 covalently modified as a carbamate. The postulated mechanism of FAAH interaction with **3** starts similarly, but since the tetrahedral intermediate features no leaving group, reversible inhibition occurs. Within this family of covalent reversible inhibitors, SAR studies have clearly shown that the activity is linked to the electrophilic character of the ketone.[35]

Embedding the sensitive carbonyl function into a cyclic structure appears to be a quite unusual strategy for the design of FAAH inhibitors. (*E*)-6-(Bromomethylene)tetrahydro-3-(1-naphthalenyl)-2*H*-pyran-2-one (**4**, Figure 1) was an early covalent inhibitor of anandamide hydrolysis.[40] A unique series of (thio)hydantoin-based FAAH inhibitors, exemplified with 3-heptyl-5,5'-diphenylimidazolidine-2,4-dione (**5**, Figure 1), has been reported by Muccioli *et al*.[41] Such molecules act as competitive inhibitors without being hydrolyzed by the enzyme. Lastly, a few lipophilic β-lactams were shown to be modest inhibitors of FAAH : 3-(4'-pentenyl)-1-(4'-pentenoyl)-2-azetidinone (**6**, Figure 1) emerged as a micromolar inhibitor.[7] Starting from this preliminary result, we have investigated the synthesis and the pharmacological properties of a new family of FAAH inhibitors, derived from acetoxy-azetidinone **7**, in order to possibly improve the activity. The structures were decorated with different acyl chains on N1 and C5-O positions, featuring a terminal phenyl (Ph), biphenyl (biPh) or alkene (Alk) motif as found on the hydrophobic scaffolds of traditional FAAH inhibitors. A series of thirty azetidinones was evaluated *in vitro* for the inhibition of human FAAH (*h*FAAH) and human MGL (*h*MGL). The most promising compounds were submitted to a docking study in a new model of *h*FAAH.

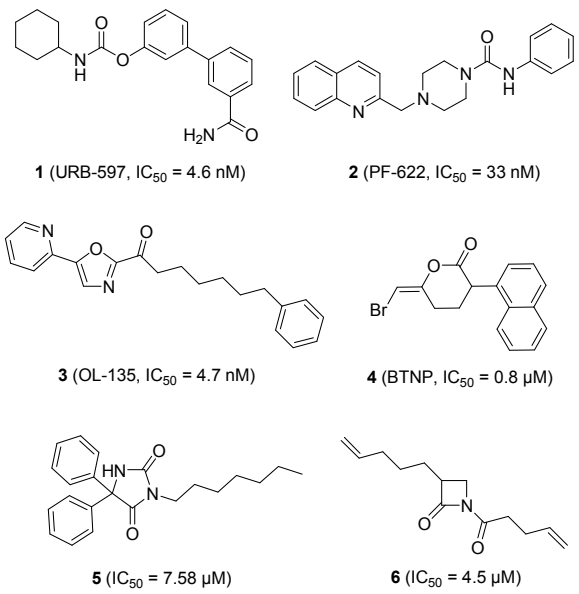
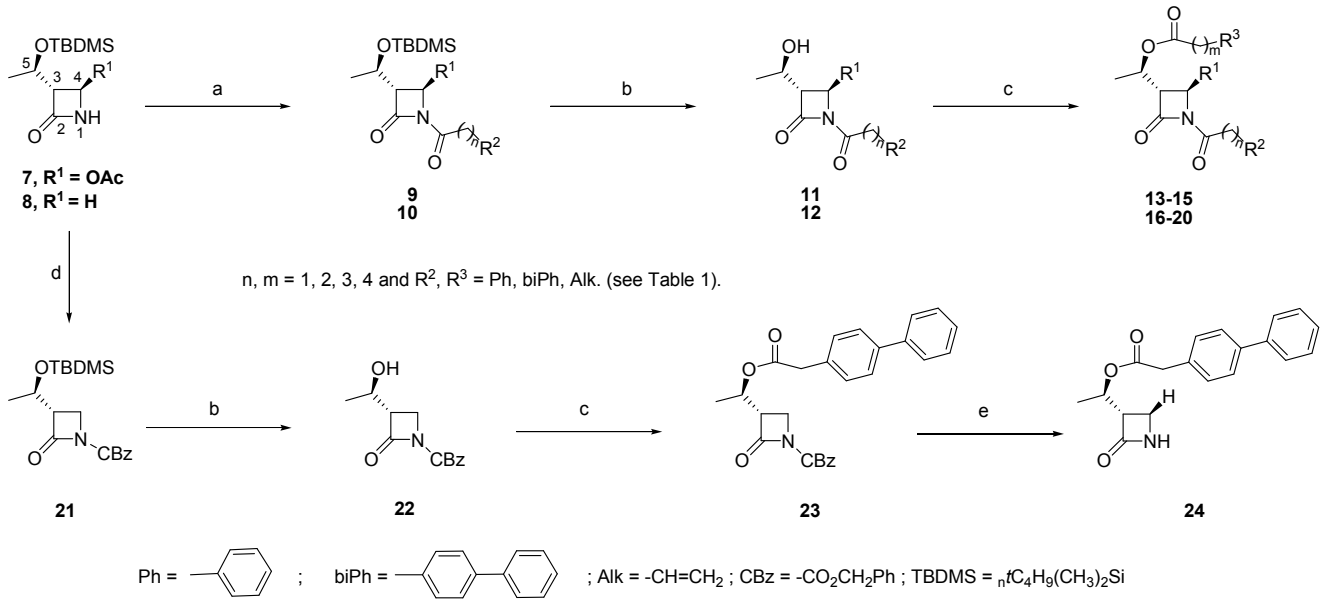


Figure 1. Structures of previously described FAAH inhibitors (the mentioned IC_{50} is the one reported by the respective authors, against rat enzyme)

IV.2 Results and discussion

IV.2.1 Synthesis

Acetoxy-azetidinone **7** is a commercially available chiral precursor of (carba)penems antibiotics.[42] This molecule offers several advantages: (i) the amide function can be easily substituted on the N1 position; (ii) after deprotection of the silyl ether group, the hydroxyl function of the side-chain can also be substituted (C5-O position); (iii) the acetoxy substituent (OAc) on the C4 position increases the heterocycle chemical reactivity (N1-C2 cleavage) by its electronwithdrawing effect; (iv) OAc is also a good leaving group. This last structural feature would make possible the occurrence of an irreversible suicide-type inhibition, if a serine hydrolase enzyme reacted on the β-lactam ring. Moreover, the chemical reactivity of the OAc substituent allows its formal elimination by a two-step sequence of reactions (substitution/reduction), giving the less hindered and more stable precursor **8** (scheme 1).



Scheme 1. Synthesis of substituted azetidinones. Reagents and conditions : (a) acyl chloride, pyridine, DCM, 45 °C, 24 h; (b) HCl, AcOH, ACN, -5 °C to rt, 3 h; (c) acyl chloride, pyridine, DCM, rt, 15 h or carboxylic acid, DCC, DMAP, DCM, rt, 15 h; (d) benzyl chloroformate, LiHMDS, -78 °C to rt, 4 h; (e) H_2, Pd/C, EtOH/AcOEt, 1 h.

A first family of lipophilic azetidinones was prepared from **7**, taking inspiration from previously described protocols (Table 1, entries 1 to 10).[43, 44] Briefly, **7** was *N*-acylated by reaction with hydrocynnamoyl chloride, 4-phenyl-butanoyl chloride or 4-pentenoyl chloride, and pyridine, in refluxing dichloromethane (DCM), to furnish respectively azetidinones **9a** (89 %), **9b** (94 %) and **9c** (80 %). The silyl ether function was deprotected by treatment with HCl-HOAc at -5 °C. The resulting alcohols **11a-c** (83-99 %) were directly engaged in esterification reactions with hydrocynnamoyl chloride, 4-phenyl-butanoyl chloride or 4-pentenoyl chloride, in the presence of pyridine at room temperature, giving the following bis-acylated compounds : **13a** (99 %), **14a** (88 %), **13b** (70 %), **14b** (52 %) and **15e** (90 %). The biphenylacetyl side chain was introduced by an alternative method: the reaction of **11a,b** with biphenylacetic acid and dicyclohexylcarbodiimide (DCC), in the presence of dimethylaminopyridine (DMAP) as catalyst. Compounds **13d** (67 %) and **14d** (77 %) were isolated.

A second family of compounds (Table 1, entries 11 to 31) was prepared from **8**.[45] This precursor could be readily obtained by substitution of **7** with thiophenolate followed by reduction with tris(trimethylsilyl)silane hydride (see supporting information). As above, **8** reacted with hydrocynnamoyl chloride, 4-phenyl-butanoyl chloride, 5-phenyl-pentanoyl

chloride, 4-pentenoyl chloride or 5-hexenoyl chloride to afford respectively the *N*-acylated azetidinones **10a** (88 %), **10b** (87 %), **10c** (74 %), **10d** (95 %) and **10e** (46 %) (see scheme 1). After *t*-butyldimethylsilyl deprotection under acidic conditions, the resulting alcohols **12a-e** (78-94 %) were esterified with various acid chlorides and pyridine (Method A), or with the corresponding carboxylic acids, DCC and DMAP (Method B). Application of the Method A to hydrocynnamoyl chloride and **12a,b** gave the azetidinones **16a** (79 %) and **17a** (89 %). From 4-phenyl-butanoyl chloride and **12a-e** were obtained respectively **16b** (75 %), **17b** (87 %), **18b** (63 %), **19b** (84 %) and **20b** (77 %). Reaction of 4-pentenoyl chloride with **12d** furnished **19e** (88 %). Applying the Method B to **12a,b** and 5-phenylvaleric acid, we produced the bis-acylated azetidinones **16c** (59 %) and **17c** (93 %). Similarly, from biphenylacetic acid and **12a-e**, we prepared the compounds **16d** (93 %), **17d** (83 %), **18d** (81 %), **19d** (68 %) and **20d** (66 %). Lastly, reaction of **12d** with 5-hexenoic acid gave the azetidinone **19f** (84 %).

For comparison purpose (see below, enzymatic tests), one representative azetidinone mono-substituted at the C5-O position was prepared in four steps (Scheme 1 and Table 1, entry 32). Amide protection of **8** with a benzyloxycarbonyl group (**21**, 99 %), silyl ether deprotection as usual (**22**, 91 %), esterification with biphenylacetic acid (**23**, 83 %) and N1 deprotection by catalytic hydrogenation afforded the azetidinone **24** (96 %; overall yield for four steps, 72 %).

All final azetidinones and intermediates were fully characterized by the usual spectroscopies (see experimental section). Typical features are exemplified with **14d** (first series, R^1 = OAc) and **19b** (second series, R^1 = H). ^{1}H NMR spectrum of **14d** shows the vicinal β-lactamic protons with the *trans* relationship at 3.28 ppm (H3, dd, J = 6.5 and 1.7 Hz) and 6.46 ppm (H4, d, J = 1.7 Hz); four carbonyl signals are visible in ^{13}C NMR at 170.4 (O-CO), 169.8 (N-CO), 169.1 (OAc) and 162.2 (β-lactam carbonyl) ppm; the IR spectrum shows the carbonyl stretchings at 1803 (β-lactam), 1740 (broad, OAc and ester) and 1717 (imide) cm^{-1}. For **19b**, the geminal β-lactamic protons H4/H4' appear in ^{1}H NMR as a typical ABX pattern at 3.53 ppm (dd, J = 7.7 and 3.7 Hz) and 3.66 ppm (dd, J = 7.7 and 6.6 Hz), while H3 gives a multiplet at 3.40 ppm; the ^{13}C NMR spectrum shows three carbonyl signals at 172.4 (O-CO), 170.3 (N-CO) and 164.4 (β-lactam CO) ppm, and the IR spectrum shows the carbonyl stretchings at 1786 (β-lactam), 1734 (ester) and 1703 (imide) cm^{-1}. In both series (R^1 = OAc or H), H5 proton of precursors **9,10** (silyl ether) and **11,12** (free alcohol) gives a multiplet (qd) around 4.3 δ in ^{1}H NMR spectra; after the *O*-acylation leading to the final compounds **13-15** and **16-20**, a deshielding of about 1 δ is observed (H5 around 5.3 δ). The

chemical and enantiomeric purity of all tested compounds has been controlled by HPLC, using C18 and AD-H columns, respectively.

Table 1. Determination of the inhibitory potential of azetidinones towards human FAAH and human MGL

entry	compound	R^1	n	R^2	m	R^3	IC_{50} *h*FAAH[a]	% inhibition (MGL)[b]	IC_{50} *h*MGL
1	**11a**	OAc	2	Ph	-	-	223.6	48	-
2	**11b**	OAc	3	Ph	-	-	182.8	61	-
3	**11c**	OAc	2	Alk	-	-	537.0	16	-
4	**13a**	OAc	2	Ph	2	Ph	2.02	100 (0)	-
5	**13b**	OAc	2	Ph	3	Ph	0.96	100 (0)	-
6	**13d**	OAc	2	Ph	1	biPh	0.826	66	-
7	**14a**	OAc	3	Ph	2	Ph	5.12	100 (0)	-
8	**14b**	OAc	3	Ph	3	Ph	3.12	100 (0)	-
9	**14d**	OAc	3	Ph	1	biPh	0.708	100 (0)	-
10	**15e**	OAc	2	Alk	2	Alk	1.9	99 (33)	133
11	**12a**	H	2	Ph	-	-	408.7	8	-
12	**12b**	H	3	Ph	-	-	nd	nd	-
13	**12c**	H	4	Ph	-	-	nd	nd	-
14	**12d**	H	2	Alk	-	-	7.9	89 (8)	-
15	**12e**	H	3	Alk	-	-	nd	nd	-
16	**16a**	H	2	Ph	2	Ph	0.157	100 (0)	-
17	**16b**	H	2	Ph	3	Ph	0.049	100 (0)	-
18	**16c**	H	2	Ph	4	Ph	0.091	100 (0)	-
19	**16d**	H	2	Ph	1	biPh	0.050	31	-
20	**17a**	H	3	Ph	2	Ph	0.057	54	-
21	**17b**	H	3	Ph	3	Ph	0.030	100 (0)	-
22	**17c**	H	3	Ph	4	Ph	0.045	59	-
23	**17d**	H	3	Ph	1	biPh	0.032	0	-
24	**18b**	H	4	Ph	3	Ph	0.449	39	-
25	**18d**	H	4	Ph	1	biPh	0.236	25	-
26	**19b**	H	2	Alk	3	Ph	0.005	89	4.06
27	**19d**	H	2	Alk	1	biPh	0.012	91	1.84
28	**19e**	H	2	Alk	2	Alk	0.098	99	23.3
29	**19f**	H	2	Alk	3	Alk	0.032	8	4.72
30	**20b**	H	3	Alk	3	Ph	0.010	85	8.51
31	**20d**	H	3	Alk	1	biPh	0.014	67	14.6
32	**24**	H	-	-	1	biPh	6.5	16	-

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

[b] Percentage of inhibition at 10^{-4} M. The percentage of inhibition at 10^{-6} M is stated between brackets.

See supporting information for the corresponding table of pI_{50} values and Standard Error.

IV.2.2 Biochemical evaluation

The azetidinones listed in Table 1 have been tested as potential inhibitors of *h*FAAH and *h*MGL. Human recombinant enzymes, developed in our laboratory[46, 47], were used in competitive hydrolytic assays using [^{3}H]-radiolabelled AEA and [^{3}H]-radiolabelled 2-OG,

respectively, as substrates. Tested compounds, enzymes and [^{3}H]-substrates were incubated at 37 °C during 10 min. The inhibition rates were evaluated by liquid scintillation counting (LSC) of the restitual hydrolysis products of the labelled substrates. The results reported in Table 1 are the means of three independent assays.

-FAAH inhibition- Collected results clearly show that the azetidinones equipped with only one acyl chain, at N1 position (entries 1 to 3 and 11 to 15) or C5-O position (entry 32), are modest or very weak inhibitors of FAAH. Amongst the compounds bearing two acyl chains, fixed at N1 and C5-O positions, the first series (R^1 = OAc, entries 4 to 10) systematically appears less active than the second one (R^1 = H, entries 16 to 31). Our initial hypothesis that the C4 acetate substituent would improve the azetidinone inhibitory effect - by increasing the chemical reactivity of the heterocycle (electronwithdrawing effect) and/or by initiating an enzymatic suicide-mechanism (leaving group effect) – turned out to be contradicted by these first results. Accordingly, the discussion focuses only on the second series of disubstituted azetidinone inhibitors **16a-d**, **17a-d**, **18b,d**, **19b-f** and **20b,d** which are potent FAAH inhibitors. The studied factors were the chain length (n, m = 1 to 4) and the nature of the end group (Ph, biPh, Alk) for both substituted positions (N1, C5-O). All compounds **16-20** revealed to be good inhibitors of *h*FAAH with IC_{50} values ranging from 0.005 μM (**19b**) to 0.45 μM (**18b**). Comparatively to our previous "hit" (structure **6**, Figure 1; IC_{50} = 4.5 μM), the activities have been significantly improved. Based on the results reported here some structure-activity relationships can be drawn. Compounds **16**, with *N*-(3-phenyl-propanoyl) chain (entries 16 to 19), and **12**, with *N*-(5-phenyl-pentanoyl) chain (entries 24 and 25), are less potent than their corresponding analogs **17**, with *N*-(4-phenyl-butanoyl) chain (entries 21 to 23). Compounds **19**, with *N*-(4-pentenoyl) chain (entries 26 to 29), are slightly more potent than their corresponding analogs **20**, with *N*-(5-hexenoyl) chain. Within sub-families, compounds named **b**, with *O*-(4-phenyl-butanoyl) chain (entries 17, 21, 26, 30), and **d**, with *O*-(biphenyl-acetyl) chain (entries 19, 23, 27, 31) are the best inhibitors. We concluded that similar activities result from the presence of 4-phenyl-butanoyl (n = 3, R^2 = Ph) and 4-pentenoyl (n = 2, R^2 = Alk) substituents at the N1 position, on the one hand, and from the presence of 4-phenyl-butanoyl (m = 3, R^3 = Ph) and biphenylacetyl (m = 1, R^3 = biPh) substituents at the C5-O position, on the other hand.

-MGL inhibition- Azetidinones **11**, **13-14** of the first series (R^1 = OAc) inhibited the enzyme at 10^{-4} M concentration (50 to 100 % inhibition), but not at 10^{-6} M concentration

(Table 1, entries 1 to 10). An IC_{50} value of 133 μM was determined for the most active compound **15e** (entry 10) which, however, shows a great selectivity for the inhibition of FAAH (IC_{50} = 1.90 μM).

Azetidinones **12**, **16-20** of the second series (R^1 = H) were also modest inhibitors of MGL (entries 11 to 25). IC_{50} values of the most active azetidinones **19b-f** and **20b,d** ranged from 1.84 to 23.3 μM (entries 26 to 31). Here again, the selectivity versus FAAH inhibition is high: for instance, **19b** (entry 26) and **20b** (entry 30) are respectively 800 and 850 times more potent against FAAH. For the other compounds, **19d-f** and **20d**, the selectivities range within 100 and 240.

-Inhibition mode- To determine the likely mechanism of FAAH inhibition, two types of experiments were performed, *i.e.* time-dependent preincubation and rapid dilution studies, both using azetidinones **19b** (IC_{50} = 0.005 μM) and **19f** (IC_{50} = 0.032 μM). Concerning the preincubation study, it is expected with an irreversible-type inhibitor that the inhibitor potency should increase upon prolonged preincubation time. Conversely, a constant IC_{50} value upon preincubation supports a reversible mechanism of inhibition.[48] Thus, **19b** and **19f** were incubated with the enzyme for 0, 15, 45 or 90 minutes, prior to substrate addition. As illustrated in Figure 2, the preincubation had no effect on the inhibiting activity of the compounds. This suggests an inhibition mode similar to those of α-keto-oxazoles (see **3**, Figure 1) or hydantoins (see **5**, Figure 1).[35, 41] On the other hand, after rapid and large dilution of the inhibitor-enzyme mixture, the recovery of enzymatic activity should be almost total if the inhibitor is reversible. For the irreversible inhibitors, the enzyme remains largely inhibited because the inhibitor is bound to the enzyme. Here, the rapid and large dilution led to a recovery of enzymatic activity in the case of **19b** and **19f**, as for 1-oxazolo[4,5-b]pyridin-2-yl-6-phenyl-1-hexanone (CAY10402)[49]**,** an analog of **3** (Figure 1). As a further control we used two irreversible FAAH inhibitors, compound **1** (Figure 1) and methyl arachidonyl fluorophosphonate (MAFP)[50] and found that the enzyme activity was still profoundly inhibited after the dilution (Figure 3a).

Furthermore, the mechanism of **19b** interaction with *h*FAAH was determined by studying the velocity of anandamide metabolism in function of increasing concentration of anandamide. The Michaelis-Menten curves (Figure 3b) and resulting kinetic parameters suggest a competitive inhibition type for this compound. Indeed, the V_{max} values in the presence of 15 or 45 nM of **19b** (12.77 ± 0.22 and 11.91 ± 0.50 $nmol.min^{-1}.mg^{-1}$, respectively

) are similar to the V_{max} value obtained in the absence of inhibitor (13.38 ± 0.25 nmol.min^{-1}.mg^{-1}) whereas the K_M values are largely increased in the presence of inhibitor.

Based on these data, to collect more information about the possible enzyme-inhibitor interactions at the atomic level, a modelling study has been performed.

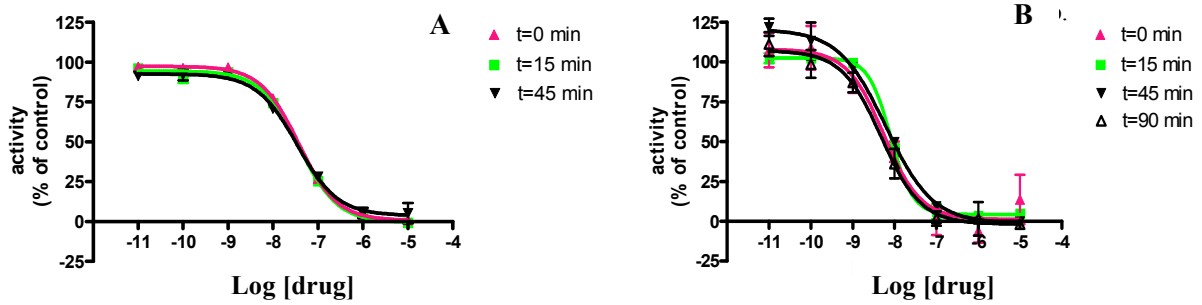


Figure. 2 Determination of the mode of inhibition of **19f** (**A**) and **19b** (**B**). The influence of the time of preincubation (0, 15, 45 and 90 min) on the inhibition curves of *h*FAAH was studied resulting in no significant variation of the IC_{50} values.

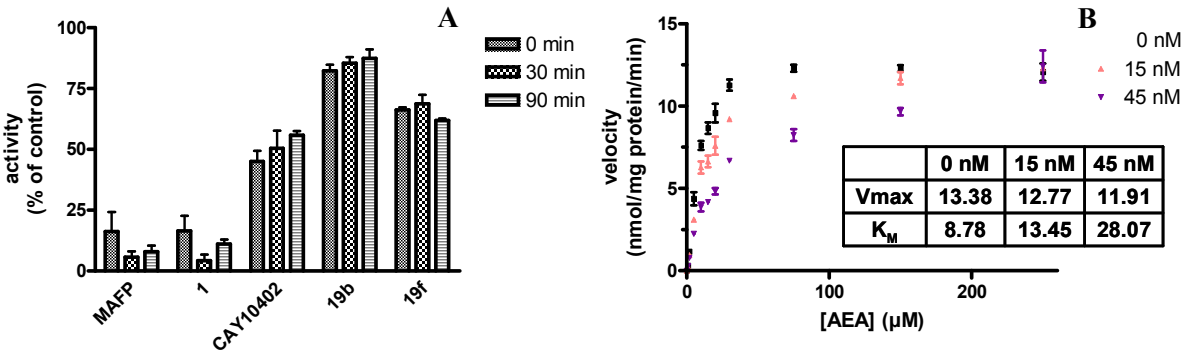


Figure. 3 (**A**) Test of reversibility: influence of a rapid and large dilution on the recovery of *h*FAAH activity (studies after 0, 30 and 90 min following the rapid and large dilution). (**B**) Determination of the mechanism of **19b** interactions with *h*FAAH. Michaelis-Menten curves and rapid dilution graphs were obtained from three independent experiments. The kinetic parameters are shown in the inset (Vmax values are given as nmol.min^{-1}.mg^{-1} of protein and K_M values are in µM)

IV.2.3 Thereotical study

-Model of the human FAAH- The crystal structure of *h*FAAH is currently not available. But recently, an engineered form of rat FAAH showing the same activity profile as the human one was crystallized (PDB code 2VYA)[51]. We therefore decided to build a model of *h*FAAH

through homology modelling using this X-ray crystal structure. Their amino acid sequence shared 80.6 % identity. The EsyPred3D program was used[52]. This automated homology modelling tool compares results from various multiple alignment algorithms to derive a "consensus" alignment between the target sequence and the template. Quality verification of the model was performed with Procheck 3.0 with a pseudo-resolution of 2.8 Å[53]. The model obtained is reliable based on the Ramachandran plot, showing 91.2 % of the residues in the core regions and 8.6 % in the allowed one (see supporting information). Moreover, 99.1, 94.8 and 100.0 % of the main chain bond lengths, main chain bond angles and the planar groups, respectively, are within the standard geometries. The RMSD (Root Mean Square Deviation) for the backbone atoms between both structures is 0.091 Å. The active site of *h*FAAH is formed by a hydrophobic tunnel, called the acyl chain binding channel (ACB), leading from the membrane-bound surface to the hydrophilic catalytic triad (Ser241, Ser217, and Lys142) (Figure 4). From the membrane, the ACB channel bifurcates into a lipophilic bulge. A second tunnel, the cytoplasmic access channel (CA), is exposed to the solvent and emerges at about 80° angle from the ACB channel. A third channel composed of three phenylalanine residues (Phe388, Phe381 and Phe192), here called the "phenyl pocket", lies close to the ACB channel.

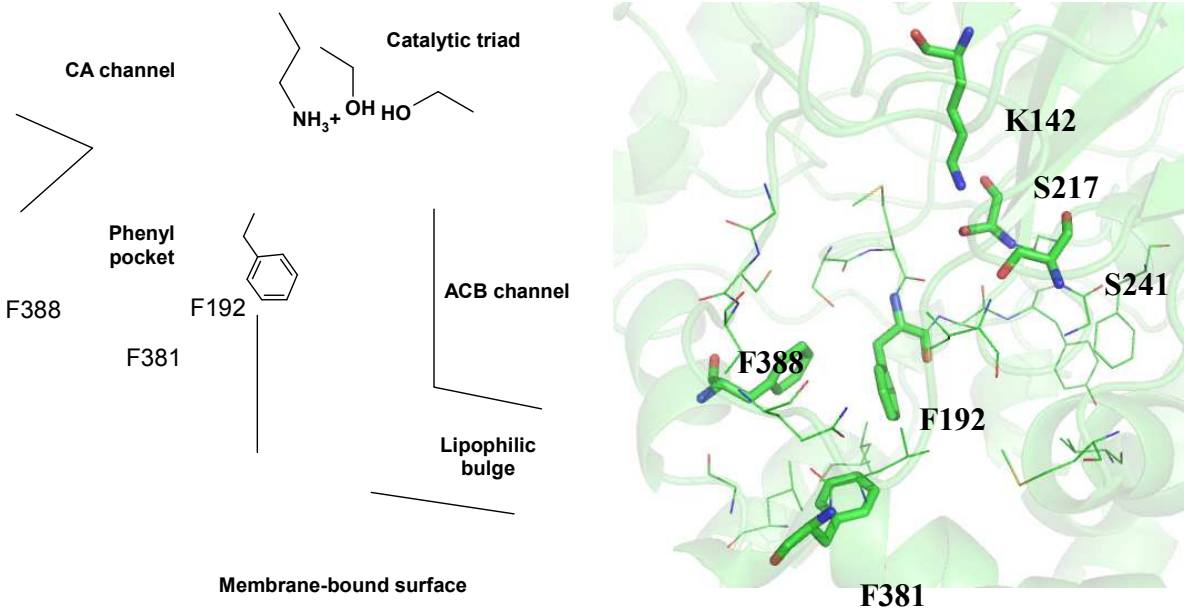


Figure. 4 Representation of the active site of the modelled human FAAH

-Docking studies- Since the above described pharmacological data suggest that these compounds are competitive inhibitors we docked the most active inhibitors, **19b** and **19d**, into the substrate binding site of the modelled *h*FAAH to further understand their binding mode.

Therefore we used the GOLD program, which we used for a previous work on FAAH[54], to dock these compounds into the active site of our human FAAH model. Recent theoretical and structural studies showed the planarity of the amide β-lactam bond and that the two imide carbonyls (called here COlactam and COexo) can adopt either *E* or *Z* configuration, the *E* configuration being the more stable in the gas phase (ΔE = 3.9 kcal/mol)[43]. We therefore allowed flipping the imide bond during the docking run. Based on the GOLD scoring function and on the occurrence of the docking poses, two preferential binding modes were retained (Figures 5-6; Table 2). We observed a *Z* or *E* configuration of the imide carbonyls following the binding mode and the studied compound. In the first binding mode (**I**) (Figure 5), the phenyl or biphenyl chain lies in the "phenyl pocket" and interacts with the three phenylalanines Phe192, Phe381 and Phe388. The catalytic serine Ser241 is close to the lactam and imide carbonyls. The alkene chain lies at the beginning of ACB channel and is close to Phe192. The observed H bonds are described in Table 2. In the second binding mode (**II**) (Figure 6), only observed for **19d**, the biphenyl and alkene chains are located in the ACB channel and "phenyl pocket", respectively. The biphenyl group interacts with Phe192.

In both binding modes, several aminoacids of the active site are involved in hydrophobic contacts with the inhibitors (see supporting information). From our docking experiments, we can explain the optimal chain length m = 3 (phenyl) or m = 1 (biphenyl), and n = 2 (alkenyl), by the stabilizing π-π interactions between the phenyl/biphenyl or alkene group and phenylalanine residues of the active site. Moreover, in both cases, mode **I** or **II**, adding an acetate moiety in the lactam cycle at C4 would lead to steric hindrance. The same binding modes were also observed for **16b** and **16d** (results not shown).

Table. 2 Characteristics of the two proposed binding modes of azetidinone compounds inside the modelled human FAAH

Compound	Binding mode	Configuration of the imide carbonyls	H bonds	Distance (Å)
19b	**I**	*Z*	COexo...OH(Ser241)	2.24
			COlactam...OH(Thr236)	3.26
			COester...NH(Val270)	2.95
19d	**II**	*Z*	COester…NH(Cys269)	3.32
	I	*E*	COlactam…OH(Ser241)	3.07

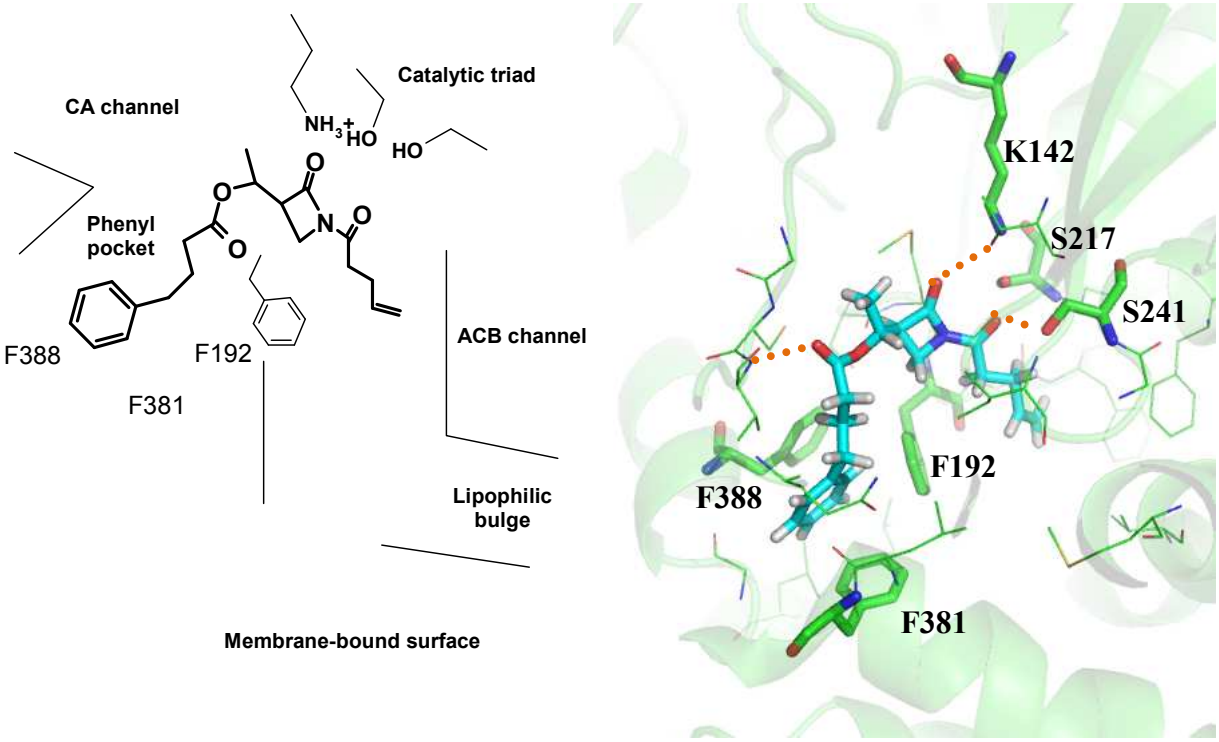


Figure. 5 Proposed binding mode **I** of **19b** into the human FAAH. In the picture on the right, H bonds are depicted by orange dotted lines

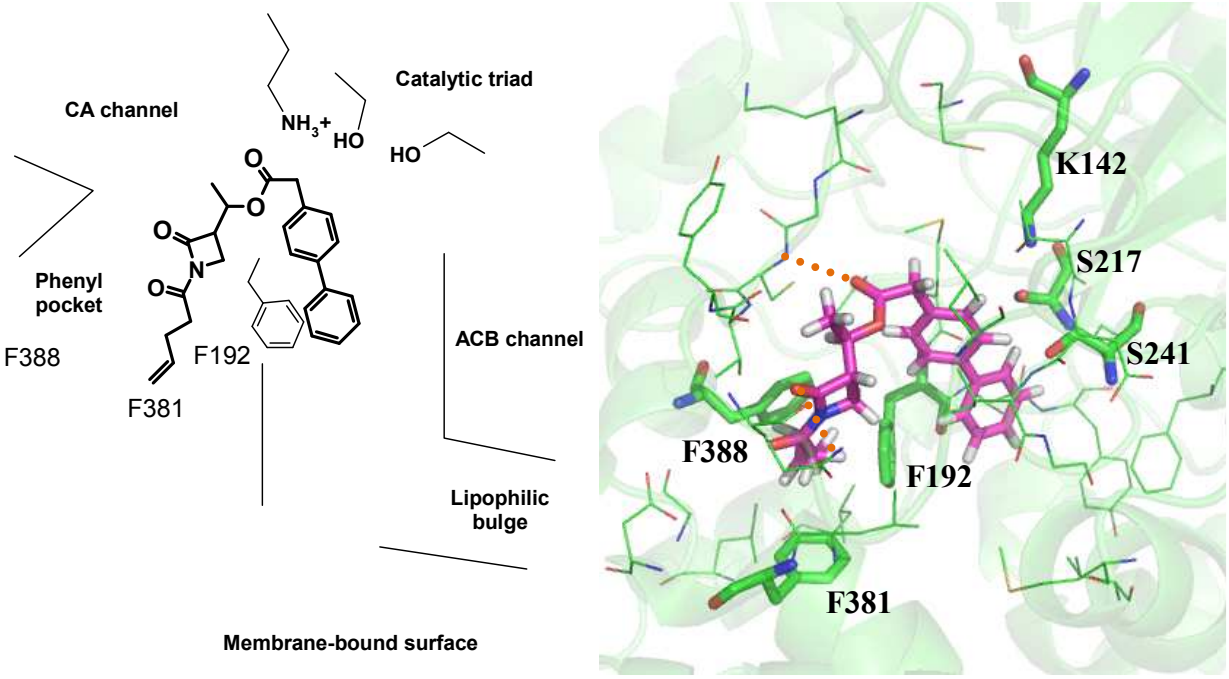


Figure. 6 Proposed binding mode **II** of **19d** into the human FAAH. In the picture on the right, H bonds are depicted by orange dotted lines

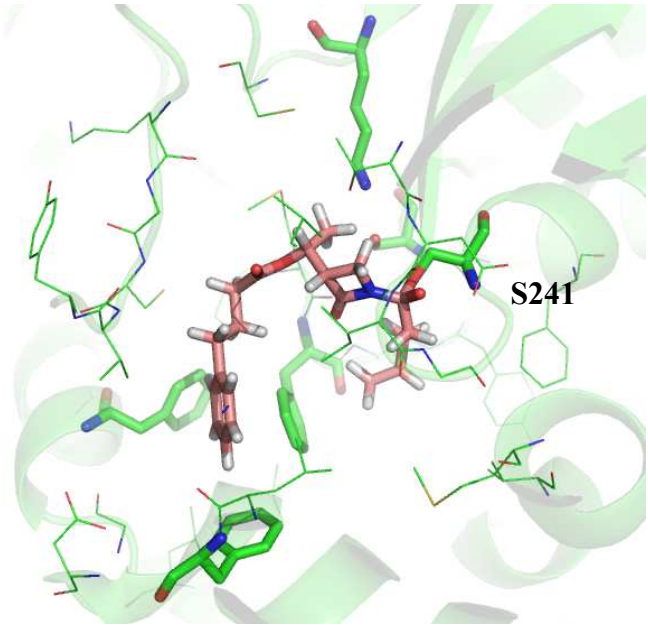
The first binding mode (Figure 5) could suggest a mechanism of action similar to that of α–keto heterocycles acting as reversible, competitive inhibitors presumably via reversible hemiketal formation with the active serine Ser241[35, 55]. In this context, we did a covalent

docking of the two putative tetrahedral intermediates of **19b**, binding Ser241 either via the lactam carbonyl (COlactam) or via the exocyclic imide carbonyl (COexo). In both cases, the position of the inhibitor is close to the one of the first binding mode with the phenyl group interacting with the "phenyl pocket" and the alkene chain lying in the ACB channel (Figure 7). The anion oxygen interacts by H bonding with the oxyanion hole, *i.e.* with the backbone of Ile238, Gly239, and Gly240. For the intermediate via the exocyclic carbonyl, both *Z* and *E* configurations are observed with a highest occurrence for *E*.

Following the second binding mode (Figure 6), the inhibitors would rather act as the (thio)hydantoin inhibitors, described recently, without tetrahedral intermediate[54].

As an internal validation of the docking methodology, the inhibitor *N*-phenyl-4-(quinolin-3-ylmethyl)piperidine-1-carboxamide (PF-750)[33] covalently attached to the Ser241 and used to generate the published crystal structure of the humanized form of rat FAAH[51], was re-docked into the empty catalytic pocket of the crystal structure using the same docking protocols. The conformation of the top scoring pose could reproduce the crystal structure conformation (data not shown), validating the docking methodology.

a)



b)

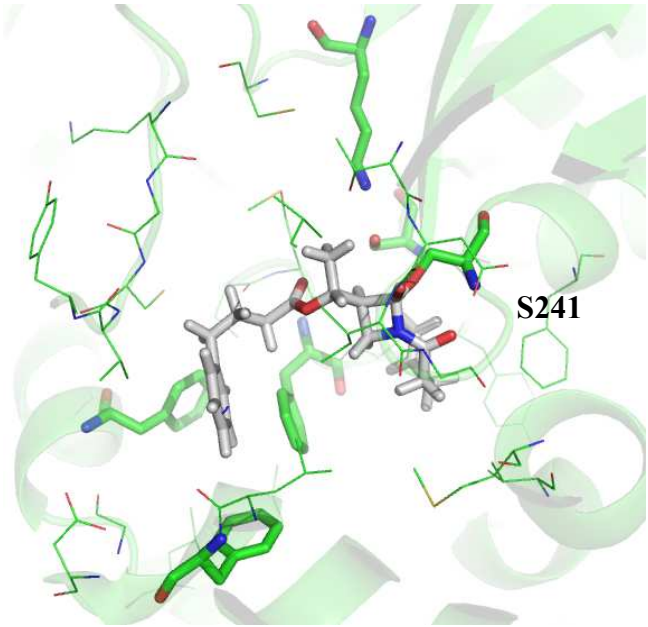


Figure. 7 Binding mode of the putative tetrahedral intermediates of **19b**, binding Ser241 either a) via the exocyclic imide carbonyl (COexo) or b) via the lactam carbonyl (COlactam) in the modelled human FAAH.

IV.3 Conclusion

Untill now, β-lactams were not considered as potential pharmacologically active compounds to interact with the endocannabinoid system in humans. In 2008, the virtual screening of a database of about 500,000 Shering-Plough compounds by using a CB_1 pharmacophore model as filter, and additional constraints for drug-like structures, allowed to select 420 compounds for further in vitro evaluation. Among them, a series of five diaryl 2-azetidinones emerged, giving an inhibition rate of ≥ 50 % at 100 nM in a CB_1 competitive binding assay. From this non-orientated approach, one β-lactam "lead" compound was identified as novel CB_1 receptor antagonist with a Ki value of 53 nM.[56]

To our knowledge, the design of potentially active β-lactams in the cannabinoid system was not reported before. Our approach was simply based on the FAAH inhibition by using the β-lactam core as electrophilic carbonyl function; this heterocycle was equipped with lateral chains mimicking the natural substrates or the known inhibitors, and susceptible to make hydrophobic contacts in the active site of the target enzyme.

Starting from the chiral 2-azetidinone **7** traditionally used for the synthesis of antibiotics, we generated a variety of lipophilic derivatives by placing alkenoyl, phenylalkanoyl and

biphenylacetyl chains on positions N1 and C5-O. Evaluation of this library of 30 azetidinones against *h*FAAH and *h*MGL revealed good to excellent and selective inhibitors of *h*FAAH versus *h*MGL, with IC_{50} values of 5-14 nM for the best representatives (**19b**, **19d**, **20b** and **20d**). Since the IC_{50} values were constant upon prolonged incubation time and as total recovery of enzymatic activity was observed after rapid and large dilution, a reversible mechanism of inhibition can be proposed. In addition, as the V_{max} values are not affected by the presence of **19b** while the K_M values are increased, the interaction between **19b** and *h*FAAH is likely to be of a competitive type. This is a quite unexpected result, since the β-lactams are prone to form (more or less) stable acyl-enzyme intermediates with serine hydrolases. Note that docking studies of two potent inhibitors into a validated homology model of *h*FAAH support well the reversible mechanism, even though they do not allow discriminating between two binding modes, with either the lactam/imide carbonyls or the ester carbonyl facing the catalytic triad. Further studies are in progress in view to clarify the role played by each carbonyl function of the inhibitors **16-20** and to identify the carbonyl function possibly responsible of the formation of a reversible tetrahedral intermediate by reaction with the active serine.

IV.4 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 purifications. (3*R*,4*R*)-4-Acetoxy-3-[(*R*)-(tert-butyldimethylsilyloxy)ethyl]-2-azetidinone **7** was obtained from Kaneka corporation (Japan). [^{3}H]-AEA (60 Ci/mmol) and [^{3}H]-2-OG (40 Ci/mmol) were 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 Reasonance (^{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 deuterate chloroform ($CDCl_3$). Chemical shifts are reported in ppm relative to the solvent signals ($CDCl_3$ 7.26 and 77.16 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 Perkin-Elmer 241 MC polarimeter, at 20 °C, in $CHCl_3$. Concentrations are given in percentage (g/100 mL). Low resolution mass spectra were

acquired using a Thermo Finnigan LCQ spectrometrer in negative mode (ESI). High Resolution Mass Spectrometry (HRMS) analyses were performed at the University of Mons Hainaut (Belgium) or at the University of Oxford (UK). Infrared (IR) spectra were recorded using 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 $60F_{254}$ and detected 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 chiral AD-H column (2.1 mm x 150 mm, 5 µm particle size) using hexane/isopropanol eluant (90:10), at a flow rate of 0.5 mL/min and on Symetry C18 (4.6 mm x 250 mm, 5µm particle size) using acetonitrile/H_2O eluant (70:30), at a flow rate of 1 mL/min (purity ≥ 97 %).

General procedure for *N*-acylation. To a stirred solution of azetidinone **7** (1 eq.) in dry dichloromethane (8.6 mL/mmol) at 20 °C, were added pyridine (2 eq.) and the suitable acyl chloride (2 eq.) under argon atmosphere. The mixture was refluxed during 24 h, then diluted in dichloromethane and the excess of acyl chloride was quenched by Na_2CO_3 (10 % aqueous solution; 8.6 mL/mmol). The organic layer was washed with 3 N HCl and brine, dried over $MgSO_4$, filtered and concentrated under vacuum. After purification by flash chromatography (cyclohexane/ethyl acetate), white solids (**9a** and **10a-b**) or colourless oils (**9b-c** and **10c-e**) were obtained.

1-(3-Phenylpropanoyl)-(3*R*,4*R*)-3-[1(*R*)-(*tert*-butyldimethylsilyloxy)-ethyl]-4-(acetoxy)-azetidin-2-one (9a). Yield : 89 % (130.1 mg from 0.35 mmol of **7**). Mp: 70.0-70.5 °C. $[\alpha]_D$ = -54.0 (c = 1.0). R_f = 0.54 (cyclohexane/ethyl acetate : 5/2). MS (ESI) : *m/z* : 442.1 ($(M + Na)^+$). ^{1}H NMR (500 MHz, $CDCl_3$) : δ = 0.05 (s, 3H), 0.10 (s, 3H), 0.84 (s, 9H), 1.34 (d, 3H, *J* = 6.4 Hz), 2.14 (s, 3H), 2.97-3.06 (m, 4H), 3.15 (dd, 1H, *J* = 1.5 Hz, *J* = 2.5 Hz), 4.31 (m, 1H), 6.62 (d, 1H, *J* = 1.5 Hz), 7.21-7.34 (m, 5H). ^{13}C NMR (125 MHz, $CDCl_3$) : δ = -5.3, -4.1, 17.9, 21.0, 21.9, 25.6, 29.8, 38.3, 64.3, 65.3, 74.3, 126.4, 128.6, 128.6, 140.2, 164.6, 169.1, 169.3. IR (cm^{-1}) : υ = 2854-2952, 1803, 1755, 1714, 1454-1495, 1308, 1251, 837. HRMS : C22H33NO5SiNa : calculated : 442.2026, found : 442.2040.

1-(4-Phenylbutanoyl)-(3*R*,4*R*)-3-[1(*R*)-(*tert*-butyldimethylsilyloxy)-ethyl]-4-(acetoxy)-azetidin-2-one (9b). Yield : 94 % (360 mg from 0.89 mmol of **7**). $[\alpha]_D$ = -40.2 (c = 1.0). R_f = 0.48 (cyclohexane/ethyl acetate : 5/2). MS (ESI) : *m/z* : 456.2 ($(M + Na)^+$), 888.9 ($(2M + Na)^+$). ^{1}H NMR (300 MHz, $CDCl_3$) : δ = 0.03 (s, 3H), 0.08 (s, 3H), 0.82 (s, 9H), 1.31 (d, 3H, *J* = 6.4 Hz), 1.99 (m, 2H), 2.11 (s, 3H), 2.63-2.78 (m, 4H), 3.12 (m, 1H), 4.29 (m, 1H), 6.59

(d, 1H, *J* = 1.1 Hz), 7.12-7.38 (m, 5H). ^{13}C NMR (75 MHz, $CDCl_3$) : δ = -5.3, -4.1, 17.8, 20.9, 21.9, 25.3, 25.6, 35.1, 35.9, 64.3, 65.1, 74.2, 126.1, 128.4, 128.5, 141.4, 164.5, 169.1, 169.8. IR (cm^{-1}) : υ = 2854-2926, 1805, 1757, 1717, 1462, 1308, 1211-1250, 839. HRMS : C23H35NO5SiNa : calculated : 456.2182, found : 456.2187.

1-(Pent-4-enoyl)-(3*R*,4*R*)-3-[1(*R*)-(*tert*-butyldimethylsilyloxy)-ethyl]-4-(acetoxy)-azetidin-2-one (9c). Yield : 80 % (515 mg from 1.74 mmol of **7**). R_f = 0.57 (cyclohexane/ethyl acetate : 5/2). MS (ESI) : *m/z* : 392.1 ($(M + Na)^+$), 760.9 ($(2M + Na)^+$). ^{1}H NMR (500 MHz, $CDCl_3$) : δ = 0.03 (s, 3H), 0.07 (s, 3H), 0.82 (s, 9H), 1.31 (d, 3H, *J* = 6.7 Hz), 2.10 (s, 3H), 2.40 (td, 2H, *J* = 7.6 Hz, *J* = 6.5 Hz), 2.74 (td, 1H, *J* = 7.6 Hz, *J* = 16.9 Hz), 2.81 (td, 1H, *J* = 7.6 Hz, *J* = 16.9 Hz), 3.12 (m, 1H), 4.29 (m, 1H), 5.01 (dd, 1H, *J* = 1.6 Hz, *J* = 10.5 Hz), 5.08 (dd, 1H, *J* = 1.6 Hz, *J* = 17.2 Hz), 5.82 (ddt, 1H, *J* = 10.5 Hz *J* = 17.2 Hz, *J* = 6.5 Hz), 6.58 (d, 1H, *J* = 1.6 Hz). ^{13}C NMR (125 MHz, $CDCl_3$) : δ = -5.5, -4.3, 17.6, 20.8, 21.7, 25.5, 27.4, 35.6, 64.1, 65.0, 74.1, 115.8, 136.2, 164.4, 168.9, 169.1. IR (cm^{-1}) : υ = 2857-2961, 1808, 1759, 1721, 1642, 1306, 835. HRMS : C18H31NO5SiNa : calculated : 392.1869, found : 392.1863.

1-(3-Phenylpropanoyl)-(3*S*)-3-[1(*R*)-(*tert*-butyldimethylsilyloxy)-ethyl]-azetidin-2-one (10a). Yield : 97 % (617 mg from 1.77 mmol of **8**). $[\alpha]_D$ = -53.7 (c = 4.1) . R_f = 0.53 (cyclohexane/ethyl acetate : 5/3). MS (ESI) : *m/z* : 384.3 ($(M + Na)^+$), 744.9 ($(2M + Na)^+$). ^{1}H NMR (300 MHz, $CDCl_3$) : δ = 0.06 (s, 3H), 0.09 (s, 3H), 0.85 (s, 9H), 1.20 (d, 3H, *J* = 6.3 Hz), 2.95-3.06 (m, 4H), 3.23 (m, 1H), 3.56 (dd, 1H, *J* = 6.7 Hz, *J* = 7.2 Hz), 3.70 (dd, 1H, *J* = 3.6 Hz, *J* = 7.2 Hz), 4.31 (m, 1H), 7.11-7.40 (m, 5H). ^{13}C NMR (75 MHz, $CDCl_3$) : δ = -5.1, -4.1, 17.9, 22.2, 25.7, 30.2, 38.3, 38.4, 56.5, 64.8, 126.3, 128.6 (2C), 140.5, 166.5, 170.2. IR (cm^{-1}) : υ = 2856-2955, 1786, 1701, 1310, 1252, 839. HRMS : C20H31NO3SiNa : calculated : 384.1971, found : 384.1974.

1-(4-Phenylbutanoyl)-(3*S*)-3-[1(*R*)-(*tert*-butyldimethylsilyloxy)-ethyl]-azetidin-2-one (10b). Yield : 87 % (286 mg from 0.87 mmol of **8**). Mp : 30.5-31.5 °C. $[\alpha]_D$ = -42.3 (c = 1.9). R_f = 0.52 (cyclohexane/ethyl acetate : 5/2). MS (ESI) : *m/z* : 376.2 ($(M + H)^+$), 398.1 ($(M + Na)^+$). ^{1}H NMR (300 MHz, $CDCl_3$) : δ = 0.06 (s, 3H), 0.09 (s, 3H), 0.85 (s, 9H), 1.20 (d, 3H, *J* = 6.3 Hz), 2.00 (m, 2H), 2.61-2.84 (m, 4H), 3.22 (m, 1H), 3.54 (dd, 1H, *J* = 6.8 Hz, *J* = 7.2 Hz), 3.69 (dd, 1H, *J* = 3.6 Hz, *J* = 7.2 Hz), 4.31 (m, 1H), 7.14-36 (m, 5H). ^{13}C NMR (75 MHz, $CDCl_3$) : δ = -5.2, -4.2, 17.8, 22.2, 25.61, 25.66, 35.2, 36.0, 38.2, 56.3, 64.7, 126.0, 128.4, 128.5, 141.5, 166.4, 170.7. IR (cm^{-1}) : υ = 2856-3026, 1784, 1697, 1454-1497, 1389,

1309, 1249, 839. HRMS : C21H34NO3Si : calculated : 376.2308, found : 376.2295; C21H33NO3SiNa : calculated : 398.2127, found : 398.2107.

1-(5-Phenylpentanoyl)-(3*S*)-3-[1(*R*)-(*tert*-butyldimethylsilyloxy)-ethyl]-azetidin-2-one (10c). Yield : 74 % (126 mg from 0.43 mmol of **8**). $[\alpha]_D$ = -37.2 (c = 1.0). R_f = 0.53 (cyclohexane/ethyl acetate : 5/2). MS (ESI) : *m/z* : 390.2 ($(M + H)^+$), 412.1 ($(M + Na)^+$). ^{1}H NMR (300 MHz, $CDCl_3$) : δ = 0.05 (s, 3H), 0.07 (s, 3H), 0.83 (s, 9H), 1.18 (d, 3H, *J* = 6.3 Hz), 1.56-1.80 (m, 4H), 2.55-2.79 (m, 4H), 3.20 (m, 1H), 3.52 (dd, 1H, *J* = 6.8 Hz, *J* = 7.2 Hz), 3.67 (dd, 1H, *J* = 3.6 Hz, *J* = 7.2 Hz), 4.30 (m, 1H), 7.07-7.45 (m, 5H). ^{13}C NMR (75 MHz, $CDCl_3$) : δ = -5.2, -4.2, 17.9, 22.2, 23.7, 25.6, 30.9, 35.6, 36.3, 38.2, 56.3, 64.7, 125.8, 128.3, 128.4, 142.1, 166.4, 170.9. IR (cm^{-1}) : υ = 2856-2951, 1784, 1701, 1454-1496, 1389, 1310, 1250, 839. HRMS : C22H36NO3Si : calculated : 390.2464, found : 390.2448; C22H35NO3SiNa : calculated : 412.2284, found : 412.2263.

1-(Pent-4-enoyl)-(3*S*)-3-[1(*R*)-(*tert*-butyldimethylsilyloxy)-ethyl]-azetidin-2-one (10d). Yield : 95 % (965 mg from 3.27 mmol of **8**). R_f = 0.76 (cyclohexane/ethyl acetate : 5/2). MS (ESI) : *m/z* : 312.3 ($(M + H)^+$). ^{1}H NMR (300 MHz, $CDCl_3$) : δ = 0.03 (s, 3H), 0.05 (s, 3H), 0.82 (s, 9H), 1.17 (d, 3H, *J* = 6.1 Hz), 2.38 (m, 2H), 2.75 (m, 2H), 3.21 (m, 1H), 3.52 (dd, 1H, *J* = 6.6 Hz, *J* = 6.6 Hz), 3.66 (dd, 1H, *J* = 3.4 Hz, *J* = 6.6 Hz), 4.29 (m, 1H), 4.97 (dd, 1H, *J* = 1.6 Hz, *J* = 10.2 Hz), 5.05 (dd, 1H, *J* = 1.6 Hz, *J* = 17.0 Hz), 5.80 (ddt, 1H, *J* = 6.5 Hz, *J* = 10.2 Hz, *J* = 17.0 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) : δ = -4.4, -5.4, 17.7, 22.0, 25.4, 27.8, 35.6, 38.1, 56.2, 64.5, 115.5, 136.4, 166.2, 170.1. IR (cm^{-1}) : υ = 2858-2930, 1788, 1705, 1311, 840. HRMS : C16H29NO3SiNa : calculated : 334.1814, found : 334.1806.

1-(Hexa-5-enoyl)-(3*S*)-3-[1(*R*)-(*tert*-butyldimethylsilyloxy)-ethyl]-azetidin-2-one (10e). Yield : 46 % (40 mg from 0.27 mmol of **8**). $[\alpha]_D$ = -34.6 (c = 1.0). R_f = 0.60 (cyclohexane/ethyl acetate : 5/3). MS (ESI) : *m/z* : 326.2 ($(M + H)^+$), 348.1 ($(M + Na)^+$). ^{1}H NMR (300 MHz, $CDCl_3$) : δ = 0.05 (s, 3H), 0.07 (s, 3H), 0.83 (s, 9H), 1.19 (d, 3H, *J* = 6.3 Hz), 1.75 (m, 2H), 2.10 (m, 2H), 2.68 (m, 2H), 3.21 (m, 1H), 3.54 (dd, 1H, *J* = 6.6 Hz, *J* = 6.6 Hz), 3.68 (dd, 1H, *J* = 3.4 Hz, *J* = 6.6 Hz), 4.30 (m, 1H), 4.96-5.06 (m, 2H), 5.77 (m, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) : δ = -5.1, -4.1, 18.0, 22.3, 23.3, 25.7, 33.2, 36.0, 38.4, 56.4, 64.8, 115.4, 137.9, 166.5, 171.0. IR (cm^{-1}) : υ = 2856-2953, 1786, 1701, 1464, 1389, 1310, 1252, 839. HRMS : C17H31NO3SiNa : calculated : 348.1971, found : 348.1985.

General procedure for deprotection. To a stirred suspension of silyl ether (1 eq.) in acetonitrile (30 mL/mmol) at -5 °C was added dropwise 12 N HCl (5eq.) and 17 N AcOH (7

eq.). The mixture was stirred for 30 min at -5 °C, and for 3 h at 0 °C. Acetonitrile was removed under vacuum, and the oily residue was diluted in ethyl acetate. The organic layer was washed with 10 % $NaHCO_3$ and brine, dried over $MgSO_4$, filtered and concentrated under vacuum. After purification by flash chromatography (dichloromethane/ethyl acetate) a white solid (**11a-b** and **12a-b**) or a colourless oil (**11c**, **12c-e** and **22**) was obtained.

1-(3-Phenylpropanoyl)-(3*R*,4*R*)-3-[1(*R*)-hydroxyethyl]-4-(acetoxy)-azetidin-2-one (11a). Yield : 93 % (822 mg from 2.93 mmol of **9a**). Mp : 116.0-117.0 °C. $[\alpha]_D$ = -76.1 (c = 2.9). R_f = 0.20 (cyclohexane/ethyl acetate : 5/3). MS (ESI) : *m/z* : 328.1 $((M + Na)^+)$. ^1H NMR (300 MHz, $CDCl_3$) : δ = 1.37 (d, 3H, *J* = 6.3 Hz), 2.16 (s, 3H), 2.62 (br s, 1H), 2.94-3.16 (m, 4H), 3.17 (dd, 1H, *J* = 1.4 Hz, *J* = 6.4 Hz), 4.18 (m, 1H), 6.32 (d, 1H, *J* = 1.4 Hz), 7.16-7.38 (m, 5H). ^{13}C NMR (75 MHz, $CDCl_3$) : δ = 21.0, 21.1, 29.9, 38.4, 64.3, 65.3, 75.9, 126.5, 128.6 (2C), 140.0, 163.2, 169.6, 170.4. IR (cm^{-1}) : υ = 3504, 2931-2974, 1803, 1755, 1716, 1454-1496, 1313. HRMS : C16H19NO5Na : calculated : 328.1161 , found : 328.1174.

1-(4-Phenylbutanoyl)-(3*R*,4*R*)-3-[1(*R*)-hydroxyethyl]-4-(acetoxy)-azetidin-2-one (11b). Yield : 99 % (83 mg from 0.25 mmol of **9b**). Mp : 81.0-81.5 °C. $[\alpha]_D$ = -74.9 (c = 2.9) . R_f = 0.21 (cyclohexane/ethyl acetate : 5/3). MS (ESI) : *m/z* : 342.2 $((M + Na)^+)$, 660.77 $((2M + Na)^+)$. ^1H NMR (300 MHz, $CDCl_3$) : δ = 1.37 (d, 3H, *J* = 6.4 Hz), 2.00 (m, 2H), 2.14 (s, 3H), 2.62 (br s, 1H), 2.64-2.88 (m, 4H), 3.13 (dd, 1H, *J* = 1.6 Hz, *J* = 5.7 Hz), 4.21 (m, 1H), 6.27 (d, 1H, *J* = 1.6 Hz), 7.12-7.38 (m, 5H). ^{13}C NMR (75 MHz, $CDCl_3$) : δ = 21.0, 21.1, 25.3, 35.1, 36.0, 64.3, 65.2, 75.9, 126.2, 128.5, 128.6, 141.3, 163.2, 170.2, 170.4. IR (cm^{-1}) : υ = 3502, 2931, 1803, 1753, 1716, 1454-1497, 1379, 1308, 1213. HRMS : C17H21NO5Na : calculated : 342.1317, found : 342.1307.

1-(Pent-4-enoyl)-(3*R*,4*R*)-3-[1(*R*)-(hydroxyethyl]-4-(acetoxy)-azetidin-2-one (11c). Yield : 83 % (490 mg from 2.3 mmol of **9c**). R_f = 0.40 (cyclohexane/ethyl acetate : 5/3). MS (ESI) : *m/z* : 278.1 $((M + Na)^+)$. ^1H NMR (500 MHz, $CDCl_3$) : δ = 1.24 (d, 3H, *J* = 6.4 Hz), 2.02 (s, 3H), 2.30 (dt, 2H, *J* = 7.4 Hz, *J* = 6.8 Hz), 2.70 (m, 1H), 3.08 (dd, 1H, *J* = 1.4 Hz, *J* = 5.4 Hz), 3.30 (br s, 1H), 4.13 (qd, 1H, *J* = 5.4 Hz, *J* = 6.4 Hz), 4.91 (dd, 1H, *J* = 1.5 Hz, *J* = 10.3 Hz), 4.98 (dd, 1H, *J* = 1.5 Hz, *J* = 17.1 Hz), 5.72 (ddt, 1H, *J* = 10.3 Hz, *J* = 17.1 Hz, *J* = 6.8 Hz), 6.33 (d, 1H, *J* = 1.4 Hz). ^{13}C NMR (125 MHz, $CDCl_3$) : δ = 20.7, 20.9, 27.4, 35.6, 63.8, 64.9, 75.2, 115.8, 136.0, 163.3, 169.5, 169.9. IR : υ = 3501, 2932-2978, 1805, 1755, 1718, 1641, 1311. HRMS : C12H17NO5Na : calculated : 278.1004, found : 278.0992.

1-(3-Phenylpropanoyl)-(3*S*)-3-[1(*R*)-hydroxyethyl]-azetidin-2-one (12a). Yield : 78 % (297 mg from 1.55 mmol of **10a**). Mp : 64.5-65.5 °C. $[\alpha]_D$ = -36.7 (c = 1.5). R_f = 0.09

(cyclohexane/ethyl acetate : 5/3). MS (ESI) : *m/z* : 270.2 ($(M + Na)^+$), 516.9 ($(2M + Na)^+$). ^1H NMR (300 MHz, $CDCl_3$) : δ = 1.28 (d, 3H, *J* = 6.4 Hz), 1.91 (br s, 1H), 2.88-3.12 (m, 4H), 3.25 (m, 1H), 3.60 (m, 2H), 4.22 (m, 1H), 7.12-7.38 (m, 5H). ^{13}C NMR (75 MHz, $CDCl_3$) : δ = 21.7, 30.2, 38.4, 39.2, 55.9, 64.9, 126.4, 128.6, 128.7, 140.4, 166.1, 170.4. IR (cm^{-1}) : υ = 3464, 2970, 1782, 1697, 1454-1496, 1387, 1313, 1236. HRMS : C14H17NO3Na : calculated : 270.1106, found : 270.1109.

1-(4-Phenylbutanoyl)-(3*S*)-3-[1(*R*)-hydroxyethyl]-azetidin-2-one (12b). Yield : 86 % (165.5 mg from 0.73 mmol of **10b**). Mp : 89.5-90.0 °C. $[\alpha]_D$ = -38.6 (c = 2.5). R_f = 0.10 (cyclohexane/ethyl acetate : 5/3). MS (ESI) : *m/z* : 262.1 ($(M + H)^+$), 284.15 ($(M + Na)^+$). ^1H NMR (500 MHz, $CDCl_3$) : δ = 1.30 (d, 3H, *J* = 6.4 Hz), 1.68 (br s, 1H), 2.00 (m, 2H) 2.68 (t, 2H, *J* = 4.5 Hz), 2.73 (t, 2H, *J* = 4.5 Hz), 3.27 (m, 1H), 3.60 (m, 2H), 4.27 (m, 1H), 7.15-7.30 (m, 5H). ^{13}C NMR (125 MHz, $CDCl_3$) : δ = 21.7, 25.7, 35.2, 36.1, 39.1, 55.8, 64.9, 126.1, 128.5, 128.6, 141.5, 166.1, 171.0. IR (cm^{-1}) : υ = 3449, 2970, 1784, 1697, 1454, 1389, 1312, 1250. HRMS : C15H19NO3Na : calculated : 284.1263, found : 284.1261.

1-(5-Phenylpentanoyl)-(3*S*)-3-[1(*R*)-hydroxyethyl]-azetidin-2-one (12c). Yield : 84 % (68.6 mg from 0.30 mmol of **10c**). $[\alpha]_D$ = -13.1 (c = 0.1). R_f = 0.10 (cyclohexane/ethyl acetate : 5/3). MS (ESI) : *m/z* : 276.2 ($(M + H)^+$), 298.1 ($(M + Na)^+$). ^1H NMR (δ ppm, 300 MHz, $CDCl_3$) : δ = 1.27 (d, 3H, *J* = 6.4 Hz), 1.55-1.80 (m, 4H), 2.49-2.77 (m, 4H), 3.25 (m, 1H), 3.58 (m, 2H), 4.21 (m, 1H), 7.04-7.33 (m, 5H). ^{13}C NMR (δ ppm, 75 MHz, $CDCl_3$) : δ = 21.6, 23.7, 30.8, 35.6, 36.4, 39.1, 55.7, 64.7, 125.8, 128.3, 128.4, 142.1, 166.3, 171.2. IR (ATR-SeZn, cm^{-1}) : 3445, 2930, 1784, 1697, 1452, 1389, 1312, 1240. HRMS : C16H21NO3Na : calculated : 298.1419, found : 298.1405.

1-(Pent-4-enoyl)-(3*S*)-3-[1(*R*)-hydroxyethyl]-azetidin-2-one (12d). Yield : 85 % (457 mg from 2.32 mmol of **10d**). R_f = 0.38 (cyclohexane/ethyl acetate : 5/4). MS (ESI) : *m/z* : 198.1 ($(M + H)^+$), 220.1 ($(M + Na)^+$). ^1H NMR (300 MHz, $CDCl_3$) : δ = 1.28 (d, 3H, *J* = 6.4 Hz), 2.38 (td, 2H, *J* = 7.3 Hz, *J* = 6.8 Hz), 2.78 (t, 2H, *J* = 7.3 Hz), 3.28 (m, 1H), 3.56 (br s, 1H), 3.61 (m, 2H), 4.24 (m, 1H), 4.99 (dd, 1H, *J* = 1.5 Hz, *J* = 10.3 Hz), 5.72 (dd, 1H, *J* = 1.5 Hz, *J* = 17.1 Hz), 5.81 (ddt, 1H, *J* = 10.3 Hz, *J* = 17.1 Hz, *J* = 6.8 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) : δ = 21.4, 27.8, 35.6, 39.1, 55.7, 64.6, 115.7, 136.3, 166.2, 170.5. IR (cm^{-1}) : υ = 3443, 2928-2972, 1786, 1701, 1641, 1315. HRMS : C10H15NO3Na : calculated : 198.1130, found : 198.1122.

1-(Hexa-5-enoyl)-(3*S*)-3-[1(*R*)-hydroxyethyl]-azetidin-2-one (12e). Yield : 94 % (20.5 mg from 0.10 mmol of **10e**). $[\alpha]_D$ = -30.2 (c = 3.0). R_f = 0.13 (cyclohexane/ethyl acetate :

5/3). MS (ESI) : *m/z* : 212.1 ((M + H)$^+$). ^{1}H NMR (300 MHz, $CDCl_3$) : δ = 1.30 (d, 3H, *J* = 6.4 Hz), 1.75 (m, 2H), 2.00 (br s, 1H), 2.11 (m, 2H), 2.70 (t, 2H, *J* = 7.5 Hz), 3.27 (m, 1H), 3.61 (d, 2H, *J* = 5.1 Hz), 4.26 (m, 1H), 4.96-5.06 (m, 2H), 5.78 (m, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) : δ = 21.7, 23.3, 33.1, 36.0, 39.1, 55.8, 64.9, 115.5, 137.8, 166.2, 171.2. IR (cm^{-1}) : υ = 3470, 2930, 1786, 1697, 1441-1456, 1389, 1312, 1259. HRMS : C11H17NO3Na : calculated : 212.12866, found : 212.12837.

1-(Benzoyloxycarbonyl)-(3*S*)-3-[1(*R*)-hydroxyethyl]-azetidin-2-one (22). Yield : 91 % (98.3 mg from 0.43 mmol of **21**). $[\alpha]_D$ = -37.0 (c = 4.1). R_f = 0.09 (cyclohexane/ethyl acetate : 5/3). MS (ESI) : *m/z* : 272.1 ((M + Na)$^+$). ^{1}H NMR (300 MHz, $CDCl_3$) : δ = 1.24 (d, 3H, *J* = 6.4 Hz), 2.65 (br s, 1H), 3.25 (m, 1H), 3.51-3.75 (m, 2H), 4.20 (m, 1H), 5.22 (s, 2H), 7.21-7.54 (m, 5H). ^{13}C NMR (75 MHz, $CDCl_3$) : δ = 21.5, 40.6, 56.8, 64.5, 68.1, 128.4, 128.7 (2C), 135.0, 149.1, 165.9. IR (cm^{-1}) : υ = 3497, 2972, 1803, 1726, 1456, 1389, 1335. HRMS : C13H15NO4Na : calculated : 272.0899, found : 272.0888.

General procedure for esterification with acyl chloride (13a-b, 14a-b, 15e, 16a-b, 17a-b, 18b, 19b and 19e, 20b). To a stirred solution of alcohol precursor (1 eq.) in dry dichloromethane (20 mL/mmol), at 20 °C, were added pyridine (2 eq.) and the suitable acyl chloride (2 eq.) under argon atmosphere. After stirring overnight, the mixture was diluted in dichloromethane and the excess of acyl chloride was quenched by 10 % aqueous Na_2CO_3. The organic layer was washed with 3 N HCl and brine, dried over $MgSO_4$, filtered and concentrated under vacuum. After purification by flash chromatography (dichloromethane/ethyl acetate) a colourless oil was obtained in all cases.

1-(3-Phenylpropanoyl)-(3*R*,4*R*)-3-[1(*R*)-(3-phenylpropanoyloxy)-ethyl]-4-(acetoxy)-azetidin-2-one (13a). Yield : 99 % (77 mg from 0.18 mmol of **11a**). $[\alpha]_D$ = -16.6 (c = 5.3). R_f = 0.44 (cyclohexane/ethyl acetate : 5/3). MS (ESI) : *m/z* : 460.3 ((M + Na)$^+$). ^{1}H NMR (300 MHz, $CDCl_3$) : δ = 1.37 (d, 3H *J* = 6.9 Hz), 2.15 (s, 3H), 2.64 (m, 2H), 2.90-2.98 (m, 2H) , 2.99-3.06 (m, 4H), 3.30 (dd, 1H, *J* = 1.7 Hz, *J* = 5.9 Hz), 5.31 (m, 1H), 6.48 (d, 1H, *J* = 1.7 Hz), 7.17-7.39 (m, 10H). ^{13}C NMR (75 MHz, $CDCl_3$) : δ = 18.2, 20.9, 29.8, 30.8, 35.8, 38.3, 62.8, 65.8, 74.8, 126.4, 126.5, 128.3, 128.6, 128.6 (2C), 139.9, 140.2, 162.3, 169.0, 169.3, 171.9. IR (cm^{-1}) : υ = 2930, 1805, 1736, 1720, 1454, 1381, 1313, 1213. HRMS : C25H27NO6Na : calculated : 460.1736, found : 460.1722.

1-(3-Phenylpropanoyl)-(3*R*,4*R*)-3-[1(*R*)-(4-phenylbutanoyloxy)-ethyl]-4-(acetoxy)-azetidin-2-one (13b). Yield : 70 % (153 mg from 0.49 mmol of **11a**). $[\alpha]_D$ = -17.7 (c = 2.6).

R_f = 0.48 (cyclohexane/ethyl acetate : 5/3). MS (ESI) : *m/z* : 474.2 ($(M + Na)^+$). ^{1}H NMR (300 MHz, $CDCl_3$) : δ = 1.41 (d, 3H, *J* = 6.5 Hz), 1.94 (m, 2H), 2.14 (s, 3H), 2.36 (t, 2H, *J* = 7.3 Hz), 2.65 (t, 2H, *J* = 7.6 Hz), 2.93-3.08 (m, 4H), 3.32 (dd, 1H, *J* = 1.6 Hz, *J* = 5.7 Hz), 5.32 (m, 1H), 6.53 (d, 1H *J* = 1.6 Hz), 7.12-7.39 (m, 10H). ^{13}C NMR (75 MHz, $CDCl_3$) : δ = 18.1, 20.7, 26.4, 29.7, 33.5, 35.0, 38.2, 62.6, 65.5, 74.6, 126.0, 126.4, 128.4 (2C), 128.5, 128.5, 139.8, 141.2, 162.3, 168.9, 169.2, 172.2. IR (cm^{-1}) : υ = 2932, 1803, 1742, 1720, 1454, 1381, 1313, 1213, 1188. HRMS : C26H29NO6Na : calculated : 474.1893, found : 474.1893.

1-(4-Phenylbutanoyl)-(3*R*,4*R*)-3-[1(*R*)-(3-phenylpropanoyloxy)-ethyl]-4-(acetoxy)-azetidin-2-one (14a). Yield : 88 % (90 mg from 0.23 mmol of **11b**). $[\alpha]_D$ = -17.1 (c = 6.2). R_f = 0.42 (cyclohexane/ethyl acetate : 5/3). MS (ESI) : *m/z* : 473.9 ($(M + Na)^+$), 925.3 ($(2M + Na)^+$). ^{1}H NMR (300 MHz, $CDCl_3$) : δ = 1.36 (d, 3H, *J* = 6.5 Hz), 2.00 (m, 2H), 2.12 (s, 3H), 2.57-2.77 (m, 6H), 2.93 (t, 2H, *J* = 7.7 Hz), 3.28 (dd, 1H, *J* = 1.7 Hz, *J* = 5.9 Hz), 5.31 (m, 1H), 6.46 (d, 1H, *J* = 1.7 Hz), 7.14-7.34 (m, 10H). ^{13}C NMR (75 MHz, $CDCl_3$) : δ = 18.2, 20.8, 25.3, 30.8, 35.0, 35.7, 36.0, 62.7, 65.8, 74.8, 126.1, 126.4, 128.3 (2C), 128.5, 128.6, 140.2, 141.1, 162.3, 169.0, 169.9, 171.8. IR (cm^{-1}) : υ = 2935-3028, 1803, 1740, 1717, 1454-1497, 1379, 1310, 1213. HRMS : C26H29NO6Na : calculated : 474.1893, found : 474.1875.

1-(4-Phenylbutanoyl)-(3*R*,4*R*)-3-[1(*R*)-(4-phenylbutanoyloxy)-ethyl]-4-(acetoxy)-azetidin-2-one (14b). Yield : 52 % (63 mg from 0.26 mmol of **11b**). $[\alpha]_D$ = -11.6 (c = 2.2). R_f = 0.48 (cyclohexane/ethyl acetate : 5/3). MS (ESI) : *m/z* : 488.4 ($(M + Na)^+$). ^{1}H NMR (300 MHz, $CDCl_3$) : δ = 1.40 (d, 3H, *J* = 6.5 Hz), 1.81-2.03 (m, 4H), 2.12 (s, 3H), 2.31 (m, 2H), 2.57-2.73 (m, 6H), 3.29 (dd, 1H, *J* = 1.8 Hz, *J* = 5.6 Hz), 5.31 (m, 1H), 6.49 (d, 1H, *J* = 1.8 Hz), 7.22-7.33 (m, 10H). ^{13}C NMR (75 MHz, $CDCl_3$) : δ = 18.3, 20.9, 25.4, 26.5, 33.6, 35.0, 35.1, 36.0, 62.7, 65.7, 74.7, 126.1, 126.2, 128.5 (4C), 141.2, 141.3, 162.4, 169.1, 169.9, 172.3. IR (cm^{-1}) : υ = 2852-3026, 1803, 1736, 1720, 1454-1496, 1381, 1307, 1213, 1058. HRMS : C27H31NO6Na : calculated : 488.2049, found : 488.2044.

1-(Pent-4-enoyl)-(3*R*,4*R*)-3-[1(*R*)-(pent-4-enoyloxy)-ethyl]-4-(acetoxy)-azetidin-2-one (15e). Yield : 90 % (950 mg from 3.14 mmol of **11c**). $[\alpha]_D$ = -21.9 (c = 5.4). R_f = 0.56 (cyclohexane/ethyl acetate : 5/2). MS (ESI) : *m/z* : 360.0 ($(M + Na)^+$). ^{1}H NMR (300 MHz, $CDCl_3$) : δ = 1.39 (d, 3H, *J* = 6.4 Hz), 2.11 (s, 3H), 2.34 (m, 2H), 2.40 (m, 4H), 2.80 (m, 2H), 3.28 (dd, 1H, *J* = 1.6 Hz, *J* = 5.8 Hz), 5.00 (dd, 1H, *J* = 1.6 Hz, *J* = 10.3 Hz), 5.02 (dd, 1H, *J* = 1.6 Hz, *J* = 10.3 Hz), 5.05 (dd, 1H, *J* = 1.6 Hz, *J* = 17.0 Hz), 5.08 (dd, 1H, *J* = 1.6 Hz, *J* = 17.0 Hz), 5.29 (qd, 1H, *J* = 5.8 Hz, *J* = 6.4 Hz), 5.78 (ddt, 1H, *J* = 6.4 Hz, *J* = 10.3 Hz, *J* = 17.0 Hz), 5.82 (ddt, 1H, *J* = 6.4 Hz, *J* = 10.3 Hz, *J* = 17.0 Hz), 6.48 (d, 1H, *J* = 1.6 Hz). ^{13}C NMR

(75 MHz, $CDCl_3$) : δ = 18.1, 20.6, 27.4, 28.6, 33.3, 35.7, 62.5, 65.6, 74.5, 115.6, 115.9, 135.9, 136.2, 162.2, 168.8, 169.2, 171.7. IR (cm^{-1}) : υ = 2982, 1806, 1742, 1722, 1642, 1313. HRMS : C17H23NO6Na : calculated : 360.1423, found : 360.1412.

1-(3-Phenylpropanoyl)-(3*S*)-3-[1(*R*)-(3-phenylpropanoyloxy)-ethyl]-azetidin-2-one (16a). Yield : 79 % (64 mg from 0.21 mmol of **12a**). $[\alpha]_D$ = -17.1 (c = 2.7). R_f = 0.41 (cyclohexane/ethyl acetate : 5/3). MS (ESI) : *m/z* : 402.1 ($(M + Na)^+$). ^1H NMR (300 MHz, $CDCl_3$) : δ = 1.29 (d, 3H, *J* = 6.4 Hz), 2.61 (t, 2H, *J* = 7.7 Hz), 2.93 (t, 2H, *J* = 7.7 Hz), 2.96-3.08 (m, 4H), 3.31 (m, 1H), 3.41 (dd, 1H, *J* = 3.6 Hz, *J* = 7.7 Hz), 3.56 (dd, 1H, *J* = 6.6 Hz, *J* = 7.7 Hz), 5.21 (m, 1H), 7.09-7.60 (m, 10H). ^{13}C NMR (75 MHz, $CDCl_3$) : δ = 18.3, 30.1, 30.9, 35.8, 38.3, 40.1, 53.6, 67.6, 126.4, 126.5, 128.3, 128.56, 128.62 (2C), 140.1, 140.2, 164.3, 170.2, 171.9. IR (cm^{-1}) : υ = 2931-3028, 1786, 1735, 1701, 1454-1497, 1383, 1315, 1238, 1132-1161. HRMS : C23H25NO4Na : calculated : 402.1681, found : 402.1675.

1-(3-Phenylpropanoyl)-(3*S*)-3-[1(*R*)-(4-phenylbutanoyloxy)-ethyl]-azetidin-2-one (16b). Yield : 75 % (79 mg from 0.27 mmol of **12a**). $[\alpha]_D$ = -9.6 (c = 3.4). R_f = 0.44 (cyclohexane/ethyl acetate : 5/3). MS (ESI) : *m/z* : 416.2 ($(M + Na)^+$), 808.7 ($(2M + Na)^+$). ^1H NMR (300 MHz, $CDCl_3$) : δ = 1.37 (d, 3H, *J* = 6.4 Hz), 1.95 (m, 2H), 2.32 (t, 2H, *J* = 7.5 Hz), 2.66 (t, 2H, *J* = 7.6 Hz), 2.90-3.11 (m, 4H), 3.41 (m, 1H), 3.32 (dd, 1H, *J* = 3.6 Hz, *J* = 7.7 Hz), 3.68 (dd, 1H, *J* = 6.8, Hz, *J* = 7.7 Hz), 5.28 (m, 1H), 7.12-7.42 (m, 10H). ^{13}C NMR (75 MHz, $CDCl_3$) : δ = 18.4, 26.6, 30.1, 33.7, 35.1, 38.3, 40.0, 53.6, 67.3, 126.2, 126.4, 128.5 (2C), 128.6 (2C), 140.2, 141.2, 164.4, 170.2, 172.4. IR (cm^{-1}) : υ = 2858-3086, 1784, 1732, 1697, 1454-1497, 1381, 1313, 1238, 1132-1190. HRMS : C24H27NO4Na : calculated : 416.1838, found : 416.1827.

1-(4-Phenylbutanoyl)-(3*S*)-3-[1(*R*)-(3-phenylpropanoyloxy)-ethyl]-azetidin-2-one (17a). Yield : 89 % (71 mg from 0.20 mmol of **12b**). $[\alpha]_D$ = -9.9 (c = 4.9). R_f = 0.40 (cyclohexane/ethyl acetate : 5/3). MS (ESI) : *m/z* : 394.0 ($(M + H)^+$), 416.1 ($(M + Na)^+$). ^1H NMR (300 MHz, $CDCl_3$) : δ = 1.31 (d, 3H, *J* = 6.4 Hz), 2.00 (m, 2H), 2.56-2.83 (m, 6H), 2.93 (t, 2H, *J* = 7.6 Hz), 3.33 (m, 1H), 3.41 (dd, 1H, *J* = 3.6, Hz, *J* = 7.7 Hz), 3.56 (dd, 1H, *J* = 6.7, Hz, *J* = 7.7 Hz), 5.24 (m, 1H), 7.01-7.59 (m, 10H). ^{13}C NMR (75 MHz, $CDCl_3$) : δ = 18.3, 25.6, 30.9, 35.1, 35.8, 36.0, 40.0, 53.5, 67.6, 126.1, 126.5, 128.3, 128.4, 128.5, 128.6, 140.1, 141.3, 164.3, 170.8, 171.8. IR (cm^{-1}) : υ = 2935-3026, 1786, 1736, 1701, 1454-1497, 1383, 1313, 1250, 1132-1190. HRMS : C24H27NO4Na : calculated : 416.1838, found : 416.1821.

1-(4-Phenylbutanoyl)-(3*S*)-3-[1(*R*)-(4-phenylbutanoyloxy)-ethyl]-azetidin-2-one (17b). Yield : 87 % (68 mg from 0.19 mmol of **12b**). $[\alpha]_D$ = -3.3 (c = 4.8). R_f = 0.41 (cyclohexane/ethyl acetate : 5/3). MS (ESI) : *m/z* : 408.0 ($(M + H)^+$), 430.1 ($(M + Na)^+$). ^1H NMR (500 MHz, $CDCl_3$) : δ = 1.35 (d, 3H, *J* = 6.4 Hz), 1.87-2.03 (m, 4H), 2.29 (t, 2H, *J* = 7.5 Hz), 2.56-2.83 (m, 6H), 3.38 (m, 1H), 3.52 (dd, 1H, *J* = 3.7 Hz, *J* = 7.7 Hz), 3.64 (dd, 1H, *J* = 6.7 Hz, *J* = 7.7 Hz), 5.28 (m, 1H), 7.12-7.32 (m, 10H). ^{13}C NMR (125 MHz, $CDCl_3$) : δ = 18.4, 25.7, 26.6, 33.7, 35.1, 35.2, 36.1, 40.0, 53.6, 67.3, 126.1, 126.2, 128.5, 128.55, 128.57 (2C), 141.2, 141.4, 164.4, 170.9, 172.4. IR (cm^{-1}) : υ = 2934, 1786, 1734, 1701, 1454-1497, 1383, 1312, 1246, 1130-1159. HRMS : C25H29NO4Na : calculated : 430.1994, found : 430.1985.

1-(5-Phenylpentanoyl)-(3*S*)-3-[1(*R*)-(4-phenylbutanoyloxy)-ethyl]-azetidin-2-one (18b). Yield : 63 % (34 mg from 0.13 mmol of **12c**). $[\alpha]_D$ = -2.2 (c = 1.8). R_f = 0.43 (cyclohexane/ethyl acetate : 5/3). MS (ESI) : *m/z* : 444.1 ($(M + Na)^+$). ^1H NMR (300 MHz, $CDCl_3$) : δ = 1.35 (d, 3H, *J* = 6.3Hz), 1.57-1.79 (m, 4H), 1.91 (m, 2H), 2.29 (t, 2H, *J* = 7.5 Hz), 2.54-2.79 (m, 6H), 3.39 (m, 1H), 3.53 (dd, 1H, *J* = 3.6 Hz, *J* = 7.5 Hz), 3.65 (dd, 1H, *J* = 6.8 Hz, *J* = 7.5 Hz), 5.28 (m, 1H), 7.08-7.38 (m, 10H). ^{13}C NMR (75 MHz, $CDCl_3$) : δ = 18.4, 23.7, 26.6, 30.9, 33.7, 35.1, 35.6, 36.5, 39.9, 53.6, 67.3, 125.9, 126.2, 128.4, 128.5, 128.6 (2C), 141.2, 142.1, 164.5, 171.1, 172.4. IR (cm^{-1}) : υ = 2858-3026, 1786, 1734, 1699, 1452-1497, 1381, 1313, 1242, 1132-1192. HRMS : C26H31NO4Na : calculated : 444.2151, found : 444.2152.

1-(Pent-4-enoyl)-(3*S*)-3-[1(*R*)-(4-phenylbutanoyloxy)-ethyl]-azetidin-2-one (19b). Yield : 84 % (89 mg from 0.31 mmol of **12d**). $[\alpha]_D$ = -1.3 (c = 3.5). R_f = 0.46 (cyclohexane/ethyl acetate : 5/3). MS (ESI) : *m/z* : 344.0 ($(M + H)^+$), 366.1 ($(M + Na)^+$). ^1H NMR (300 MHz, $CDCl_3$) : δ = 1.34 (d, 3H, *J* = 6.4 Hz), 1.92 (m, 2H), 2.30 (t, 2H, *J* = 7.6 Hz), 2.39 (m, 2H), 2.63 (t, 2H, *J* = 7.6 Hz), 2.77 (t, 2H, *J* = 7.4 Hz), 3.40 (m, 1H), 3.53 (dd, 1H, *J* = 3.7 Hz, *J* = 7.7 Hz), 3.66 (dd, 1H, *J* = 6.6 Hz, *J* = 7.7 Hz), 4.96-5.09 (m, 2H), 5.28 (m, 1H), 5.81 (m, 1H), 7.07-7.36 (m, 5H). ^{13}C NMR (75 MHz, $CDCl_3$) : δ = 18.4, 26.5, 27.9, 33.7, 35.0, 35.8, 39.9, 53.6, 67.2, 115.9, 126.1, 128.5 (2C), 136.4, 141.2, 164. 4, 170.3, 172.4. IR (cm^{-1}) : υ = 2864-3026, 1786, 1734, 1703, 1454, 1381, 1313, 1238, 1132-1191. HRMS : C20H25NO4Na : calculated : 366.1681, found : 366.1685.

1-(Pent-4-enoyl)-(3*S*)-3-[1(*R*)-(pent-4-enoyloxy)-ethyl]-azetidin-2-one (19e). Yield : 88 % (500 mg from 2.03 mmol of **12d**). $[\alpha]_D$ = -0.25 (c = 4.9). R_f = 0.63 (cyclohexane/ethyl acetate : 5/3). MS (ESI) : *m/z* : 280.0 ($(M + H)^+$), 302.1 ($(M + Na)^+$). ^1H NMR (500 MHz,

$CDCl_3$) : δ = 1.34 (d, 3H, *J* = 6.5 Hz), 2.37 (m, 6H), 2.78 (m, 2H), 3.39 (m, 1H), 3.52 (dd, 1H, *J* = 3.7 Hz, *J* = 7.8 Hz), 3.65 (dd, 1H, *J* = 6.5 Hz, *J* = 7.8 Hz), 4.99 (m, 2H), 5.03 (m, 1H), 5.06 (m, 1H), 5.26 (m, 1H), 5.77 (ddt, 1H, *J* = 5.9 Hz, *J* = 10.2 Hz, *J* = 16.2 Hz), 5.81 (ddt, 1H, *J* = 6.5 Hz, *J* = 10.2 Hz, *J* = 16.8 Hz). ^{13}C NMR (125 MHz, $CDCl_3$) : δ = 18.2, 27.8, 28.7, 33.4, 35.7, 39.8, 53.4, 67.2, 115.6, 115.7, 136.1, 136.2, 164.2, 170.2, 171.7. IR (cm^{-1}) : υ = 2927-2979, 1785, 1738, 1702, 1320. HRMS : C15H21NO4Na : calculated : 302.1368, found : 302.1358.

1-(Hexa-5-enoyl)-(3*S*)-3-[1(*R*)-(4-phenylbutanoyloxy)-ethyl]-azetidin-2-one (20b). Yield : 77 % (20 mg from 0.07 mmol of **12e**). $[\alpha]_D$ = -2.6 (c = 1.0). R_f = 0.43 (cyclohexane/ethyl acetate : 5/3). MS (ESI) : *m/z* : 358.0 ($(M + H)^+$), 380.1 ($(M + Na)^+$). ^{1}H NMR (300 MHz, $CDCl_3$) : δ = 1.35 (d, 3H, *J* = 6.4 Hz), 1.74 (m, 2H), 1.92 (m, 2H), 2.10 (m, 2H), 2.29 (t, 2H, *J* = 7.5 Hz), 2.56-2.74 (m, 4H), 3.39 (m, 1H), 3.53 (dd, 1H, *J* = 3.7 Hz, *J* = 7.7 Hz), 3.66 (dd, 1H, *J* = 6.6 Hz, *J* = 7.7 Hz), 4.93-5.02 (m, 2H), 5.28 (m, 1H), 5.77 (m, 1H), 7.10-7.36 (m, 5H). ^{13}C NMR (75 MHz, $CDCl_3$) : δ = 18.4, 23.2, 26.6, 33.1, 33.7, 35.1, 35.9, 40.0, 53.6, 67.3, 115.6, 126.2, 128.6 (2C), 137.7, 141.2, 164.5, 171.1, 172.4. IR (cm^{-1}) : υ = 2934-2976, 1786, 1734, 1701, 1454, 1381, 1313, 1250, 1132-1190. HRMS : C21H27NO4Na : calculated : 380.1838, found : 380.1827.

General procedure for esterification with carboxylic acid (16c-d, 17c-d, 18d, 19d and 19f, 20d and 23). To a stirred solution of alcohol precursor, DCC (1.1 eq.) and DMAP (cat.) in dry dichloromethane (13 mL/mmol), at 20 °C, was added a solution of the suitable carboxylic acid (1.1 eq.) in dry dichloromethane (7 mL/mmol) under argon atmosphere. After stirring overnight, the mixture was cooled in an ice-bath for precipitation of urea, filtered and concentrated under vacuum. After purification by flash chromatography (dichloromethane/ethyl acetate) white solids (**13d**, **14d**, **16d**, **17d**, **18d**, **19d**, **20d** and **23**) or colourless oils (**16c**, **17c** and **19f**) were obtained.

1-(3-Phenylpropanoyl)-(3*R*,4*R*)-3-[1(*R*)-(biphenylacetyloxy)-ethyl]-4-(acetoxy)-azetidin-2-one (13d). Yield : 67 % (35 mg from 0.16 mmol of **11a**). Mp : 98.0-103.0 °C. $[\alpha]_D$ = -15.1 (c = 2.3). R_f = 0.28 (cyclohexane/ethyl acetate : 5/2). MS (ESI) : *m/z* : 522.2 ($(M + Na)^+$), 1020.9 ($(2M + Na)^+$). ^{1}H NMR (300 MHz, $CDCl_3$) : δ = 1.42 (d, 3H, *J* = 6.5 Hz), 2.14 (s, 3H), 2.76-2.94 (m, 4H), 3.29 (dd, 1H, *J* = 1.7 Hz, *J* = 5.6 Hz), 3.64 (d, 1H, *J* = 17.1 Hz, AB system), 3.70 (d, 1H, *J* = 17.1 Hz, AB system), 5.33 (m, 1H), 6.46 (d, 1H, *J* = 1.7 Hz), 7.13-7.66 (m, 14H). ^{13}C NMR (75 MHz, $CDCl_3$) : δ = 18.3, 20.9, 29.8, 38.3, 41.0, 62.8, 66.2, 74.8, 126.5, 127.2, 127.4 (2C), 128.6, 128.6, 128.9, 129.8, 132.6, 140.0, 140.2, 140.7, 162.2,

169.1, 169.2, 170.5. IR (cm^{-1}) : υ = 2931-3029, 1803, 1740, 1718, 1454-1489, 1381, 1313, 1213. HRMS : C30H29NO6Na : calculated : 522.1893, found : 522.1899.

1-(4-Phenylbutanoyl)-(3*R*,4*R*)-3-[1(*R*)-(biphenylacetyloxy)-ethyl]-4-(acetoxy)-azetidin-2-one (14d). Yield : 77 % (62 mg from 0.16 mmol of **11b**). Mp : 87.5-89.0 °C. $[\alpha]_D$ = -8.2 (c = 4.7). R_f = 0.32 (cyclohexane/ethyl acetate : 5/2). MS (ESI) : *m/z* : 536.1 ($(M + Na)^+$), 1048.6 ($(2M + Na)^+$). ^1H NMR (300 MHz, $CDCl_3$) : δ = 1.43 (d, 3H, *J* = 6.5 Hz), 1.85-2.00 (m, 2H), 2.12 (s, 3H), 2.48-2.73 (m, 4H), 3.28 (dd, 1H, *J* = 1.7 Hz, *J* = 5.6 Hz), 3.64 (d, 1H, *J* = 15.5 Hz, AB system), 3.70 (d, 1H, *J* = 15.5 Hz, AB system), 5.35 (m, 1H), 6.46 (d, 1H, *J* = 1.7 Hz), 7.15-7.70 (m, 14H). ^{13}C NMR (75 MHz, $CDCl_3$) : δ = 18.3, 20.9, 25.2, 35.0, 36.0, 41.0, 62.7, 66.2, 74.7, 126.1, 127.1, 127.4 (2C), 128.5 (2C), 128.9, 129.7, 132.6, 140.2, 140.7, 141.2, 162.2, 169.1, 169.8, 170.4. IR (cm^{-1}) : υ = 2854-3082, 1803, 1740, 1717, 1452-1489, 1381, 1312, 1213. HRMS : C31H31NO6Na : calculated : 536.2049, found : 536.2062.

1-(3-Phenylpropanoyl)-(3*S*)-3-[1(*R*)-(5-phenylpentanoyloxy)-ethyl]-azetidin-2-one (16c). Yield : 59 % (48 mg from 0.20 mmol of **12a**). $[\alpha]_D$ = -9.9 (c = 1.8). R_f = 0.44 (cyclohexane/ethyl acetate : 5/3). MS (ESI) : *m/z* : 430.1 ($(M + Na)^+$). ^1H NMR (300 MHz, $CDCl_3$) : δ = 1.35 (d, 3H, *J* = 6.5 Hz), 1.58-1.76 (m, 4H), 2.32 (m, 2H), 2.63 (m, 2H), 2.96-3.09 (m, 4H), 3.38 (m, 1H), 3.52 (dd, 1H, *J* = 3.6 Hz, *J* = 7.7 Hz), 3.66 (dd, 1H, *J* = 6.5 Hz, *J* = 7.7 Hz), 5.25 (m, 1H), 7.14-7.40 (m, 10H). ^{13}C NMR (75 MHz, $CDCl_3$) : δ = 18.4, 24.6, 30.2, 30.9, 34.3, 35.6, 38.3, 40.1, 53.6, 67.3, 125.9, 126.4, 128.5 (2C), 128.6 (2C), 140.2, 142.0, 164.4, 170.3, 172.5. IR (cm^{-1}) : υ = 2932-3026, 1785, 1734, 1701, 1454-1497, 1387, 1315, 1238-1255, 1132-1175. HRMS : C25H29NO4Na : calculated : 430.1994, found : 430.1990.

1-(3-Phenylpropanoyl)-(3*S*)-3-[1(*R*)-(biphenylacetyloxy)-ethyl]-azetidin-2-one (16d). Yield : 93 % (84 mg from 0.20 mmol of **12a**). Mp : 61.5-62.0 °C. $[\alpha]_D$ = -18.2 (c = 3.7). R_f = 0.38 (cyclohexane/ethyl acetate : 5/3). MS (ESI) : *m/z* : 464.2 ($(M + Na)^+$), 904.8 ($(2M + Na)^+$). ^1H NMR (300 MHz, $CDCl_3$) : δ = 1.29 (d, 3H, *J* = 6.4 Hz), 2.77-2.96 (m, 4H), 3.31 (m, 1H), 3.42 (dd, 1H, *J* = 3.7 Hz, *J* = 7.7 Hz), 3.54 (dd, 1H, *J* = 7.2 Hz, *J* = 7.7 Hz), 3.58 (s, 2H), 5.22 (m, 1H), 7.09-7.60 (m, 14H). ^{13}C NMR (75 MHz, $CDCl_3$) : δ = 18.5, 30.2, 38.4, 39.9, 41.3, 53.7, 67.9, 126.5, 127.3, 127.5 (2C), 128.7 (2C), 129.0, 129.7, 132.8, 140.3, 140.4, 140. 7, 164.3, 170.3, 170.5. IR (cm^{-1}) : υ = 2906-3058, 1786, 1734, 1701, 1454-1489, 1387, 1315, 1251, 1132-1157. HRMS : C28H27NO4Na : calculated : 464.1838 , found : 464.1845.

1-(4-Phenylbutanoyl)-(3*S*)-3-[1(*R*)-(5-phenylpentanoyloxy)-ethyl]-azetidin-2-one (17c). Yield : 93 % (76 mg from 0.19 mmol of **12b**). $[\alpha]_{20/D}$ = -2.3 (c = 4.0). R_f = 0.41

(cyclohexane/ethyl acetate : 5/3). MS (ESI) : *m/z* : 421.9 ($(M + H)^+$), 444.1 ($(M + Na)^+$). ^{1}H NMR (300 MHz, $CDCl_3$) : δ = 1.35 (d, 3H, *J* = 6.4 Hz), 1.56-1.73 (m, 4H), 2.01 (m, 2H), 2.31 (m, 2H), 2.62 (m, 2H), 2.66-2.84 (m, 4H), 3.38 (m, 1H), 3.51 (dd, 1H, *J* = 3.7 Hz, *J* = 7.7 Hz), 3.63 (dd, 1H, *J* = 6.6 Hz, *J* = 7.7 Hz), 5.27 (m, 1H), 7.08-7.45 (m, 10H). ^{13}C NMR (75 MHz, $CDCl_3$) : δ = 18.4, 24.6, 25.7, 30.8, 34.2, 35.1, 35.5, 36.0, 39.9, 53.5, 67.2, 125.9, 126.1, 128.4 (2C), 128.5 (2C), 141.3, 142.0, 164.4, 170.8, 172.5. IR (cm^{-1}) : υ = 2856-3026, 1786, 1734, 1701, 1454, 1383, 1313, 1250, 1130. HRMS : C26H31NO4Na : calculated : 444.2151, found : 444.2141.

1-(4-Phenylbutanoyl)-(3*S*)-3-[1(*R*)-(biphenylacetyloxy)-ethyl]-azetidin-2-one (17d). Yield : 83 % (72 mg from 0.19 mmol of **12b**). Mp : 96.0-96.5 °C. $[\alpha]_D$ = -14.8 (c = 4.7). R_f = 0.40 (cyclohexane/ethyl acetate : 5/3). MS (ESI) : *m/z* : 456.0 ($(M + H)^+$), 478.1 ($(M + Na)^+$). ^{1}H NMR (500 MHz, $CDCl_3$) : δ = 1.37 (d, 3H, *J* = 6.4 Hz), 1.95 (m, 2H), 2.57-2.76 (m, 4H), 3.37 (m, 1H) 3.48 (dd, 1H, *J* = 3.7 Hz, *J* = 7.7 Hz), 3.59 (dd, 1H, *J* = 6.6 Hz, *J* = 7.7 Hz), 3.65 (s, 2H), 5.31 (m, 1H), 6.98-7.76 (m, 14H). ^{13}C NMR (125 MHz, $CDCl_3$) : δ = 18.4, 25.5, 35.1, 36.0, 39.7, 41.1, 53.5, 67.8, 126.1, 127.1, 127.38, 127.43, 128.4, 128.5, 128.9, 129.6, 132.6, 140.2, 140.6, 141.3, 164.2, 170.4, 170.7. IR (cm^{-1}) : υ = 2936-3028, 1786, 1736, 1697, 1452-1489, 1389, 1313, 1248, 1132-1155. HRMS : C29H29NO4Na : calculated : 478.1994, found : 478.1994.

1-(5-Phenylpentanoyl)-(3*S*)-3-[1(*R*)-(biphenylacetyloxy)-ethyl]-azetidin-2-one (18d). Yield : 66 % (39 mg from 0.12 mmol of **12c**). Mp : 70.5-71.3 °C. $[\alpha]_D$ = -1.1 (c = 7.0). R_f = 0.44 (cyclohexane/ethyl acetate : 5/3). MS (ESI) : *m/z* : 492.1 ($(M + Na)^+$). ^{1}H NMR (300 MHz, $CDCl_3$) : δ = 1.36 (d, 3H, *J* = 6.4 Hz), 1.52-1.73 (m, 4H), 2.50-2.75 (m, 4H), 3.38 (m, 1H), 3.48 (dd, 1H, *J* = 3.6 Hz, *J* = 7.7 Hz), 3.56-3.65 (m, 3H), 5.31 (m, 1H), 7.07-7.64 (m, 14H). ^{13}C NMR (75 MHz, $CDCl_3$) : δ = 18.4, 23.7, 30.8, 35.6, 36.4, 39.7, 41.2, 53.5, 67.8, 125.8, 127.1, 127.4, 127.5, 128.4, 128.5, 128.9, 129.6, 132.7, 140.2, 140.6, 142.1, 164.3, 170.4, 170.9. IR (cm^{-1}) : υ = 2854-3028, 1786, 1736, 1699, 1452-1489, 1389, 1315, 1246, 1132-1159. HRMS : C30H31NO4Na : calculated : 492.2151, found : 492.2133.

1-(Pent-4-enoyl)-(3*S*)-3-[1(*R*)-(biphenylacetyloxy)-ethyl]-azetidin-2-one (19d). Yield : 68 % (70 mg from 0.26 mmol of **12d**). Mp : 92.5-93.0 °C. $[\alpha]_D$ = -9.8 (c = 2.5). R_f = 0.44 (cyclohexane/ethyl acetate : 5/3). MS (ESI) : *m/z* : 414.1 ($(M + Na)^+$). ^{1}H NMR (300 MHz, $CDCl_3$) : δ = 1.36 (d, 3H, *J* = 6.4 Hz), 2.36 (m, 2H), 2.70 (m, 2H), 3.39 (m, 1H), 3.48 (dd, 1H, *J* = 3.7 Hz, *J* = 7.7 Hz), 3.61 ((dd, 1H, *J* = 6.6 Hz, *J* = 7.7 Hz), 3.64 (s, 2H), 5.00 (m, 2H), 5.30 (m, 1H), 5.79 (m, 1H), 7.27-7.70 (m, 9H). ^{13}C NMR (75 MHz, $CDCl_3$) : δ = 18.4, 27.9,

35.8, 39.8, 41.2, 53.6, 67.8, 115.8, 127.1, 127.4, 128.9 (2C), 129.6, 132.7, 136.4, 140.2, 140.6, 164.3, 170.3, 170.4. IR (cm^{-1}) : υ = 2916, 1788, 1734, 1701, 1488, 1387, 1315, 1238-1259. HRMS : C24H25NO4Na : calculated : 414.1681, found : 414.1692.

1-(Pent-4-enoyl)-(3*S*)-3-[1(*R*)-(hexa-5-enoyloxy)-ethyl]-azetidin-2-one (19f). Yield : 84 % (65 mg from 0.26 mmol of **12d**) . $[\alpha]_D$ = -0.5 (c = 4.0). R_f = 0.41 (cyclohexane/ethyl acetate : 5/3). MS (ESI) : *m/z* : 316.1 $((M + Na)^+)$. ^1H NMR (300 MHz, $CDCl_3$) : δ = 1.32 (d, 3H, *J* = 6.4 Hz), 1.66 (m, 2H), 2.04 (m, 2H), 2.26 (t, 2H, *J* = 7.5 Hz), 2.38 (m, 2H), 2.76 (t, 2H, *J* = 7.5 Hz), 3.39 (m, 1H), 3.51 (dd, 1H, *J* = 3.6 Hz, *J* = 7.7 Hz), 3.64 (dd, 1H, *J* = 6.8 Hz, *J* = 7.7 Hz), 4.85-5.12 (m, 4H), 5.22 (m, 1H), 5.76 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) : δ = 18.4, 24.1, 28.0, 33.0, 33.6, 35.9, 39.9, 53.6, 67.2, 115.7, 115.9, 136.4, 137.5, 164.5, 170.4, 172.5. IR (cm^{-1}) : υ = 2935-2978, 1788, 1736, 1701, 1381, 1315, 1238, 1134-1168. HRMS : C16H23NO4Na : calculated : 316.1525, found : 316.1515.

1-(Hexa-5-enoyl)-(3*S*)-3-[1(*R*)-(biphenylacetyloxy)-ethyl]-azetidin-2-one (20d). Yield : 66 % (39 mg from 0.06 mmol of **12e**). Mp : 44.2-45.1 °C. $[\alpha]_D$ = -11.7 (c = 1.1). R_f = 0.43 (cyclohexane/ethyl acetate : 5/3). MS (ESI) : *m/z* : 428.1 $((M + Na)^+)$. ^1H NMR (300 MHz, $CDCl_3$) : δ = 1.36 (d, 3H, *J* = 6.4 Hz), 1.71 (m, 2H), 2.06 (m, 2H), 2.61 (m, 2H), 3.39 (m, 1H), 3.49 (dd, 1H, *J* = 3.7 Hz, *J* = 7.7 Hz), 3.61 (dd, 1H, *J* = 7.2 Hz, *J* = 7.7 Hz), 3.64 (s, 2H), 4.90-5.06 (m, 2H), 5.30 (m, 1H), 5.74 (m, 1H), 7.23-7.65 (m, 9H). ^{13}C NMR (75 MHz, $CDCl_3$) : δ = 18.4, 23.2, 33.0, 35.9, 39.8, 41.2, 53.5, 67.8, 115.5, 127.1, 127.4 (2C), 128.9, 129.6, 132.7, 137.7, 140.3, 140.6, 164.3, 170.5, 171.0. IR (cm^{-1}) : υ = 2934-2976, 1786, 1736, 1701, 1450-1489, 1389, 1315, 1252, 1132-1194. HRMS : C25H27NO4Na : calculated : 428.1838, found : 428.1825.

1-(Benzyloxycarbonyl)-(3*S*)-3-[1(*R*)-(biphenylacetyloxy)-ethyl]-azetidin-2-one (23). Yield : 83 % (75 mg from 0.20 mmol of **22**). Mp : 90.8-91.6 °C. $[\alpha]_D$ = -19.8 (c = 0.6). R_f = 0.37 (cyclohexane/ethyl acetate : 5/3). MS (ESI) : *m/z* : 466.1 $((M + Na)^+)$. ^1H NMR (300 MHz, $CDCl_3$) : δ = 1.35 (d, 3H, *J* = 6.3 Hz), 3.39 (m, 1H), 3.51 (dd, 1H, *J* = 3.6 Hz, *J* = 7.0 Hz), 3.60-3.70 (m, 3H), 5.19 (s, 2H), 5.29 (m, 1H), 7.26-7.67 (m, 14H). ^{13}C NMR (75 MHz, $CDCl_3$) : δ = 18.4, 41.2, 41.4, 54.5, 68.0, 68.2, 127.2 (2C), 128.4 (2C), 128.7, 128.8, 128.9, 129.7, 132.7, 135.0, 140.2, 140.8, 148.9, 163.6, 170.6. IR (cm^{-1}) : 2920-3059, 1813, 1772, 1730, 1456-1489, 1389, 1329, 1128. HRMS : C27H25NO5Na : calculated : 466.1630, found : 466.1609.

1-(Benzyloxycarbonyl)-(3*S*)-3-[1(*R*)-(*tert*-butyldimethylsilyloxy)-ethyl]-azetidin-2-one (21). To a stirred solution of lithium hexamethyldisilazide (436 µL, 0.44 mmol) in tetrahydrofurane (2 mL), at -78 °C was added **8** (100 mg, 0.44 mmol) in tetrahydrofurane (2 mL) under argon atmosphere. The mixture was stirred for 30 min at -78 °C; then benzyl chloroformate (75 µL, 0.52 mmol) was added. After stirring during 1 h, at low temperature, the solution was allowed to warm up and stirred for 1 h at 20 °C. After dilution in dichloromethane, the organic layer was washed with brine, dried over $MgSO_4$, filtered and concentrated under vacuum. Purification by flash chromatography (cyclohexane/ethyl acetate) gave **21** as a white solid. Yield : 99 % (157 mg from 0.44 mmol of **8**). Mp : 44.6-46.0 °C. R_f = 0.46 (cyclohexane/ethyl acetate : 5/3). MS (ESI) : *m/z* : 386.1 ($(M + Na)^+$). ^1H NMR (300 MHz, $CDCl_3$) : δ = 0.03 (s, 3H), 0.06 (s, 3H), 0.81 (s, 9H), 1.17 (d, 3H, *J* = 6.3 Hz), 3.22 (m, 1H), 3.58 (dd, 1H, *J* = 3.5 Hz, *J* = 6.5 Hz), 3.73 (dd, 1H, *J* = 6.4 Hz, *J* = 6.5 Hz), 4.29 (m, 1H), 5.25 (s, 2H), 7.27-7.48 (m, 5H). ^{13}C NMR (75 MHz, $CDCl_3$) : δ = -5.1, -4.1, 17.9, 22.3, 25.7, 39.8, 57.3, 64.8, 68.0, 128.4, 128.5, 128.7, 135.4, 149.3, 165.8. IR (cm^{-1}) : υ = 2854-2924, 1801, 1726, 1464, 1387, 1323-1339, 1259. HRMS : C19H29NO4SiNa : calculated : 386.1764, found : 386.1776.

(3*S*)-3-[1(*R*)-(Biphenylacetyloxy)-ethyl]-azetidin-2-one (24). To a stirred solution of **23** (56 mg, 0.13 mmol) in ethyl acetate (2.5 mL) and ethanol (3 mL), was added 10 % Pd/C (5.6 mg). After stirring under hydrogen atmosphere (P = 1 atm), during 1 hour at room temperature, the mixture was filtered through a short pad of Celite and concentrated under vacuum. **24** was obtained without further purification as a white solid. Yield : 96 % (38 mg from 0.13 mmol of **23**). Mp : 119.5-120.8 °C. $[\alpha]_D$ = -27.2 (c = 2.0). R_f = 0.06 (cyclohexane/ethyl acetate : 5/3). MS (ESI) : *m/z* : 309.8 ($(M + H)^+$), 332.0 ($(M + Na)^+$). ^1H NMR (300 MHz, $CDCl_3$) : δ = 1.37 (d, 3H, *J* = 6.3 Hz), 3.14 (dd, 1H, *J* = 2.3 Hz, *J* = 5.5 Hz), 3.32 (dd, 1H, *J* = 5.4 Hz, *J* = 5.5 Hz), 3.35-3.43 (m, 1H), 3.65 (s, 2H), 5.25 (m, 1H), 5.96 (br s, 1H), 7.36 (m, 3H), 7.44 (m, 2H), 7.56 (m, 4H). ^{13}C NMR (75 MHz, $CDCl_3$) : δ = 18.5, 39.3, 41.2, 56.5, 69.1, 127.1, 127.3, 127.4, 128.9, 129.7, 133.0, 140.1, 140.7, 167.7, 170.8. IR (cm^{-1}) : υ = 3248, 2922-2978, 1755, 1732, 1489, 1250, 1136-1155. HRMS : C19H19NO3Na : calculated : 332.1263, found : 332.1249.

In vitro assays for human FAAH. Tubes containing the enzyme[47] (10 mM Tris-HCl, 1 mM EDTA, 0.1 % (w/v) BSA, pH 7.4, 165 µL), test compounds in DMSO or DMSO alone for controls (10 µL) and [^3H]-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 buffer only were used as 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.

In vitro assays for human MGL activity. Tubes containing purified enzyme[46] (10 mM Tris-HCl, 1 mM EDTA, 0.1 % (w/v) BSA, pH 8.0, 165 µL), test compounds in DMSO or DMSO alone for controls (10 µL) and [^{3}H]-2-OG (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 buffer only were used as control for chemical hydrolysis (blank) and this value was systematically subtracted.

Preincubation studies. Tubes containing enzyme (10 mM Tris-HCl, 1 mM EDTA, 0.1 % (w/v) BSA, pH 7.4, 165 µL) and test compounds in DMSO or DMSO alone (10 µL) were preincubated 90, 45, 15 and 0 min at room temperature prior to addition of [^{3}H]-AEA (50,000 dpm, 2 µM final concentration, 25 µL). 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 buffer only were used as control for chemical hydrolysis (blank) and this value was systematically subtracted.

Reversibility studies. In a total volume of 15 µL, human FAAH (27.5 µg) and inhibitors (or DMSO for controls) at concentrations allowing inhibition of the enzyme before dilution and no inhibition after the 100-fold dilution, were preincubated during 1 h at room temperature. The mixtures were then diluted 100-fold by adding assay buffer. Immediatly after, an aliquot (165µL) was taken and [^{3}H]-AEA (50,000 dpm, 2 µM final concentration, 25 µL) was added. Two samples were taken at 30 and 90 min after the dilution too. Each aliquots

were incubated at 37 °C for 30 min and 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 buffer only were used as control for chemical hydrolysis (blank) and this value was systematically subtracted.

Determination of inhibitor interactions with *h*FAAH. Tubes containing enzyme (10 mM Tris-HCl, 1 mM EDTA, 0.1 % (w/v) BSA, pH 7.4, 165 µL; except for 150 µM of AEA, 159.5 µL and 250 µM of AEA, 139.5 µL) and test compounds in DMSO or DMSO alone (10 µL) were incubated at 37 °C with increasing concentrations of [^{3}H]-AEA (50,000 dpm, 1, 2, 5, 10, 15, 20, 30, 75, 150 and 250 µM final concentration, 25 µL; except for 150 µM, 30.5 µL and 250 µM, 50.5 µL). 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 buffer only were used as control for chemical hydrolysis (blank) and this value was systematically subtracted.

Docking studies. Docking of the inhibitors into the active site of FAAH was performed using the GOLD program. GOLD is based on a genetic algorithm, performing docking of flexible ligands into proteins with partial flexibility in the neighbourhood of the active site. Default settings were used for the genetic algorithm parameters. 20 solutions were generated and ranked by GOLD score. The GOLD fitness function is made up of four components: protein-ligand hydrogen bond energy, protein-ligand van der Walls energy, ligand internal van der Walls energy and ligand torsional strain energy. The figures were produced using PyMOL[57] and Ligplot[58].

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Supporting information available: Synthesis of compound **8**, pI_{50} and standard deviation of each tested compound, representatives 'dose-response' curves, docking showing aminoacids involved in hydrophobic contacts and Ramachadran plot of the modelled human FAAH. This material is available free of charge via the internet at http://pubs.acs.org.

IV.5 References

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Chapter 5

V SAR and LC/MS studies of β-lactamic inhibitors of human Fatty acid amide hydrolase: Evidence of a nonhydrolytic process.

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The discovery of the reversible mode of inhibition was quite surprising: β-lactams are known for acting as irreversible or suicide-type inhibitors. Three possibilities explain a reversible inhibition: i) a tetrahedral intermediate is formed but is reversed and does not evolve towards a stable acyl-enzyme complex; ii) an acyl-enzyme intermediate is formed but is hydrolyzed slowly like the natural substrate; iii) there is no covalent bond between the inhibitor and the enzyme but a very good affinity.

To explore this atypical feature, and to understand how a β-lactam can be a reversible inhibitor, we proceeded in two steps. Firstly, we synthesized a set of five compounds in order to identify which carbonyl group is essential for the inhibition, and secondly, we checked whether our compounds are slow substrates of FAAH.

All the results were published in the Journal of Medicinal Chemistry, in 2011.

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SAR and LC/MS studies of *β*-Lactamic inhibitors of human Fatty Acid Amide Hydrolase (*h*FAAH): Evidence of a non-hydrolytic process

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The endocannabinoid hydrolyzing enzyme FAAH uses a non-classical 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.

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V.1 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 localisation 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 to enhance 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 being 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, iii) a non-covalent interaction based on a 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 a 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 nucleophilic addition on the carbonyl function of **3**[28-29], confirmed the covalent irreversible and reversible FAAH inhibitions, respectively.

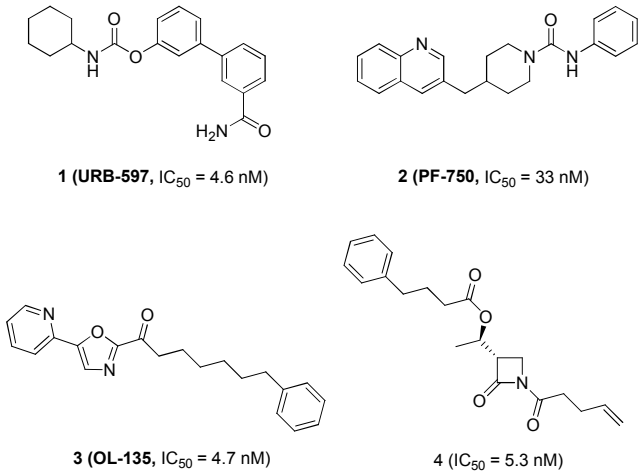


Chart 1. Chemical structure of previously described FAAH-inhibitors.

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 towards 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.

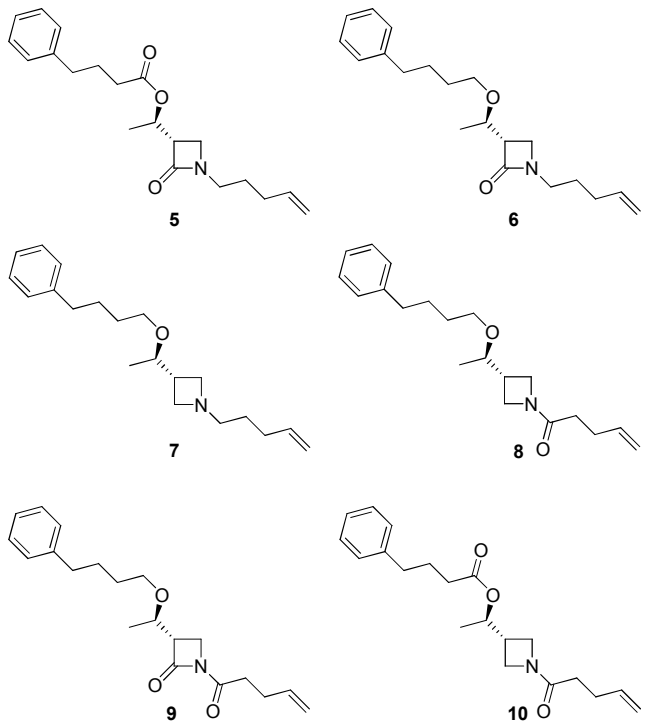
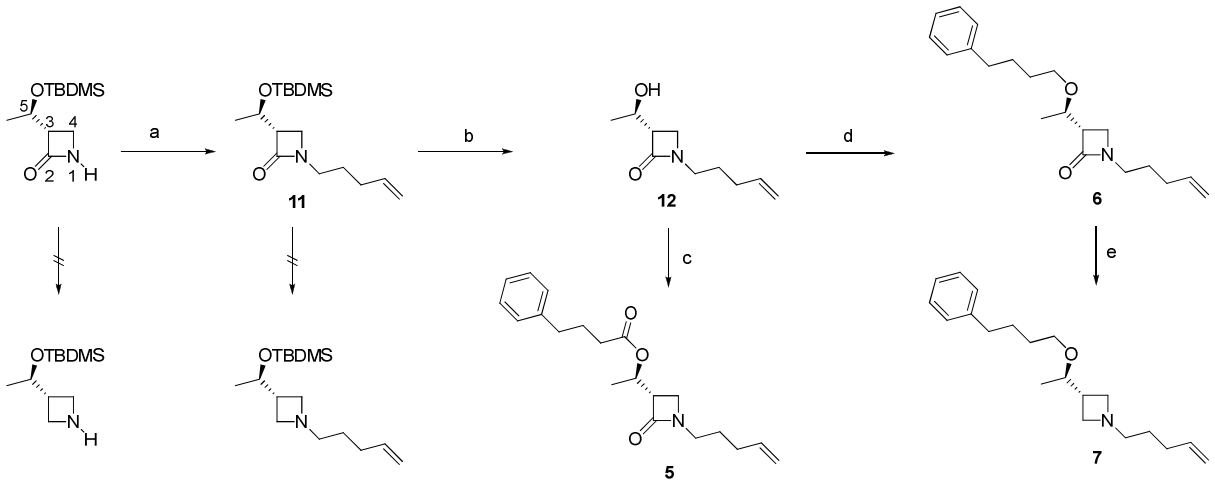


Chart 2. Analogues of compound **4**.

V.2 Chemistry

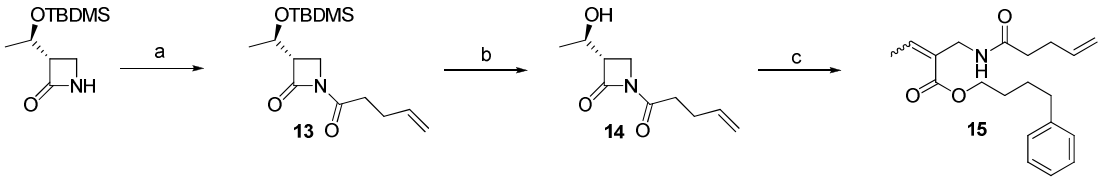
Based on the structure of our lead compound **4**, potentially six derivatives can be imagined that would possess two (**5**, **9**, **10**), one (**6**, **8**) or zero (**7**) carbonyl(s) (Chart 2) instead of the three carbonyls of **4**. Among these six analogues that we planned to synthesize, **10** 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 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; (iii) the *O*-alkylation reaction did not occur when the N1 position was acylated because the

anionic intermediate drove to a rearrangement; this imposed that the N1 position had to be protected with an alkyl group in the case of treatment with a strong base.

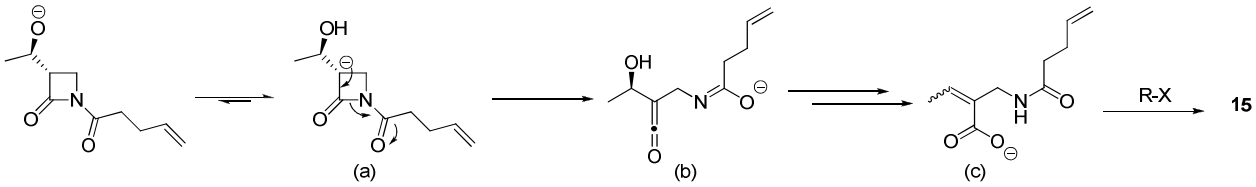


Scheme 1. Reagents and conditions: (a) 5-bromopent-1-ene, KOH, Bu_4NHSO_4, KI, THF, rt, 24 h; (b) TBAF, AcOH, THF, rt, 1 h; (c) 4-phenylbutanoyl chloride, pyridine, DCM, rt, 15 h; (d) 4-phenyl-1-butyl bromide, KI, NaH, DMF, 0 °C to rt, 4 h; (e) $LiAlH_4$, $AlCl_3$, Et_2O, 0 °C to 40 °C, 4 h.

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 functionalisation of the C5-O position (Scheme 1). The starting material was *N*-alkylated by reaction with 4-pentenyl bromide and KOH, in 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 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 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).

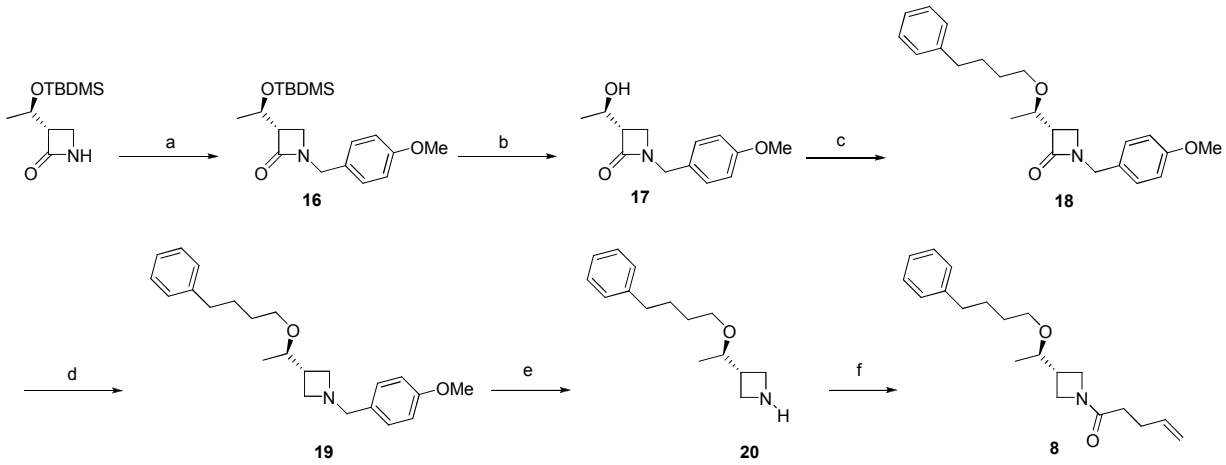


Scheme 2. Reagents and conditions: (a) 4-pentenoyl chloride, pyridine, DCM, 45 °C, 24 h; (b) HCl, AcOH, ACN, 0 °C, 3 h; c) 4-phenyl-1-butyl bromide, KI, NaH, DMF, 0 °C to rt, 4 h.



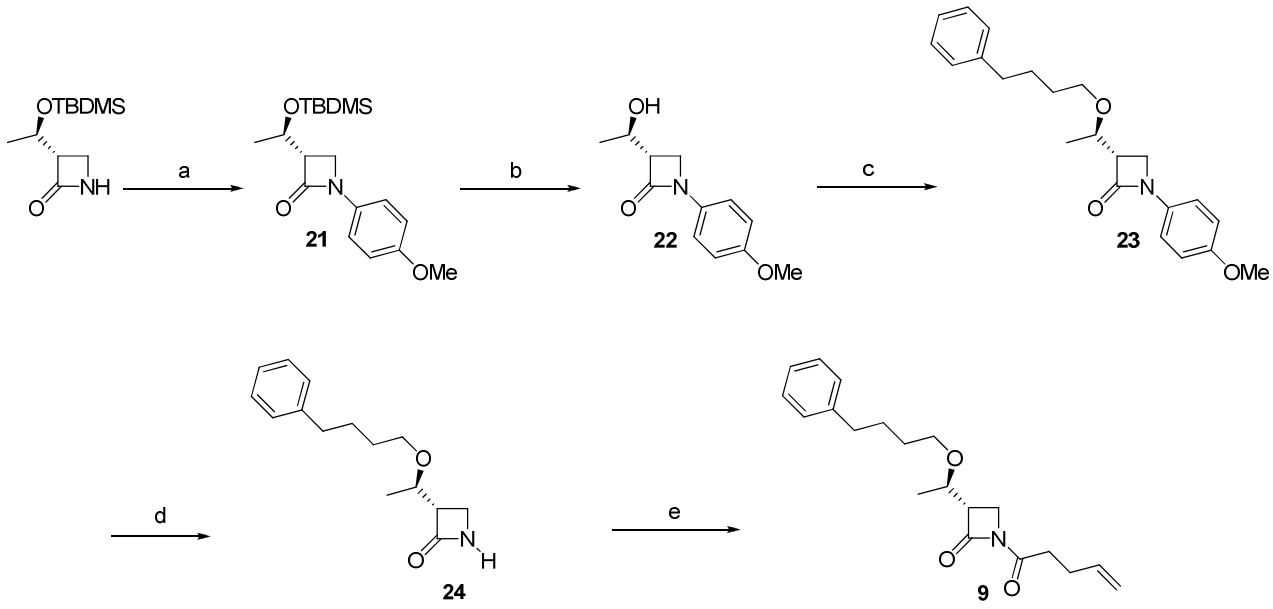
Scheme 3. Proposed mechanism for the formation of **15** in anhydrous conditions.

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 (a) might be cleaved to furnish the hydroxyketene intermediate (b) leading to the α,β-unsaturated carboxylate (c) 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 above, to furnish compound **18** (91%) (Scheme 4). Azetidinone **18** was then reduced in azetidine **19** (73%) using monochlorohydroalane as 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 drove to compound **8** (54%).



Scheme 4. 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 °C to 40 °C, 4 h; (e) CAN, H_2O, ACN, 70 °C, 1 h ; (f) 4-pentenoic acid, DIEA, PyBOP, DMF, rt, 15 h.

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 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 some contamination with a diastereoisomer (partial epimerisation 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 presence of pyridine, giving azetidinone **9** (47%) (Scheme 5).



Scheme 5. 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.

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 ^{1}H 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 splitted into two but that a coalescence of signals occurred when raising the temperature to 75 °C, confirming the presence of conformers (see supporting information).

V.3 Biochemical evaluations

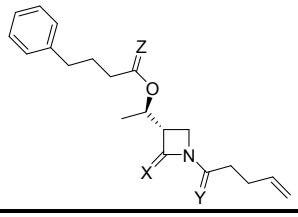
The inhibition activity of compounds **5** to **9** was evaluated on human recombinant FAAH. Then, in order to determine whether our azetidinones are hydrolyzed by FAAH or not,

incubation mixtures were analyzed by HPLC/MS using a high resolution mass spectrometer (LTQ-orbitrap) as LC detector. First, hydrolytic products from compounds **4** and **9** were analyzed after incubation in 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** et **2**, Chart 1), known for being irreversible and selective FAAH inhibitors. The results were then compared to similar experiments performed in presence of human recombinant FAAH.

V.3.1 FAAH inhibition

Human recombinant enzyme, developed in our laboratory,[43] was used in a competitive hydrolytic assay using [^{3}H]-AEA as 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**); (iv) the deletion of the C5-O carbonyl has no influence on the activity (compare compounds **9** and **4**). Based on 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.

Table 1. Determination of the Inhibition Activity Towards Human FAAH



compound	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)

V.3.2 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 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 artefacts 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 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.

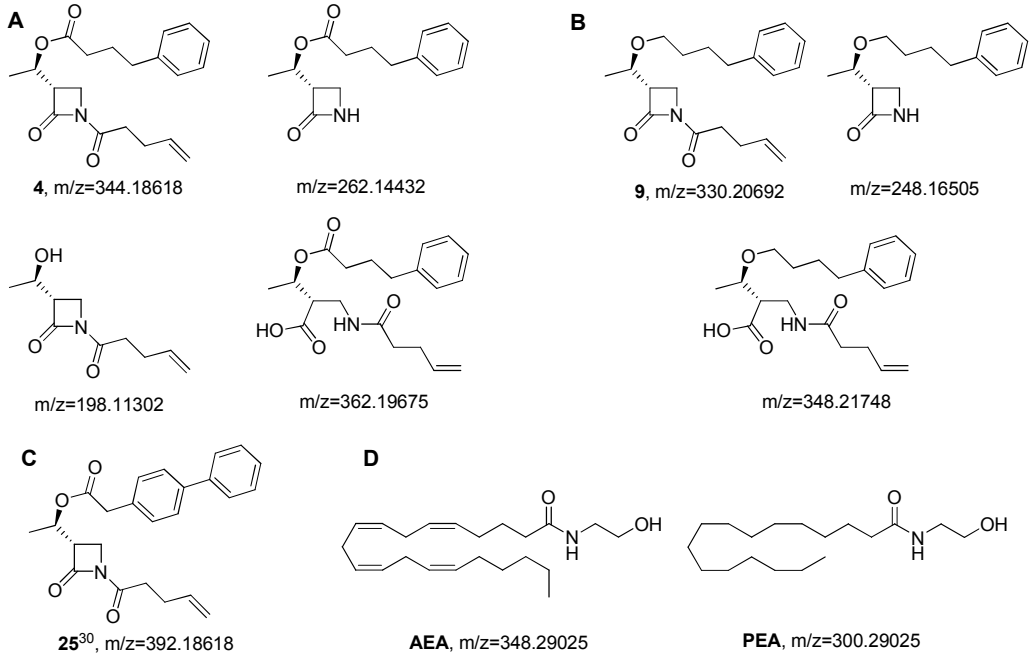


Chart 3. Ions detected in the HPLC-MS analysis

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) **25**, 1-(Pent-4-enoyl)-(3*S*)-3-[1(*R*)-(biphenylacetyloxy)-ethyl]-azetidin-2-one[30] was used as internal standard for the experiments performed with **4** and **9**. (D) Structure and exact mass for anandamide (AEA) and *N*-palmitoylethanolamine (PEA) (used as internal standard for experiments involving anandamide) $[M+H]^+$ ions.

V.3.3 Assay with liver homogenate

Mammalian liver contains numbers 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 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 for details). 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**, most probably due to the replacement of ester bond (at the C5-O position) with an ether bond.

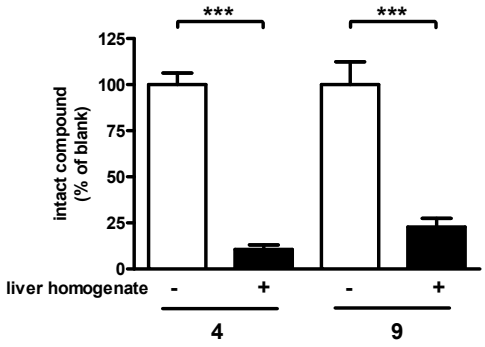


Figure 1. HPLC-MS analysis of **4** and **9** hydrolysis by liver homogenate. Detection of m/z=344.18618 and m/z=330.20692 ions corresponding to the $[M+H]^+$ ions of **4** and **9**, respectively, after incubation with mouse liver homogenate. The data are reported to the signal obtained for the chemical hydrolysis ((-) liver homogenate) which was determined using heat-inactivated liver homogenate. *** $P < 0.0001$ compared to denatured liver (student t-test). Data are shown as mean ± s.e.m of 3 independent experiments performed in duplicate.

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

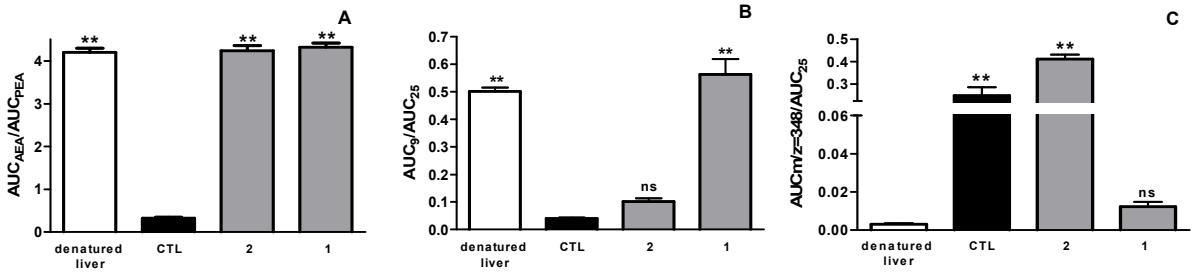


Figure 2. Study of the influence of FAAH inhibitors on the liver homogenate hydrolysis of AEA and **9**. AEA (m/z = 348.29025) (A) and **9** (m/z=344.18618) (B) were incubated at 10^{-5} M with liver homogenates in the presence (or not) of the FAAH inhibitors **1** and **2** (5.10^{-5} M). Heat-denaturated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

To determine whether the hydrolysis of compound **9** by liver homogenate is FAAH-dependent or not, the same experiment was performed in 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 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 than in the control. Accordingly, the ion due to the azetidinone ring opening (m/z = 348.21748), was detected in a large amount in presence of **2** whereas it was almost not detected in presence of **1** (Figure 2C). These results, at first surprising, may be explained by

the reported lower selectivity of **1** towards FAAH compared to **2**[24, 44], and suggest that **9** is not hydrolyzed by FAAH but by other hydrolase(s) present in the liver.

V.3.4 Assay with recombinant hFAAH

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.

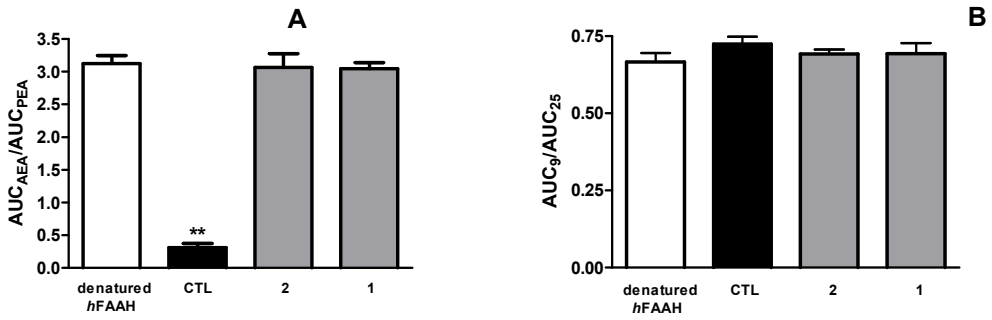


Figure 3. Study of the role of FAAH in the hydrolysis of **9**. AEA (m/z = 348.29025) (A) and **9** (m/z=344.18618) (B) were incubated at 10^{-5} M with recombinant *h*FAAH in the presence (or not) of the FAAH inhibitors **1** and **2** (5.10^{-5} M). Heat-denaturated enzyme preparation was used as control 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 denatured *h*FAAH (ANOVA one-way, Dunnett's post test). Data are shown as mean ± s.e.m of 2 (AEA) or 3 (**9**) independent experiments performed in duplicate.

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 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).

V.4 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] moities which interaction with 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 non-covalent mechanism.[48] Indeed, from X-ray studies of co-crystal structures, the authors concluded to a non-covalent inhibition mechanism 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 **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 presence of **1** and was almost completely hydrolyzed in 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 non-hydrolytic 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 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 questioning. Indeed, the fact that a *β*-lactam ring is left intact in presence of a serine hydrolase is quite unusual. The experiments we performed with liver homogenates, *i. e.* in 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 towards the C2-N1 bond cleavage with the release of the four-membered cyclic strain as 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 towards the active serine Ser241 (from previous modelisation studies)[30], may undergo the nucleophilic attack ((a), figure. 4). 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 ((b), figure. 4). Due to 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, non-processing inhibition.

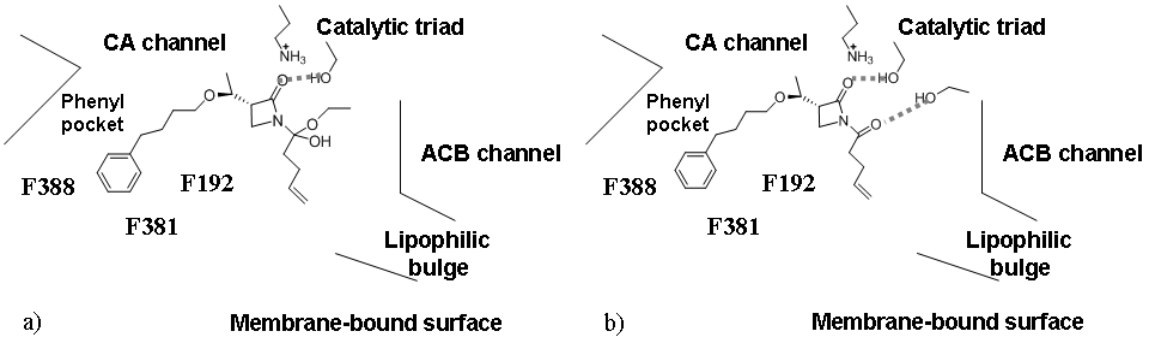


Figure 4. Schematic proposed binding mode between **9** and FAAH

V.5 Conclusion

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 behaviour of *β*-lactams **4** and **9** in the presence of *h*FAAH and other hydrolases from 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.

V.6 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 non-deuterated 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 Perkin-Elmer 241 MC polarimeter, at 20 °C, in $CHCl_3$, except for

the 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 ionisation (ESI). High Resolution Mass Spectrometry (HRMS) analyses were performed at the University College London (UK). Infrared (IR) spectra were recorded using 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 chiralpak IA column (4.6 mm x 250 mm, 5 µm particle size) using hexane/isopropanol eluant (95:05), at a flow rate of 1.0 mL/min and on symmetry C18 column (4.6 mm x 250 mm, 5µm particle size) using a gradient of acetonitrile/H_2O eluant (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 sulphate (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, and the inorganic precipitate was filtered off, washed with tetrahydrofuran and the filtrate was concentrated under vacuum. After purification by flash chromatography (cyclohexane/ethyl acetate), a colourless 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 $((M + Na)^+)$. ^1H 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 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 colourless 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 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 colourless 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 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 colourless 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: $C_{23}H_{29}NO_3Na$: 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 colourless 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 colourless 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 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 HCl 3 N, dried over $MgSO_4$, filtered and concentrated under vacuum. After purification by flash chromatography (cyclohexane/ethyl acetate), a colourless 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 colourless 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 into brackets. ^{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 during 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 colourless 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 brackets. ^{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 during 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 colourless 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 into brackets. ^{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, 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 buffer only were used as 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, 0.1 % (w/v) BSA, pH 7.4, 190 µL) for 90 min at 37 °C in the presence of *h*FAAH (90µg/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) were 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 C-18 Supelguard pre-column and a Supelcosil LC-18 column (3 µM, 4 x 150mm) (Sigma-Aldrich). Chromatographic conditions (0.5mL/min) were as follow: (**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 0.5mL/min. The MS analyses were performed in the positive mode with an APCI ionisation source. The capillary and APCI vaporiser temperatures were set at 250 and 400°C, respectively.

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Supporting information available: experimental procedures and spectroscopic details of compounds **15**, **16**, **17**, **21** and **22**, discussion about the rearrangement which drove to **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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To clarify the reversible mode of action of our inhibitors, we imagined two compounds (**4b** and **4e**) which present a good leaving group on the crucial carbonyl. That property could transform these inhibitors into irreversible ones by acylating the active serine of the catalytic site, and will allow us to discriminate the covalent from the non covalent reversible mechanism. To complete this study, we prepared a series of compounds where we inserted heteroatoms in the lead structure (**19b** in chapter 4, **4** in chapter 5 and **4a** in chapter 6) to modulate logP value and the polar surface area (PSA). The modulation of these parameters brings important information to anticipate *in vivo* experiments.

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Chapter 6

VI An unprecedented reversible mode of action of β-lactams for the inhibition of human Fatty Acid Amide Hydrolase (hFAAH)

An unprecedented reversible mode of action of β-lactams for the inhibition of human Fatty Acid Amide Hydrolase (hFAAH)

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A series of compounds was prepared to clarify the reversible mechanism of β-lactamic hFAAH inhibitors on the one hand, and to modulate some of their physicochemical parameters on the other hand. In particuliar, two compounds (**4b** and **4e**) were designed for presenting a potential good leaving group on the crucial carbonyl with a view to possibly acylating the active serine of the hFAAH catalytic triad. Reversibility studies showed that these two compounds retain the reversible mode of inhibition, suggesting a non covalent interaction between our β-lactams and hFAAH. Finally, pharmacological evaluations of bioisosteres of our lead compound (**4a**, IC_{50} = 5.3 nM) revealed that logP values and PSA could be optimized without altering the FAAH inhibition (IC_{50} values from 3.65 to 70.9 nM).

Adapted from a submitted article, in journal of medicinal chemistry, **2012**

VI.1 Introduction

Since a decade, FAAH inhibitors development is one of the most extensive research fields in the world of endocannabinoids.[1-6] Different kinds of electrophilic functions have been broadly studied in view of designing pharmacophores such as α-keto-oxazole (**1**, Chart 1)[7], carbamate (**2**, Chart 1)[8] and urea (**3**, Chart 1)[9]. Structure activity relationship (SAR) studies performed on a large number of published lead compounds allowed improvements in activity and selectivity of FAAH inhibitors. Moreover, *in vitro* and/or *in vivo* assays confirmed their potential therapeutic interest. FAAH inhibitors were demonstrated to increase the level of anandamide, an endogenous ligand of cannabinoid receptors CB_1 and CB_2, by blocking its FAAH-catalyzed degradation.[8,10-13] Rising the amount of anandamide leads to prolonged beneficial physiological effects due to activation of the cannabinoid receptors. In contrast, it was demonstrated that the cannabinoid receptors activation by exogenous ligand, *e.g.* cannabinoids like Δ^9-THC (tetrahydrocannabinol) or synthetic agonists, maximizes the side effects arising from central nervous system disturbance.[14,15] This is why, taking advantage of the endocannabinoid system, FAAH inhibitors are presently in development for treating inflammation[16,17] and pain[18-20], sleep disorders[21] and CNS diseases[22]. Recently, some clinical trials were achieved with the best representatives of some families, notably PF-04457845 (**3**).[23] This compound is involved in a phase II study aimed to collect pharmacodynamic, pharmacokinetic and toxicity data, on the one hand and to evaluate its analgesic efficiency in knee osteoarthritis and its effects on sleep, on the other hand.

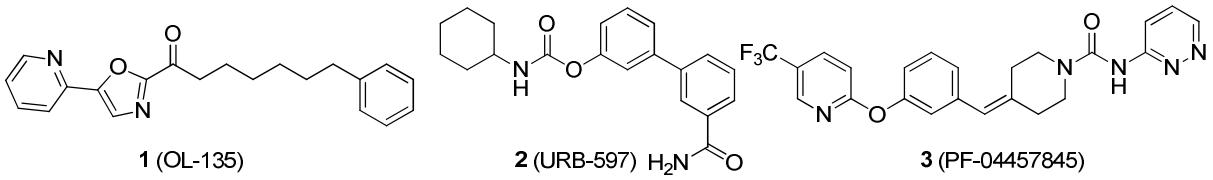


Chart 1. Most studied FAAH inhibitors

Our recent results prompt us to continue investigations about β-lactamic derivatives for FAAH inhibition. Indeed, we have described a new class of inhibitors based on the azetidin-2-one (*i.e.* β-lactam) template and disclosed the lead compound **4a** (*i.e.* 1-(pent-4-enoyl)-3(S)-[1(R)-(4-phenylbutanoyloxy)-ethyl]-azetidin-2-one, Chart 2) which exhibits a nanomolar activity, and an unusual competitive, fully reversible mode of action.[24] From SAR studies and LC/HRMS analyses, we could establish that the exocyclic carbonyl of the imide function is essential for the inhibition, but not the β-lactam carbonyl, and we could exclude that the

reversibility of inhibition results from the processing of our inhibitors by FAAH.[25] Thus, β-lactamic inhibitors such as **4a** are not slow substrates of FAAH; they are remaining unchanged in the test solutions. To further explore the reversible mode of inhibition, we designed new compounds based on bioisosteric modifications (**4b**-**4e**, Chart 2). With the aim of modulating the logP and polar surface area (PSA) values, two parameters related to solubility and permeability, we synthesized a small library of analogues of **4a**. For that purpose, heteroatoms were inserted in our lead structure **4a** (Chart 2). Because of the great importance of the exocyclic carbonyl of the imide function, carbamate (**4b**), urea (**4c**) and thiourea (**4d**) functions were investigated. These modifications may cause several effects considering lipophilicity, conformational arrangement and the potential occurrence of hydrogen bonds. In addition, they also may change the chemical reactivity and therefore the mode of inhibition. Indeed, compound **4b** for instance, presents a potential leaving group on the exocyclic carbonyl. In the case of a nucleophilic attack of the active serine onto the exocyclic carbonyl, this feature could drive to the formation of a stable acyl-enzyme intermediate, by releasing the allylic alcohol moiety.

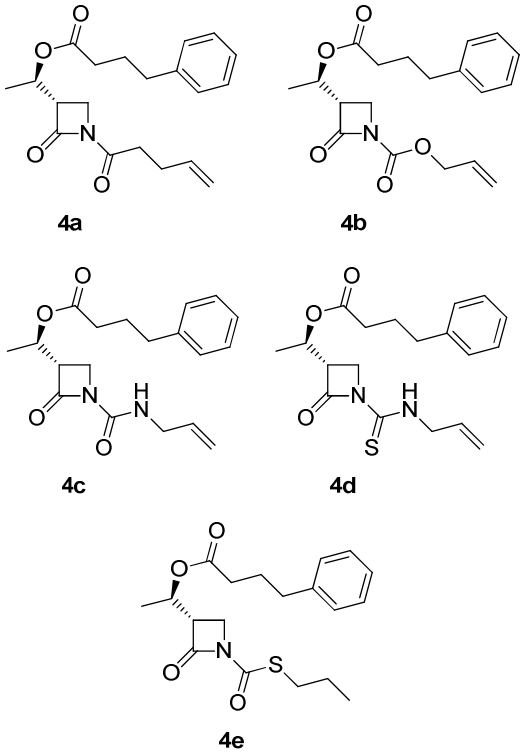


Chart 2. Bioisosteric structures of the imide function

Thus, we will be able to conclude whether our inhibitors are covalently bind to FAAH via the exocyclic carbonyl – in the case of an irreversible inhibition – or not – in the case of a still reversible inhibition. To further develop this idea of possibly transforming a reversible

inhibitor into an irreversible one, an additional compound was designed (**4e**, Chart 2) which presents a better leaving group than **4b** and can be easily prepared from a commercial reagent (namely propyl chlorothioformate).

Finally, to complete the SAR study, the ester side-chain was also modified by introducing heteroatoms at various positions (**5-7**, Chart 3).

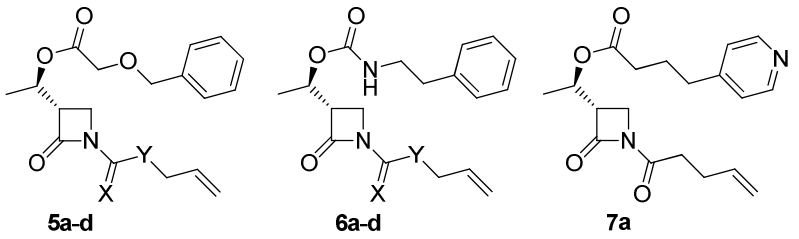
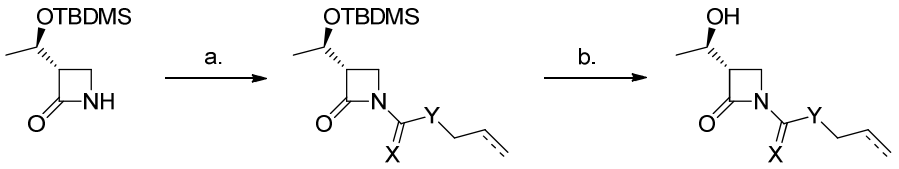


Chart 3. Bioisosteric structures of the ester chain.

The effects of all these structural modifications on FAAH inhibition were evaluated *in vitro,* in a competitive hydrolytic assay on human FAAH, and in reversibility assays to evaluate not only the activity but also the mode of inhibition. The selectivity for FAAH versus MAGL was also examined.

VI.2 Chemistry

Compounds synthesis started with precursor **8**, described in our previous publication, which is obtained in two steps from commercially available (3*R*,4*R*)-4-acetoxy-3-[(*R*)-1-(tert-butyldimethylsilyloxy)ethyl]azetidin-2-one. We established a three-step sequence to prepare our library: i) *N*-functionalization, ii) deprotection of silyl ether and iii) *O*-functionalization. First of all, compounds **9a-e** were obtained by a *N*-acylation step adapted to the nature of each chain (Scheme 1). Compound **9a** was prepared using 4-pentenoyl chloride in the presence of pyridine in refluxing DCM (80 %) while **9b-e** were obtained by reaction with allyl chloroformate (**9b**, 82 %), allyl isocyanate (**9c**, 57 %), allyl isothiocyanate (**9d**, 79 %) and propyl chlorothioformate (**9e**, 49 %), after deprotonation of **8** in the presence of LiHMDS in THF at -78 °C. Then, silyl ether protection was removed in acidic conditions leading to compounds **10a-e** in moderate to good yields (45 to 89 %, Scheme 1).



8

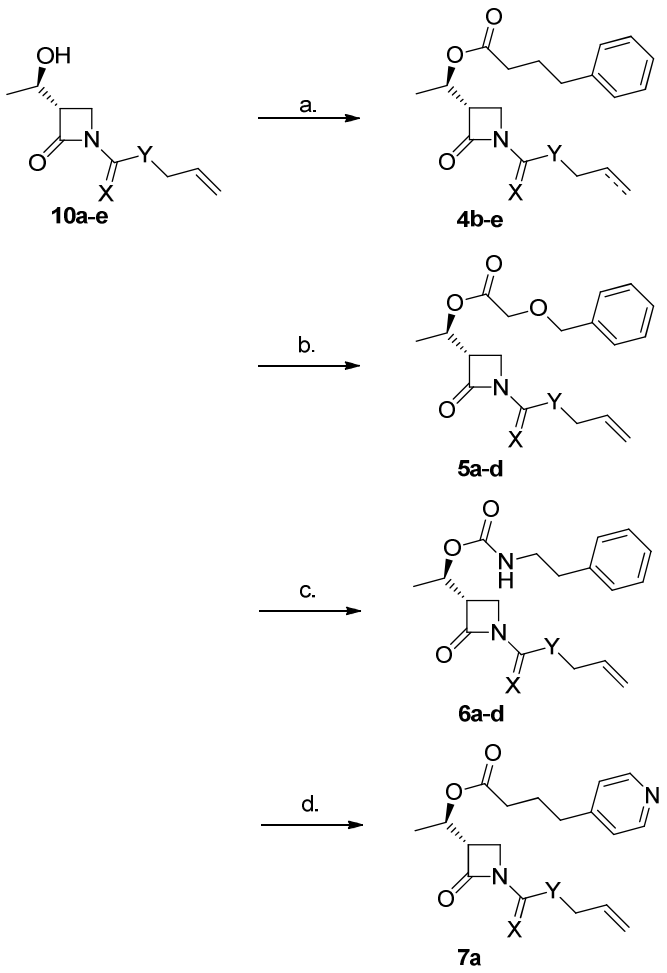
9a, X= O, Y= CH_2
b, X= O, Y= O
c, X= O, Y= NH
d, X= S, Y= NH
e, X=O, Y= S

10a-e

Scheme 1.

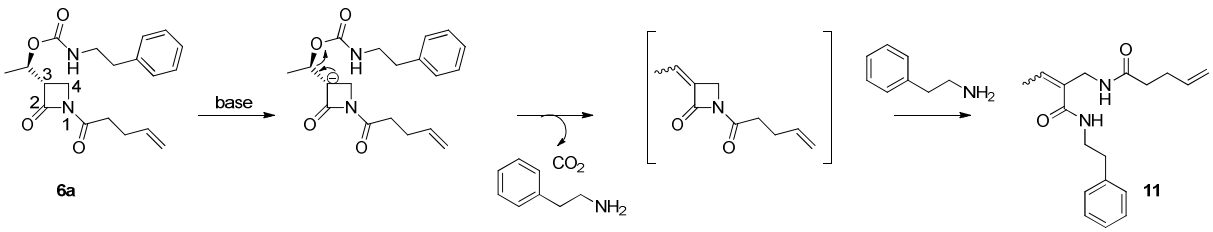
Reagents and conditions: (a) 4-pentenoyl chloride, pyridine, DCM, 45 °C, 24 h or allyl chloroformate, allyl isocyanate, allyl isothiocyanate or propyl chlorothioformate, LiHMDS, THF, -78 °C, 4 h; (b) HCl, AcOH, ACN, 0 °C, 3 h.

The resulting alcohols were engaged in the last step to give the final compounds (Scheme 2). On the one hand, each alcohol (**10a-e**) was esterified by one or two usual methods of esterification: i) **4b** (63 %), **4c** (88 %), **4d** (89 %) and **4e** (76 %) were synthesized in the presence of pyridine with 4-phenylbutanoyl chloride at room temperature; ii) **5a-d** and **7a** were obtained by using 2-(benzyloxy)acetic acid or 4-(pyridine-4-yl)butanoic acid in the presence of dicyclohexylcarbodiimide (DCC) and dimethylaminopyridine (DMAP) in good yields (**5a** (89 %), **5b** (58 %), **5c** (93 %), **5d** (74 %), **7a** (64 %), Scheme 2). On the other hand, compounds **10a-d** were functionalized with phenethyl isocyanate using very mild conditions. Indeed, the conversion into the corresponding alkoxide was not possible and any basic conditions could not be used because of the acidity of H-3 proton. This property drove to a rearrangement which gave *in fine* compound **11** (Scheme 3) by an elimination step followed by azetidinone ring opening. Thus, the carbamate bond was created in the presence of $Ti(OtBu)_4$ as a lewis acid which catalyzed the nucleophilic attack of the hydroxyl function upon the isocyanate carbonyl function.[26] By this method, compounds were obtained with good yields, in one hour at room temperature (**6a** (81 %), **6b** (64 %), **6c** (70 %), **6d** (84 %), Scheme 2).



Scheme 2.

Reagents and conditions: (a) 4-phenylbutanoyl chloride, pyridine, DCM, rt, 15 h; (b) 2-(benzyloxy)acetic acid, DCC, DMAP, DCM, rt, 15 h; (c) phenethylisocyanate, $Ti(OtBu)_4$, DCM, rt, 1h, (d) 4-(pyridin-4-yl)butanoic acid, DCC, DMAP, DCM, rt, 15 h.



Scheme 3. proposed mechanism for the formation of **11** (see supporting information).

VI.3 Pharmacology

The library of compounds was tested for FAAH inhibition using human recombinant FAAH as source of enzyme. Some of them were also evaluated as potential MAGL inhibitors to check the selectivity and to study the effect of the exocyclic C=S function of compounds **4-6d**. Indeed, MAGL is a serine hydrolase involving three cysteine residues in its active site. According to the HSAB (hard and soft acids and bases) theory, a privileged interaction could occur between the C=S (electrophile) and SH (nucleophile) functions. In addition, the inhibition mode was investigated in the case of two compounds (**4b** and **4e**, Chart 2) which present a potential good leaving group. All the collected results were analyzed taking the relative logP and PSA calculated values in consideration (Table 1).

VI.3.1 hFAAH inhibition

Human recombinant enzyme, developed in our laboratory,[27] was used in a competitive hydrolytic assay using [^{3}H]-AEA as 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 [^{3}H]-ethanolamine resulting from the hydrolysis of labelled AEA. Considering the results summarized in table 1, it appeared clearly that the insertion of a sulphur atom (thiocarbonyl) replacing the exocyclic oxygen atom of the imide function, leads to a loss of activity compared to the corresponding bioisosteres (**4d** compared to **4a-c**, **5d** to **5a-c** and **6d** to **6a-c**, IC_{50} = 145.8, 1042 and 959 nM, respectively). This structural moiety suspected to interact with the active serine, is thus less active as a thiocarbonyl function. Comparatively to our previous hit (**4a**, IC_{50}=5.32 nM, Table 1 and Chart 2), the other bioisosters were also active in the nanomolar range. They appeared to be good to excellent inhibitors of FAAH, from 70.9 nM (**5c**) to 3.65 nM (**4b**), except **7a**, which exhibited a moderate activity (IC_{50} = 310.1 nM).

Insertion of heteroatoms results in fluctuations of the logP values: increased ones with sulphur atoms (notably **4d** and **4e**, 3.47 and 4.11, respectively) and equalled (**4b**, 3.31) or lowered ones with nitrogen or oxygen atoms (from 2.65 to 1.41, **6d** and **5c**, respectively). We could not really correlate inhibitory potency and logP values but we could observe that fluctuations in the lipophilicity of some compounds did not really alter their activity compared to **4a**: for instance, **4c** presents a similar activity with a lower logP value (IC_{50} = 5.32 and 5.56 nM, and logP = 3.25 and 2.59, respectively); **5a-b** and **6a-c** differ by a logP decrease of one

logarithmic unit at least and retain excellent activities (*e.g.* **6c**, IC_{50} = 28.6 nM and logP = 1.76) and finally, compound **5c** displays the lowest logP value and a good activity (IC_{50} = 70.9 nM and logP = 1.41). Inversely to logP values, PSA tend to increase with heteroatoms insertion. But, alike logP fluctuations, no direct correlation could be done. We observed good to excellent activities for different modulations of PSA.

Table 1. determination of inhibitory potential on hFAAH and hMAGL, logP and PSA parameters.

compound	logP[a]	PSA[a,b]	pI50 (hFAAH)	IC_{50}[c] on hFAAH	IC_{50}[c] on hMAGL
4a	3.25	76.57	-8.27 ± 0.05	5.32	4060
4b	3.31	72.91	-8.44 ± 0.03	3.65	10300
4c	2.59	75.71	-8.26 ± 0.03	5.56	98410
4d	3.47	90.73	-6.84 ± 0.03	145.8	60% inh at 10^{-3} M
4e	4.11	88.98	-7.92 ± 0.07	12.1	18960
5a	2.08	72.91	-7.51 ± 0.02	30.9	nd
5b	2.14	82.14	-7.52 ± 0.06	30.1	nd
5c	1.41	84.94	-7.15 ± 0.02	70.9	56850
5d	2.30	99.96	-5.98 ± 0.02	1042	121700
6a	2.43	75.71	-7.48 ± 0.03	33.2	nd
6b	2.49	84.94	-7.92 ± 0.02	12.1	nd
6c	1.76	87.74	-7.54 ± 0.02	28.6	nd
6d	2.65	102.76	-6.02 ± 0.05	959	25% inh at 10^{-3} M
7a	2.03	76.57	-6.51 ± 0.02	310.1	nd

[a] calculated via Marvinsketch.

[b] calculated by the atom-based method (topological PSA or TPSA) of Ertl, Rohde and Seltzer.[28] It consists in the summation of tabulated values corresponding to commonly used polar fragments (N and O) and slightly less one (S).

[c] in nM, from three independent experiments.

VI.3.2 hMAGL inhibition

Human recombinant enzyme, developed in our laboratory,[29] was used in a competitive hydrolytic assay using [^{3}H]-2-OG as substrate. Tested compounds, enzyme and [^{3}H]-2-OG were preincubated at room temperature during 30 min and incubated at 37 °C during 10 min. The extent of inhibition was evaluated by liquid scintillation counting of [^{3}H]-glycerol resulting from the hydrolysis of labelled 2-OG. All the tested compounds presented a very low activity, both those with a thiocarbonyl function (**4-6d**) and the other ones (**4b**, **4c** and **5c**). IC_{50} values ranged from 10.3 µM (**4b**) to 121.7 µM (**5d**) and compound **4d** and **6d**

inhibited 60 % and 25 % of MAGL at 10^{-3} M, respectively. Comparatively to the lead compound (**4a**), there is a high selectivity for the inhibition of FAAH: for instance, compounds **4b** and **4c** are respectively 2800 and 18000 times more potent against FAAH.

VI.3.3 Mode of inhibition

To address the reversibility question, we selected two inhibitors featuring good leaving-groups near the exocyclic carbonyl (**4b** and **4e**, Chart 2). Wash-out experiments were undertaken to measure the recovery of enzyme activity after a rapid and large dilution of the inhibitor-enzyme mixtures. Results were collected after 0, 30 and 90 min.

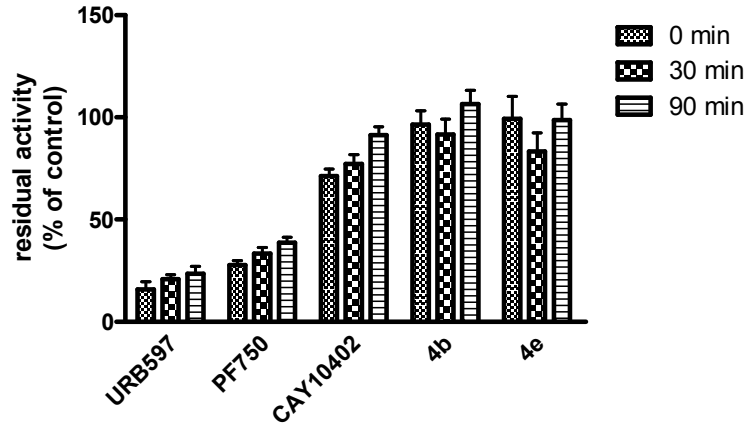


Figure 1. Test of reversibility: influence of a rapid and large dilution on the recovery of hFAAH activity (studies after 0, 30 and 90 min following the rapid and large dilution).

The enzyme activity should be almost totally recovered if the inhibitor is reversible, but the enzyme should remain largely inhibited in the case of an irreversible inhibition. Here, the rapid and large dilution led to the recovery of activity for **4b** and **4e**, similarly to 1-oxazolo[4,5-b]pyridin-2-yl-6-phenyl-1-hexanone (CAY10402)[30]**,** an analog of **1** (Chart 1). As a further control we used two known irreversible FAAH inhibitors, URB-597[31] (**2**, Chart 1) and PF-750[32] (*N*-phenyl-4-(quinolin-2-ylmethyl)piperidine-1-carboxamide) an analogue of **3** (Chart 1); we found that the enzyme activity is still largely inhibited after the dilution experiment (Figure 1).

VI.4 Discussion and conclusion

The competitive, reversible inhibition of our inhibitors appears unique in the β-lactam literature, to the best of our knowledge. Whereas β-lactams are widely known to inhibit traditional serine hydrolases (*i.e.* serine enzymes featuring the Ser-Asp-His catalytic triad)[33] in an irreversible manner (*DD*-peptidases[34], β-lactamases[35,36], elastases[37-41]), a reversible mechanism occurs between our compounds and hFAAH of which the catalytic pocket involves the unusual Ser-Ser-Lys catalytic triad.[42] We have firmly established that our β-lactams are not processed by the enzyme[25] and that, after rapid and large dilution of enzyme-inhibitor mixtures, the enzyme recovers its full activity.

From our previous studies, we know that the key moiety for interaction with hFAAH is the exocyclic carbonyl group of the imide function.[25] As a matter of fact, the replacement of this carbonyl by a thiocarbonyl led to an important decrease of the inhibition activity (compounds **4d**, **5d** and **6d**). As shown in Figure 2, the reversible inhibition of hFAAH could result from the formation of a high affinity complex between the enzyme and the β-lactamic inhibitor (step 1), eventually leading to a covalent tetrahedral intermediate by nucleophilic attack of the active serine on the C=X bond of the inhibitor (step 2). In the absence of good leaving-group on this intermediate (Y is CH_2), the step 2 could be reversible. Such modes of action (*i.e.* steps 1 and 2) have been recently demonstrated with co-crystal structures: reversible covalent interaction with OL-135 (**1**) and other α-keto-oxazoles[19,43,44] and reversible non covalent interaction with ketobenzimidazoles[45,46]. We speculated that the presence of a potential leaving group on the tetrahedral intermediate (Y is a heteroatom), could draw the reaction towards the acyl-enzyme intermediate (step 3). Depending on the stability of this intermediate versus hydrolysis, the resulting inhibition could be irreversible or slowly reversible, when the inhibitor behaves as a bad substrate.

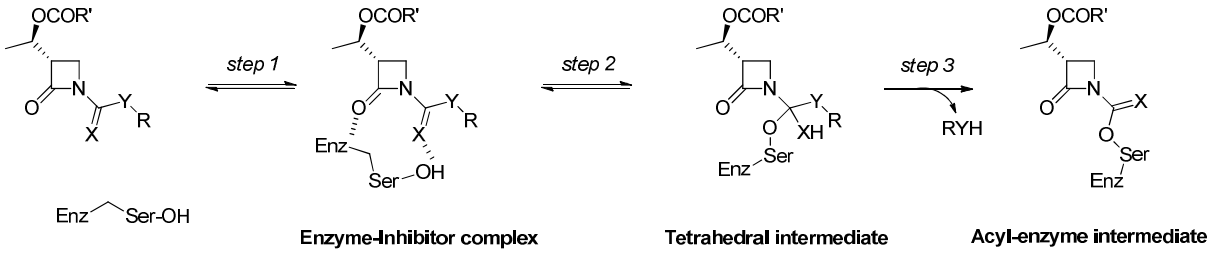


Figure 2. Possible mechanisms of inhibition (X= O, S; Y= CH_2, NH, O, S).

Irreversible inhibition of hFAAH, due to the formation of a stable acyl-enzyme intermediate, is the recognized mode of action of URB-597 (**2**)[47,48], PF-04457845 (**3**)[12,32,49] and related

carbamates and ureas. Independently of the presence of a potential leaving group in their structures, our β-lactamic inhibitors act systematically as reversible inhibitors. Our results strongly suggest a similar mode of action as ketobenzimidazoles[45] or benzothiazole[50], *i.e.* an inhibition due only to the high affinity of our β-lactams with aminoacid residues of the active site (step 1). Since we showed the importance of the imide function in our previous study, strong hydrogen bonds between both endo and exo carbonyls and the catalytic triad could be admitted. Moreover, we observed a decrease of activity when the phenyl ring of the ester chain is replaced by a pyridine ring (**7a**). This observation suggests a loss of affinity in a hydrophobic cavity where pyridine, probably protonated, does not stabilize the interaction. All the tested compounds were highly selective for the hFAAH inhibition versus hMAGL. The hypothesis of a favorable interaction between the thiocarbonyl group of β-lactams **4d**, **5d** and **6d** and cystein residues of the hMAGL catalytic pocket, was not verified.

Finally, we could not correlate LogP values, PSA and inhibition potency. Modification of these physicochemical parameters did not seem to influence greatly the inhibition of FAAH. However, we showed that it was possible to improve physicochemical parameters, *i.e.* solubility and permeability factors, without altering the inhibition of hFAAH. Indeed, the best representatives of this study conserved a nanomolar activity against hFAAH with lower LogP value and PSA. This information is important for candidates selection in view of *in vivo* experiments with β-lactamic hFAAH inhibitors.

VI.5 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 purifications. [^{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 Reasonance (^{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. Chemical shifts are reported in ppm relative to the signals of residual non-deuterated solvents ($CDCl_3$ 7.26 and 77.16 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 Atago Ap-100 polarimeter, at 25 °C, in $CHCl_3$. 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 ionisation (ESI). High Resolution Mass Spectrometry (HRMS) analyses were performed using a QExactive (Thermo Scientific). Infrared (IR) spectra were recorded using FTIR-8400S Shimadzu apparatus. Products were analyzed as thin films deposited on a Se-Zn crystal by evaporation of CH_2Cl_2 solutions. TLC analysis was performed on Merck silicagel $60F_{254}$ 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 chiralpak IA column (4.6 mm x 250 mm, 5 µm particle size) using hexane/isopropanol eluant (95:5), at a flow rate of 1.0 mL/min and on symmetry C18 column (4.6 mm x 250 mm, 5µm particle size) using a gradient of acetonitrile/H_2O eluant (50:50 to 100:0), at a flow rate of 1.2 mL/min (purity ≥ 97 %).

General procedure for coupling chloro(thio)formate with amide function (9b and 9e): To a stirred solution of azetidinone (1 eq.) **8** in dry THF (7 mL/mmol) cooled at -78 °C, was dropwise added a solution of LiHMDS (1.1 eq.) in dry THF (1 M), under argon atmosphere. The mixture was stirred for 30 min at -78 °C and the chloro(thio)formate was (1.1 eq.) was added. After 1 h at -78 °C, the solution was allowed to warm up to r.t. and additionally stirred for 1 h. The reaction was quenched, at low temperature, with brine and diluted with DCM. The aqueous layers were several extracted with DCM. The organic layers were combined, dried over MgSO4 filtered and concentrated under vacuum. After purification by flash chromatography (chex/AcOEt), colourless oils were obtained (**9b** and **9e**).

1-(Propyl-3-enoxy)carbonyl-(3S)-3-[(1R)-(*tert*-butyldimethylsilyloxy)ethyl]azetidin-2-one (9b): Purification by flash chromatography (chex/AcOEt 5:2) gave **9b** (112 mg, 82%) as a colourless oil: *R*f =0.54 (chex/AcOEt, 5:3); ^{1}H NMR (500MHz, $CDCl_3$): δ=0.02 (s, 3H), 0.04 (s, 3H), 0.81 (s, 9H), 1.15 (d, J =6.3 Hz, 3H), 3.20 (m, 1H), 3.55 (m, 1H), 3.70 (dd, J =3.5 Hz, J =6.5 Hz, 1H), 4.26 (m, 1H), 4.60-4.76 (m, 2H), 5.20-5.38 (m, 2H), 5.84-5.96 ppm (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$): δ=-5.1, -4.2, 14.2, 17.9, 22.3, 25.7, 39.7, 57.2, 64.8, 66.9, 119.1, 131.5, 149.1, 165.8 ppm; IR: υ =2852-2976, 1805, 1730, 1375, 1325, 1277, 1259, 839 cm^{-1}; MS (ESI): m/z (%): 314.09 (16) $[M+H]^+$, 336.13 (41) $[M+Na]^+$, 648.93 (100) $[2M+Na]^+$; HRMS-ESI: m/z $[M+H]^+$ calcd for $C_{15}H_{28}NO_4Si$: 314.17821, found: 314.17859.

1-(Propyl-3-sulfanyl)carbonyl-(3S)-3-[(1R)-(*tert*-butyldimethylsilyloxy)ethyl]azetidin-2-one (9e): Purification by flash chromatography (chex/AcOEt 5:1) gave **9e** (71 mg, 49%) as a

colourless oil: *R*f =0.63 (chex/AcOEt, 5:2); ^{1}H NMR (500MHz, $CDCl_3$): δ=0.04 (s, 3H), 0.05 (s, 3H), 0.82 (s, 9H), 0.97 (t, *J* =7.4 Hz, 3H), 1.17 (d, *J* =6.3 Hz, 3H), 1.65 (m, 2H), 2.85-2.99 (m, 2H), 3.23 (m, 1H), 3.61 (m, 1H), 3.75 (dd, *J* =3.5 Hz, *J* =6.8 Hz, 1H), 4.26-4.33 ppm (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$): δ=-5.1, -4.2, 13.3, 17.9, 22.3, 23.0, 25.7, 31.1, 40.0, 56.7, 64.7, 165.5, 165.7 ppm; IR: υ =2856-2949, 1786, 1668, 1306, 1252, 1136, 1076, 1018, 839 cm^{-1}; MS (ESI): m/z (%): 332.17 (60) $[M+H]^+$, 354.15 (89) $[M+Na]^+$, 685.32 (100) $[2M+Na]^+$; HRMS-ESI: m/z $[M+H]^+$ calcd for $C_{15}H_{30}NO_3SSi$: 332.17102, found: 332.17127.

General procedure for coupling iso(thio)cyanate with amide function (9c and 9d): To a stirred solution of azetidinone (1 eq.) **8** in dry THF (7 mL/mmol) cooled at -78 °C, was dropwise added a solution of LiHMDS (1.1 eq.) in dry THF (1 M), under argon atmosphere. The mixture was stirred for 30 min at -78 °C and the iso(thio)cyanate was (1.1 eq.) was added. After 1 h at -78 °C, the solution was allowed to warm up to r.t. and additionally stirred for 1 h. The reaction was quenched, at low temperature, with brine and diluted with DCM. The aqueous layers were several extracted with DCM. The organic layers were combined, dried over MgSO4 filtered and concentrated under vacuum. After purification by flash chromatography (chex/AcOEt), colourless oils were obtained (**9c** and **9d**).

1-(Propyl-3-enamino)carbonyl-(3S)-3-[(1R)-(*tert*-butyldimethylsilyloxy)ethyl]azetidin-2-one (9c): Purification by flash chromatography (DCM/AcOEt 98:2) gave **9c** (78 mg, 57%) as a colourless oil: *R*f =0.42 (chex/AcOEt, 5:3); ^{1}H NMR (500MHz, $CDCl_3$): δ=-0.04 (s, 3H), -0.02 (s, 3H), 0.75 (s, 9H), 1.10 (d, *J* =6.3 Hz, 3H), 3.17 (m, 1H), 3.51 (m, 1H), 3.63 (dd, *J* =3.1 Hz, *J* =6.3 Hz, 1H), 3.74-3.80 (m, 1H, AB system), 3.82-3.89 (m, 1H, AB system), 4.20 (m, 1H), 5.00-5.13 (m, 2H), 5.74 (m, 1H), 6.52 ppm (br t, *J* =5.7 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$): δ=-5.3, -4.3, 17.3, 22.1, 25.5, 38.9, 41.9, 56.5, 64.5, 116.0, 133.9, 150.4, 168.2 ppm; IR: υ =2856-2957, 1765, 1699, 1531, 1464, 1337, 1259, 1140, 1076, 839 cm^{-1}; MS (ESI): m/z (%): 313.19 (100) $[M+H]^+$, 625.38 (38) $[2M+H]^+$; HRMS-ESI: m/z $[M+H]^+$ calcd for $C_{15}H_{29}N_2O_3Si$: 313.19420, found: 313.19431.

1-(Propyl-3-enamino)thiocarbonyl-(3S)-3-[(1R)-(*tert*-butyldimethylsilyloxy)ethyl]-azetidin-2-one (9d): Purification by flash chromatography (chex/AcOEt 5:1) gave **9d** (113 mg, 79%) as a colourless oil: *R*f =0.69 (chex/AcOEt, 5:3); ^{1}H NMR (500MHz, $CDCl_3$): δ=0.01 (s, 3H), 0.03 (s, 3H), 0.79 (s, 9H), 1.17 (d, *J* =6.3 Hz, 3H), 3.17 (m, 1H), 3.72 (dd, *J* =6.9 Hz, *J* =6.0 Hz 1H), 3.84 (dd, *J* =3.2 Hz, *J* =6.9 Hz, 1H), 4.13-4.21 (m, 1H), 4.23-4.34 (m, 2H), 5.13-5.24 (m, 2H), 5.83 (m, 1H), 8.41 ppm (br s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$): δ=-5.2, -4.1, 17.8, 22.2, 25.6, 42.3, 46.9, 55.1, 64.7, 117.4, 132.2, 166.5, 178.3 ppm; IR: υ = 2858-2953, 1755, 1533, 1342, 1323, 1254, 1140, 1074, 1020, 839 cm^{-1}; MS (ESI): m/z (%):

329.17 (100) $[M+H]^+$, 351.15 (80) $[M+Na]^+$; HRMS-ESI: m/z $[M+H]^+$ calcd for $C_{15}H_{29}N_2O_2SSi$: 329.17135, found: 329.17153.

General procedure for silyl ether deprotection (10a-e): To a stirred suspension of silyl ether (1 eq.) in acetonitrile (30 mL/mmol) at -5 °C was added dropwise 12 N HCl (5eq.) and 17 N AcOH (7 eq.). The mixture was stirred for 30 min at -5 °C, and for 3 h at 0 °C. Acetonitrile was removed under vacuum, and the oily residue was diluted in ethyl acetate. The organic layer was washed with 10 % $NaHCO_3$ and brine, dried over $MgSO_4$, filtered and concentrated under vacuum. After purification by flash chromatography (ethyl acetate 100 %) a white solid (**10c**) or colourless oils (**10b, d-e**) were obtained.

1-(Propyl-3-enoxy)carbonyl-(3S)-3-[(1R)-hydroxyethyl]azetidin-2-one (10b): Purification by flash chromatography (AcOEt 100%) gave **10b** (241 mg, 76%) as a colourless oil: *R*f =0.57 (AcOEt 100%); ^{1}H NMR (500MHz, $CDCl_3$): δ=1.24 (d, J =6.4 Hz, 3H), 2.85 (br s, 1H), 3.22-3.31 (m, 1H), 3.60-3.71 (m, 2H), 4.22 (m, 1H), 4.67 (m, 2H), 5.20-5.41 (m, 2H), 5.91 (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$): δ=21.5, 40.6, 56.8, 64.6, 67.1, 119.3, 131.3, 149.0, 165.8; IR: υ =3447, 2912-2988, 1790, 1732, 1379, 1319, 1252, 1128, 1047 cm^{-1}; MS (ESI): m/z (%): 199.98 (2) $[M+H]^+$, 222.10 (16) $[M+Na]^+$, 420.81 (100) $[2M+Na]^+$; HRMS-CI: m/z $[M+H]^+$ calcd for $C_9H_{14}NO_4$: 200.09288, found: 200.09274.

1-(Propyl-3-enamino)carbonyl-(3S)-3-[(1R)-hydroxyethyl]azetidin-2-one (10c): Purification by flash chromatography (AcOEt 100%) gave **10c** (24 mg, 47%) as a colourless oil: *R*f =0.29 (AcOEt 100%); ^{1}H NMR (500MHz, $CDCl_3$): δ=1.27 (d, J =6.4 Hz, 3H), 2.73 (br s, 1H), 3.28 (m, 1H), 3.65 (m, 2H), 3.82-3.92 (m, 2H), 4.20 (m, 1H), 5.10-5.21 (m, 2H), 5.81 (m, 1H), 6.58 ppm (br s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$): δ=21.7, 40.0, 42.2, 56.3, 64.7, 116.6, 133.8, 150.6, 168.2 ppm; IR: υ = 3354, 2926-2961, 1755, 1678, 1528, 1331, 1286, 1265, 1138 cm^{-1}; MS (ESI): m/z (%): 199.11 (34) $[M+H]^+$, 221.09 (100) $[M+Na]^+$; HRMS-ESI: m/z $[M+H]^+$ calcd for $C_9H_{15}N_2O_3$: 199.10772, found: 199.10769.

1-(Propyl-3-enamino)thiocarbonyl-(3S)-3-[(1R)-hydroxyethyl]azetidin-2-one (10d): Purification by flash chromatography (AcOEt 100%) gave **10d** (66 mg, 89%) as a white solid: *R*f =0.26 (chex/AcOEt 5:3); ^{1}H NMR (500MHz, $CDCl_3$): δ=1.30 (d, J =6.4 Hz, 3H), 2.10 (br s, 1H), 3.25 (m, 1H), 3.82 (m, 2H), 4.20-4.32 (m, 3H), 5.18-5.29 (m, 2H), 5.89 (m, 1H), 8.43 ppm (br s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$): δ=21.7, 43.1, 47.2, 54.5, 64.8, 117.9, 132.1, 166.1, 178.2 ppm; IR: υ =2934, 1749, 1539, 1340, 1238, 1134 cm^{-1}; MS (ESI): m/z (%): 215.09 (25) $[M+H]^+$, 237.07 (100) $[M+Na]^+$; HRMS-ESI: m/z $[M+H]^+$ calcd for $C_9H_{15}N_2O_2S$: 215.08487, found: 215.08501.

1-(Propyl-3-sulfanyl)carbonyl-(3S)-3-[(1R)-hydroxyethyl]azetidin-2-one (10e): Purification by flash chromatography (AcOEt 100%) gave **10e** (21 mg, 45%) as a colourless oil: *R*f =0.21 (chex/AcOEt 5:3); ^{1}H NMR (500MHz, $CDCl_3$): δ=0.98 (t, *J* =7.4 Hz, 3H), 1.29 (d, *J* =6.4 Hz, 3H), 1.66 (m, 2H), 2.02 (br s, 1H), 2.94 (m, 2H), 3.30 (m, 1H), 3.68-3.73 (m, 2H), 4.26 ppm (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$): δ=13.4, 21.7, 23.0, 31.2, 40.9, 56.2, 64.8, 165.2, 165.8 ppm; IR: υ =2959, 1780, 1664, 1306, 1238, 1130 cm^{-1}; MS (ESI): m/z (%): 218.08 (12) $[M+H]^+$, 240.06 (100) $[M+Na]^+$, 457.14 (75) $[2M+Na]^+$; HRMS-ESI: m/z $[M+H]^+$ calcd for $C_9H_{16}NO_3S$: 218.08454, found: 218.08466.

General procedure for esterification with acyl chloride (4b-e): To a stirred solution of alcohol precursor (1 eq.) in dry dichloromethane (20 mL/mmol), at 20 °C, were added pyridine (2 eq.) and 4-phenylbutanoyl chloride (2 eq.) under argon atmosphere. After stirring overnight, the mixture was diluted in DCM and the excess of acyl chloride was quenched by 10 % aqueous Na_2CO_3. The organic layer was washed with 3 N HCl and brine, dried over $MgSO_4$, filtered and concentrated under vacuum. After purification by flash chromatography (DCM/EtOAc), a colourless oil was obtained in all cases.

1-(Propyl-3-enoxy)carbonyl-(3S)-3-[(1R)-(4-phenylbutanoyloxy)ethyl]azetidin-2-one (4b): Purification by flash chromatography (DCM/AcOEt 98:2) gave **4b** (44 mg, 63%) as a colourless oil: $[\alpha]_D$ = -0.32 (c = 0.60); *R*f =0.43 (DCM/AcOEt 95:5); ^{1}H NMR (300MHz, $CDCl_3$): δ=1.34 (d, *J* =6.4 Hz, 3H), 1.92 (m, 2H), 2.31 (m, 2H), 2.62 (m, 2H), 3.40 (m, 1H), 3.59 (dd, *J* =3.6 Hz, *J* =7.0 Hz, 1H), 3.70 (m, 1H), 4.69 (d, *J* =5.7 Hz, 2H), 5.21-5.42 (m, 3H), 5.92 (m, 1H), 7.12-7.32 ppm (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$): δ=18.5, 26.5, 33.7, 35.1, 41.4, 54.5, 67.26, 67.34, 119.4, 126.1, 128.5, 128.6, 131.2, 141.3, 149.0, 163.7, 172.5 ppm; IR: υ =2912-2959, 1805, 1798, 1728, 1454, 1377, 1327, 1186, 1126 cm^{-1}; MS (ESI): m/z (%): 346.04 (3) $[M+H]^+$, 368.19 (100) $[M+Na]^+$; HRMS-ESI: m/z $[M+Na]^+$ calcd for $C_{19}H_{23}NO_5Na$: 368.1474, found: 368.1464.

1-(Propyl-3-enamino)carbonyl-(3S)-3-[(1R)-(4-phenylbutanoyloxy)ethyl]azetidin-2-one (4c): Purification by flash chromatography (DCM/AcOEt 98:2) gave **4c** (61 mg, 88%) as a colourless oil: $[\alpha]_D$ = -0.12 (c = 0.51); *R*f =0.36 (chex/AcOEt 5:3); ^{1}H NMR (500MHz, $CDCl_3$): δ=1.32 (d, *J* =6.4 Hz, 3H), 1.90 (m, 2H), 2.28 (m, 2H), 2.60 (m, 2H), 3.40 (m, 1H), 3.57 (dd, *J* =3.2 Hz, *J* =6.8 Hz, 1H), 3.69 (m, 1H), 3.86 (m, 2H), 5.08-5.20 (m, 2H), 5.26 (m, 1H), 5.71-5.88 (m, 1H), 6.51 (br t, *J* =5.6 Hz, 1H), 7.10-7.29 ppm (m, 5H); ^{13}C NMR (125 MHz, $CDCl_3$): δ=18.5, 26.6, 33.8, 35.1, 40.6, 42.2, 54.0, 67.1, 116.6, 126.2, 128.5, 128.6, 133.8, 141.2, 150.3, 166.4, 172.4 ppm; IR: υ =2868-2918, 1769, 1734, 1703, 1533, 1339,

1136 cm^{-1}; MS (ESI): m/z (%): 345.18 (7) $[M+H]^+$, 367.16 (100) $[M+Na]^+$, 711.8 (6) $[2M+Na]^+$; HRMS-ESI: m/z $[M+H]^+$ calcd for $C_{19}H_{25}N_2O_4$: 345.18088, found: 345.18124.

1-(Propyl-3-enamino)thiocarbonyl-(3S)-3-[(1R)-(4-phenylbutanoyloxy)ethyl]azetidin-2-one (4d): Purification by flash chromatography (DCM/AcOEt 99:1) gave **4d** (48 mg, 89%) as a colourless oil: $[\alpha]_D$ = 0.31 (c = 0.48); *R*f =0.56 (chex/AcOEt 5:2); ^{1}H NMR (300MHz, $CDCl_3$): δ=1.35 (d, *J* =6.4 Hz, 3H), 1.92 (m, 2H), 2.31 (m, 2H), 2.62 (m, 2H), 3.33-3.43 (m, 1H), 3.71-3.79 (m, 1H), 3.88 (m, 1H), 4.12-4.34 (m, 3H), 5.14-5.37 (m, 2H), 5.78 (m, 1H), 7.12-7.33 (m, 5H), 8.38 ppm (br s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ=18.5, 26.6, 33.7, 35.1, 43.8, 47.2, 52.3, 67.0, 117.9, 126.1, 128.5 (2C), 132.0, 141.2, 164.6, 172.4, 178.0 ppm; IR: υ =2912-2932, 1755, 1734, 1533, 1339, 1321, 1132 cm^{-1}; MS (ESI): m/z (%): 361.16 (16) $[M+H]^+$, 383.14 (100) $[M+Na]^+$, 743.29 (6) $[2M+Na]^+$; HRMS-ESI: m/z $[M+H]^+$ calcd for $C_{19}H_{25}N_2O_3S$: 345.18088, found: 345.18124.

1-(Propyl-3-sulfanyl)carbonyl-(3S)-3-[(1R)-(4-phenylbutanoyloxy)ethyl]azetidin-2-one (4e): Purification by flash chromatography (DCM/AcOEt 99:1) gave **4d** (27 mg, 76%) as a colourless oil: *R*f =0.50 (chex/AcOEt 5:2); ^{1}H NMR (300MHz, $CDCl_3$): δ=1.00 (t, *J* =7.4 Hz, 3H), 1.35 (d, *J* =6.4 Hz, 3H), 1.67 (m, 2H), 1.93 (m, 2H), 2.30 (m, 2H), 2.63 (m, 2H), 2.94 (m, 2H), 3.42 (m, 1H), 3.62 (dd, *J* =3.6 Hz, *J* =7.3 Hz, 1H), 3.75 (dd, *J* =6.5 Hz, *J* =7.2 Hz, 1H), 5.28 (m, 1H), 7.15-7.31 ppm (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$): δ=13.4, 18.5, 23.0, 26.5, 31.2, 33.7, 35.1, 41.7, 54.0, 67.3, 126.1, 128.5, 128.6, 141.3, 163.4, 165.8, 172.4 ppm; IR: υ =2925-2962, 1788, 1732, 1663, 1454, 1308, 1240, 1130 cm^{-1}; MS (ESI): m/z (%): 364.16 (4) $[M+H]^+$, 386.14 (100) $[M+Na]^+$; HRMS-ESI: m/z $[M+H]^+$ calcd for $C_{19}H_{26}NO_4S$: 364.15771, found: 364.15797.

General procedure for esterification with carboxylic acid (5b-d and 7a): To a stirred solution of alcohol precursor, DCC (1.1 eq.) and DMAP (cat.) in dry DCM (13 mL/mmol), at 20 °C, was added a solution of the suitable carboxylic acid (1.1 eq.) in dry dichloromethane (7 mL/mmol) under argon atmosphere. After stirring overnight, the mixture was cooled in an ice-bath for precipitation of urea, filtered and concentrated under vacuum. After purification by flash chromatography (DCM/EtOAc), a colourless oil was obtained in all cases.

1-(Pent-4-enoyl)-(3S)-3-[(1R)-(2-(benzyloxy)acetoyloxy)ethyl]azetidin-2-one (5a): Purification by flash chromatography (DCM/AcOEt 98:2) gave **5a** (78 mg, 89%) as a colourless oil: $[\alpha]_D$ = -0.15 (c = 0.52);*R*f =0.38 (chex/AcOEt 5:3); ^{1}H NMR (300MHz, $CDCl_3$): δ=1.39 (d, *J* =6.4 Hz, 3H), 2.39 (m, 2H), 2.76 (m, 2H), 3.42 (m, 1H), 3.52 (dd, *J* =3.7 Hz, *J* =7.8 Hz, 1H), 3.66 (dd, *J* =6.7 Hz, *J* =7.8 Hz, 1H), 4.06 (s, 2H), 4.60 (s, 2H), 5.02 (m, 2H), 5.35 (m, 1H), 5.81 (m, 1H), 7.21-7.41 ppm (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$):

δ=18.4, 27.9, 35.9, 40.0, 53.5, 67.1, 68.2, 73.4, 115.9, 128.1, 128.2, 128.6, 136.4, 136.9, 164.1, 169.5, 170.3 ppm; IR: υ =2849-2957, 1788, 1701, 1339, 1317, 1190, 1115 cm^{-1}; MS (ESI): m/z (%): 346.20 (4) $[M+H]^+$, 368.16 (100) $[M+Na]^+$; HRMS-ESI: m/z $[M+Na]^+$ calcd for $C_{19}H_{23}NO_5Na$: 368.1474, found: 368.1469.

1-(Propyl-3-enoxy)carbonyl-(3S)-3-[(1R)-(2-(benzyloxy)acetoyloxy)ethyl]azetidin-2-one (5b): Purification by flash chromatography (DCM/AcOEt 98:2) gave **5b** (31 mg, 58%) as a colourless oil: $[\alpha]_D$ = -0.45 (c = 1.27); *R*f =0.16 (chex/AcOEt 5:2); ^{1}H NMR (300MHz, $CDCl_3$): δ=1.38 (d, *J* =6.4 Hz, 3H), 3.42 (m, 1H), 3.58 (dd, *J* =3.6 Hz, *J* =7.1 Hz, 1H), 3.70 (m, 1H), 4.06 (s, 2H), 4.59 (s, 2H), 4.68 (d, *J* =5.7 Hz, 2H), 5.18-5.44 (m, 3H), 5.82-5.99 (m, 1H), 7.21-7.37 ppm (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$): δ=18.2, 41.3, 54.2, 66.9, 67.1, 68.1, 73.3, 119.3, 127.9, 128.0, 128.4, 130.9, 136.8, 148.7, 163.2, 169.3 ppm; IR: υ =2932, 1811, 1759, 1734, 1377, 1329, 1261, 1124 cm^{-1}; MS (ESI): m/z (%): 348.14 (2) $[M+H]^+$, 370.13 (100) $[M+Na]^+$; HRMS-ESI: m/z $[M+H]^+$ calcd for $C_{18}H_{22}NO_6$: 348.14416, found: 348.14460.

1-(Propyl-3-enamino)carbonyl-(3S)-3-[(1R)-(2-(benzyloxy)acetoyloxy)ethyl]azetidin-2-one (5c): Purification by flash chromatography (DCM/AcOEt 98:2) gave **5c** (35 mg, 93%) as a colourless oil: $[\alpha]_D$ = -0.36 (c = 1.27); *R*f =0.27 (chex/AcOEt 5:2); ^{1}H NMR (300MHz, $CDCl_3$): δ=1.38 (d, *J* =6.3 Hz, 3H), 3.43 (m, 1H), 3.58 (dd, *J* =3.0 Hz, *J* =6.9 Hz, 1H), 3.72 (m, 1H), 3.89 (m, 2H), 4.06 (s, 2H), 4.60 (s, 2H), 5.10-5.23 (m, 2H), 5.35 (m, 1H), 5.76-5.88 (m, 1H), 6.52 (br s, 1H), 7.28-7.39 ppm (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$): δ=18.4, 40.7, 42.3, 53.8, 67.1, 68.1, 73.5, 116.7, 128.2, 128.2, 128.7, 133.8, 137.0, 150.3, 166.1, 169.6 ppm; IR: υ =2912, 1767, 1703, 1528, 1340, 1275, 1202, 1124 cm^{-1}; MS (ESI): m/z (%): 347.16 (26) $[M+H]^+$, 369.14 (100) $[M+Na]^+$; HRMS-ESI: m/z $[M+H]^+$ calcd for $C_{18}H_{23}N_2O_5$: 347.16015, found: 347.16032.

1-(Propyl-3-enamino)thiocarbonyl-(3S)-3-[(1R)-(2-(benzyloxy)acetoyloxy)ethyl]azetidin-2-one (5d): Purification by flash chromatography (DCM/AcOEt 98:2) gave **5d** (37 mg, 74%) as a colourless oil: $[\alpha]_D$ = 0.08 (c = 1.78); *R*f =0.43 (chex/AcOEt 5:2); ^{1}H NMR (300MHz, $CDCl_3$): δ=1.37 (d, *J* =6.3 Hz, 3H), 3.37 (m, 1H), 3.71 (dd, *J* =3.2 Hz, *J* =7.5 Hz, 1H), 3.87 (m, 1H), 4.06 (s, 2H), 4.19-4.29 (m, 2H), 4.59 (s, 2H), 5.15-5.27 (m, 2H), 5.34 (m, 1H), 5.87 (m, 1H), 7.26-7.44 (m, 5H), 8.36 ppm (br s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ=18.2, 43.7, 47.0, 52.0, 66.9, 67.8, 73.3, 117.8, 127.9, 128.0, 128.4, 131.8, 136.7, 164.0, 169.3, 177.8 ppm; IR: υ =2920, 1755, 1531, 1339, 1244, 1196, 1122 cm^{-1}; MS (ESI): m/z (%): 363.14 (94) $[M+H]^+$, 385.12 (100) $[M+Na]^+$; HRMS-ESI: m/z $[M+H]^+$ calcd for $C_{18}H_{23}N_2O_4S$: 363.13730, found: 363.13710.

1-(Pent-4-enoyl)-(3S)-3-[(1R)-(4-(pyridine-4-yl)butanoyl)ethyl]azetidin-2-one (7a): Purification by flash chromatography (DCM/AcOEt 9:1) gave **7a** (45 mg, 64%) as a colourless oil: $[\alpha]_D$ = 0.10 (c = 0.50); *R*f =0.36 (DCM/MeOH 9:1); ^{1}H NMR (500MHz, $CDCl_3$): δ=1.32 (d, *J* =6.4 Hz, 3H), 1.90 (m, 2H), 2.28 (t, *J* =7.4 Hz, 2H), 2.35 (m, 2H), 2.60 (m, 2H), 2.73 (m, 2H), 3.39 (m, 1H), 3.50 (dd, *J* =3.7 Hz, *J* =7.7 Hz, 1H), 3.63 (dd, *J* =6.8 Hz, *J* =7.6 Hz, 1H), 4.93-5.06 (m, 2H), 5.25 (m, 1H), 5.77 (m, 1H), 7.07 (d, *J* =6.0 Hz, 2H), 8.46 ppm (d, *J* =6.0 Hz, 2H); ^{13}C NMR (125 MHz, $CDCl_3$): δ=18.3, 25.3, 27.9, 33.4, 34.2, 35.8, 39.8, 53.5, 67.4, 115.9, 123.9, 136.3, 149.8, 150.2, 164.4, 170.3, 171.9 ppm; IR: υ =2922, 1786, 1734, 1701, 1603, 1313, 1238, 1132 cm^{-1}; MS (ESI): m/z (%): 345.18 (100) $[M+H]^+$, 367.16 (80) $[M+Na]^+$; HRMS-ESI: m/z $[M+H]^+$ calcd for $C_{19}H_{25}N_2O_4$: 345.18088, found: 345.18088.

General procedure for coupling isocyanate with alcohol function (6a-d): To a stirred solution of the alcohol precursor (1 eq.) in dry DCM (7 mL/mmol) at r.t., was dropwise added the phenethylisocyanate under argon atmosphere, and finally $Ti(OtBu)_4$. After 1 h, the solution was quenched, at low temperature, with a saturated aqueous solution of NH_4Cl and diluted with DCM. The aqueous layers were several extracted with DCM and the organic layers were combined, dried over MgSO4 filtered and concentrated under vacuum. After purification by flash chromatography (chex/AcOEt), white solids (**6b** and **6d**) or colourless oils were obtained (**6a** and **6c**).

1-(Pent-4-enoyl)-(3S)-3-[(1R)-((2-phenylethylamino)carbonyloxy)-ethyl]azetidin-2-one (6a): Purification by flash chromatography (DCM/AcOEt 9:1) gave **6a** (61 mg, 81%) as a colourless oil: $[\alpha]_D$ = -0.19 (c = 0.77); *R*f =0.71 (AcOEt); ^{1}H NMR (500MHz, $CDCl_3$): δ=1.33 (d, *J* =6.3 Hz, 3H), 2.29-2.48 (m, 2H), 2.79 (m, 4H), 3.25-3.46 (m, 4H), 3.62 (m, 1H), 4.74 (br s, 1H), 4.95-5.10 (m, 2H), 5.15 (m, 1H), 5.82 (m, 1H), 7.04-7.42 ppm (m, 5H); ^{13}C NMR (125 MHz, $CDCl_3$): δ=18.7, 28.0, 35.8, 36.0, 40.0, 42.2, 53.8, 67.9, 115.9, 126.6, 128.7, 128.8, 136.5, 138.7, 155.2, 164.6, 170.4 ppm; IR: υ =2922, 1786, 1699, 1529, 1379, 1315, 1240, 1196, 1134 cm^{-1}; MS (ESI): m/z (%): 345.18 (4) $[M+H]^+$, 367.16 (100) $[M+Na]^+$; HRMS-ESI: m/z $[M+H]^+$ calcd for $C_{19}H_{25}N_2O_4$: 345.18088, found: 345.18129.

1-(Propyl-3-enoxy)carbonyl-(3S)-3-[(1R)-((2-phenylethylamino)carbonyloxy)-ethyl]-azetidin-2-one (6b): Purification by flash chromatography (DCM/AcOEt 9:1) gave **6b** (22 mg, 64 %) as a white solid: $[\alpha]_D$ = -0.51 (c = 1.32); *R*f =0.78 (AcOEt); ^{1}H NMR (500MHz, $CDCl_3$): δ=1.35 (d, *J* =6.3 Hz, 3H), 2.81 (t, *J* =6.9 Hz, 2H), 3.36 (m, 1H), 3.43 (m, 2H), 3.58 (dd, *J* =3.5 Hz, *J* =6.9 Hz, 1H), 3.68 (m, 1H), 4.71 (m, 3H), 5.14 (m, 1H), 5.25-5.44 (m, 2H), 5.94 (m, 1H), 7.14-7.20 (m, 2H), 7.21-7.26 (m, 1H), 7.27-7.34 ppm (m, 2H); ^{13}C NMR (125

MHz, $CDCl_3$): δ=18.7, 36.1, 41.6, 42.3, 54.8, 67.3, 68.0, 119.5, 126.7, 128.8, 128.9, 131.3, 138.7, 149.0, 155.3, 163.9 ppm; IR: υ =2912-2932, 1807, 1717, 1705, 1518, 1379, 1327, 1259, 1128 cm^{-1}; MS (ESI): m/z (%): 347.16 (3) $[M+H]^+$, 369.14 (49) $[M+Na]^+$, 715.29 (100) $[2M+Na]^+$; HRMS-ESI: m/z $[M+H]^+$ calcd for $C_{18}H_{23}N_2O_5$: 347.16015, found: 347.16044.

1-(Propyl-3-enamino)carbonyl-(3S)-3-[(1R)-((2-phenylethylamino)carbonyloxy)-ethyl]-azetidin-2-one (6c): Purification by flash chromatography (DCM/AcOEt 9:1) gave **6c** (29 mg, 70%) as a colourless oil: $[\alpha]_D$ = -0.73 (c = 1.17); *R*f =0.45 (chex/AcOEt 5:3); ^1H NMR (300MHz, $CDCl_3$): δ=1.33 (d, *J* =6.4 Hz, 3H), 2.80 (t, *J* =6.9 Hz, 2H), 3.34-3.47 (m, 3H), 3.56 (dd, *J* =3.0 Hz, *J* =6.8 Hz, 1H), 3.68 (m, 1H), 3.95 (m, 2H), 4.72 (br s, 1H), 5.03-5.27 (m, 3H), 5.71-5.93 (m, 1H), 6.56 (br s, 1H), 7.08-7.36 ppm (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$): δ=18.7, 36.1, 40.7, 42.3 (2C), 54.2, 67.7, 116.7, 126.7, 128.8, 128.9, 133.8, 138.7, 150.4, 155.3, 166.7 ppm; IR: υ =2980, 1765, 1699, 1645, 1531, 1497, 1454, 1337, 1248, 1136 cm^{-1}; MS (ESI): m/z (%): 346.18 (9) $[M+H]^+$, 368.16 (100) $[M+Na]^+$; HRMS-ESI: m/z $[M+H]^+$ calcd for $C_{18}H_{24}N_3O_4$: 346.17613, found: 346.17636.

1-(Propyl-3-enamino)thiocarbonyl-(3S)-3-[(1R)-((2-phenylethylamino)carbonyloxy)-ethyl]-azetidin-2-one (6d): Purification by flash chromatography (DCM/AcOEt 95:5) gave **6d** (56 mg, 84%) as a white solid: $[\alpha]_D$ = 0.25 (c = 0.60); *R*f =0.35 (AcOEt); ^1H NMR (300MHz, $CDCl_3$): δ=1.33 (d, *J* =6.4 Hz, 3H), 2.80 (t, *J* =6.9 Hz, 2H), 3.26-3.36 (m, 1H), 3.37-3.48 (m, 2H), 3.72 (dd, *J* =3.2 Hz, *J* =7.4 Hz, 1H), 3.84 (m, 1H), 4.20-4.34 (m, 2H), 4.75 (br s 1H), 5.09-5.34 (m, 3H), 5.76-5.98 (m, 1H), 7.09-7.36 (m, 5H), 8.42 ppm (br s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ=18.7, 36.0, 42.2, 43.8, 47.2, 52.5, 67.5, 117.9, 126.6, 128.7, 128.9, 132.0, 138.6, 155.2, 164.8, 178.1 ppm; IR: υ =2976, 1749, 1715, 1697, 1524, 1497, 1337, 1242, 1130 cm^{-1}; MS (ESI): m/z (%): 362.15 (16) $[M+H]^+$, 384.13 (100) $[M+Na]^+$; HRMS-ESI: m/z $[M+H]^+$ calcd for $C_{18}H_{24}N_3O_3S$: 362.15329, found: 362.15359.

In vitro assays for human FAAH. Tubes containing the enzyme[27] (10 mM Tris-HCl, 1 mM EDTA, 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 buffer only were used as 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.

In vitro assays for human MGL activity. Tubes containing purified enzyme[29] (10 mM Tris-HCl, 1 mM EDTA, 0.1 % (w/v) BSA, pH 8.0, 165 µL), test compounds in DMSO or DMSO alone for controls (10 µL) and [^{3}H]-2-OG (50,000 dpm, 2 µM final concentration, 25 µL) were preincubated at 20 °C for 30 min and 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 lower chloroform phase were counted for radioactivity by liquid scintillation counting. In all experiments, tubes containing buffer only were used as control for chemical hydrolysis (blank) and this value was systematically subtracted.

Reversibility studies. In a total volume of 15 µL, human FAAH (27.5 µg) and inhibitors (or DMSO for controls) at concentrations allowing inhibition of the enzyme before dilution and no inhibition after the 100-fold dilution, were preincubated during 1 h at room temperature. The mixtures were then diluted 100-fold by adding assay buffer. Immediatly after, an aliquot (175µL) was taken and [^{3}H]-AEA (50,000 dpm, 2 µM final concentration, 25 µL) was added. Two samples were taken at 30 and 90 min after the dilution too. Each aliquots were incubated at 37 °C for 30 min and 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 buffer only were used as control for chemical hydrolysis (blank) and this value was systematically subtracted.

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ethyl]-4-acetoxy-2-azetidinone). Dr Geoffray Labar is acknowledged for the preparation of *h*FAAH, and Bouazza Es Saadi for technical assistance.

VI.6 References

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Chapter 7

VII General conclusions and perspectives

This thesis took place with the will to lead a medicinal chemistry project encompassing organic synthesis and pharmacological evaluations. Fortunately, these five years of research brought to a successful conclusion: it is possible and fascinating! Taking inspiration from structures initially described for inhibiting *DD*-peptidases and published in our laboratory (A. Urbach thesis, 2006), the project evolved in a surprising manner. The dual expertise of organic chemistry and pharmacology laboratories we had at our disposal, offered the opportunity to work rapidly and rationally in an iterative way, for providing appropriate answers to the rising questions. All syntheses were done at Louvain la Neuve, at the Institute of Condensed Matter and Nanosciences (IMCN) and pharmacological evaluations in Louvain en Woluwe at the Louvain Drug Research Institute (LDRI), each place harbouring specialized and competent people who allowed us to better understand from where our difficulties came and how to solve them.

The project began with the synthesis of two families of compounds, based on the β-lactam template and inspired from A. Urbach thesis. Initially, A. Urbach designed and synthesized large ring-bridged azetidinones to inhibit *DD*-peptidases and/or β-lactamases. To reach such special structures, β-lactamic intermediates featuring two arms with a terminal alkene function (Figure 1) were prepared in order to form the large ring by a final ring-closing metathesis reaction (RCM). These intermediates were tested in a FAAH inhibition assay to evaluate their potential activity on non-bacterial serine hydrolase. By chance, some compounds revealed to be moderate to good inhibitors of FAAH.

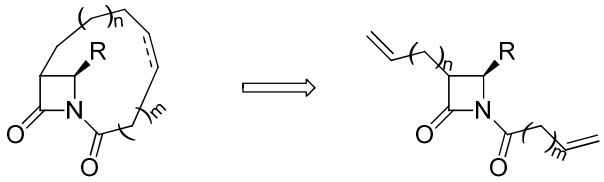

Figure 1. β-Lactamic intermediates synthesized by A. Urbach to prepare large ring-bridged azetidinones

Starting from this fortuitous discovery, we designed new compounds and decorated the β-lactam ring with various lipophilic groups connected by chains of different lengths.[1] This first part of the work drove us to clear results: i) The family with an acetate substituent at C4 position (R_3 = OAc, Figure 2) is systematically less active against FAAH than the corresponding unsubstituted derivatives (R_3=H) ; ii) amongst the C4-free position (R_3 = H;

Figure 2) family, the best representatives were obtained with a pentenoyl chain at N1 position and a 4-phenylbutanoyl (**1**, Figure 2) or a biphenylacetoyl (**2**, Figure 2) chains at C5-O position. In this study, we culminated with the compound **1** (**19b** in chapter IV, **4** in chapter V and **4a** in chapter VI) which exhibits an IC_{50} value of 5.32 nM. Preliminary experiments were done to assess the mode of action of this family. Thus, kinetics (Michaelis-Menten curves), preincubation and rapid dilution studies were performed with compounds **1** and **2**. Unambiguously, we learnt that our compounds are competitive inhibitors, *i.e.* they fit into the catalytic site of hFAAH in competition with the natural substrate, and act in a reversible manner. This last observation was quite surprising and exciting because β-lactams have a long story of irreversible inhibitors, since the discovery of Penicillin G. Docking studies in the FAAH catalytic site were performed and resulted in the proposal of the optimal conformation. Our compounds seem to expose the imide function towards the catalytic triad, while the two hydrophobic chains are respectively turned to lipophilic pockets.

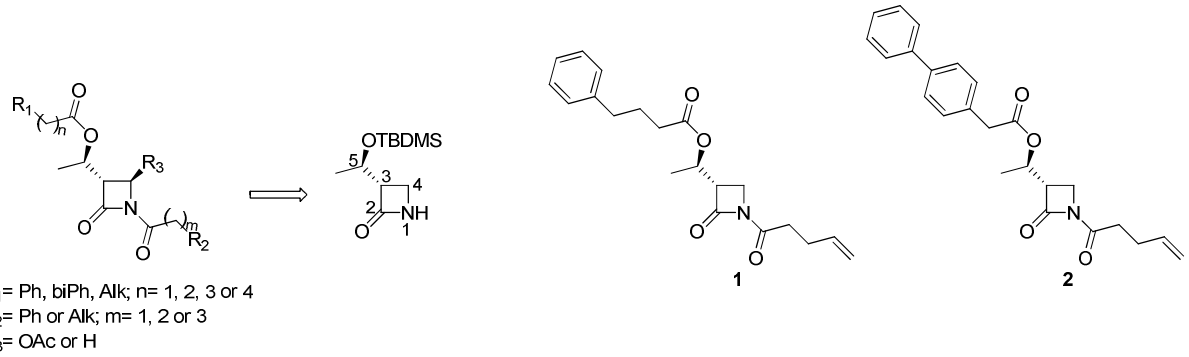

Figure 2. Strategy of synthesis of the two first families (R_3= OAc or H) prepared in 2008 and 2009 to inhibit hFAAH. The lead compound **1** inhibits hFAAH with an IC_{50} value of 5.3 nM and its biphenylic analogue **2** with an IC_{50} value of 12nM.

Obviously, this unexpected discovery drew the direction to the thesis outcome and all the further works were performed with a view to understand this reversible inhibition.

The most conclusive and beautiful, but also difficult, manner to demonstrate the mode of interaction between an inhibitor and its target enzyme is certainly the co-crystallization for X-ray diffraction analysis of a monocrystal. During our thesis, co-crystals from an engineered form of h/rFAAH[2] and three principal inhibitors, widely used as pharmacological tools, were described and published (Figure 3 and 4). The authors were able to confirm the previous knowledge about their mode of action: **3** (URB-597)[3] and **4** (PF-750)[4] were trapped as stable acyl-enzyme intermediates (Figures 3, 4A and 4B) after expelling their respective leaving

group, while **5** (OL-135)[5-7] was trapped as a tetrahedral intermediate (Figure 3 and 4C). Very recently, a team published a co-crystal structure between the reversible inhibitor **6** (Figure 3 and 4D) and FAAH.[8,9] The authors described therein a non-covalent inhibition as they trapped the inhibitor in the catalytic site but without covalent interaction (Figure 4D).

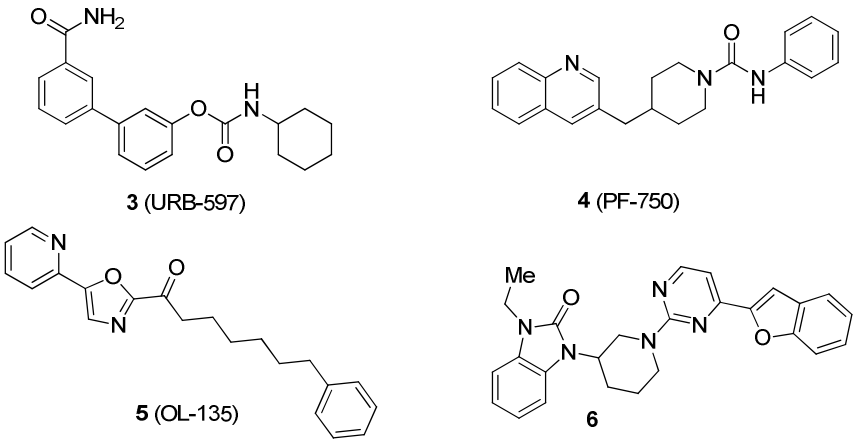

Figure 3. Structures of FAAH inhibitors with various modes of inhibition: irreversible (**3** and **4**), covalent reversible (**5**) and non-covalent reversible (**6**)

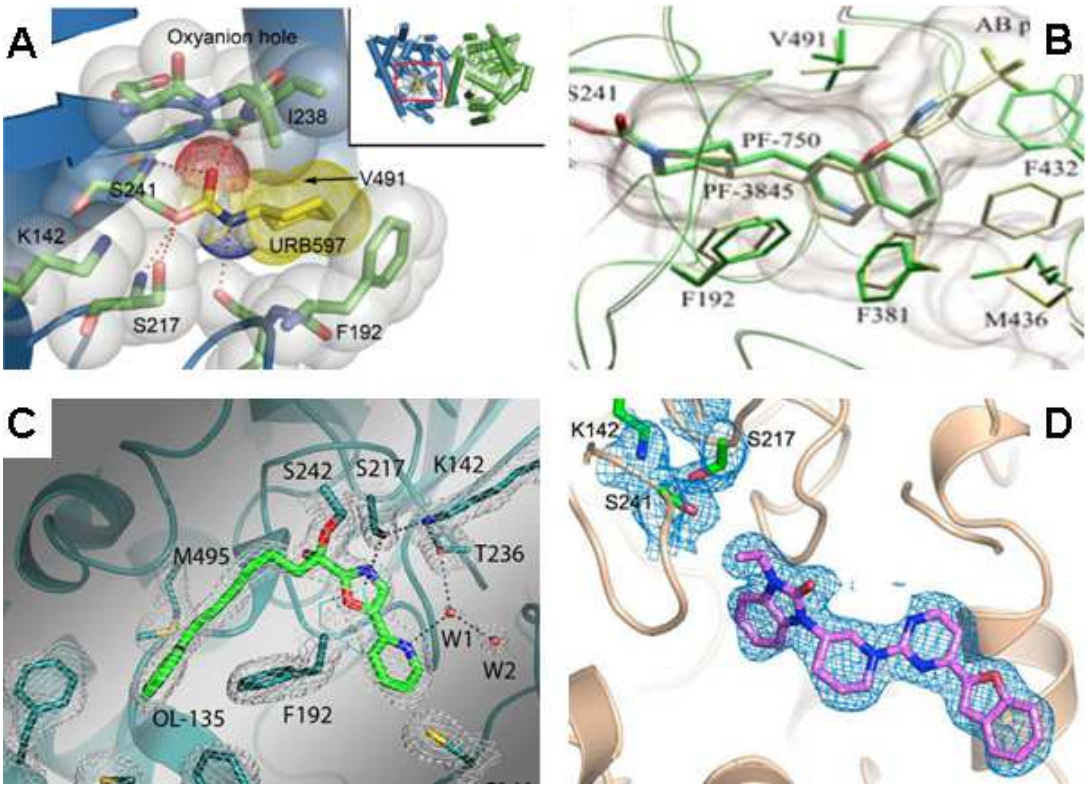

Figure 4. Binding modes of various FAAH inhibitors, according to published results of the respective authors. **A**) acyl-enzyme intermediate with URB597 (**3**),[3] **B**) acyl-enzyme

intermediate with PF-750 (**4**),[4] **C**) tetrahedral intermediate with OL-135 (**5**)[5] and **D**) non-covalent interaction with **6**[9].

Unfortunately, a co-crystallization study was not possible in our case because the home-made purified human FAAH enzyme we used is not pure enough and we did not succeed to crystallize our enzyme (unpublished work of G. Labar). Accordingly, we tried to explain the reversibility of the β-lactamic inhibitors by using our own tools; *i.e.* organic chemistry and pharmacology. Three possibilities can be considered to explain reversibility: i) like compound **5** (OL-135, Figure 4C) our compounds are covalently attached to the active serine. But as there is no good leaving group, the equilibrium is reversed; ii) an acyl-enzyme intermediate is formed thanks to the expulsion of a leaving group, but is finally hydrolyzed, like the natural substrate; iii) like compound **6** (Figure 4D) no covalent interaction occurs.

To clarify the question, we proceeded step by step, following a process of elimination. Thus, we firstly thought to synthesize analogues of compound **1** by the systematic replacement of C=O functions by CH_2 groups, in order to highlight the importance of each carbonyl in the eventual nucleophilic attack by the active serine and/or in the interaction into the catalytic site.[10] Five compounds were obtained by different ways of synthesis and the relevance of pharmacological results was undeniable (Figure 5).

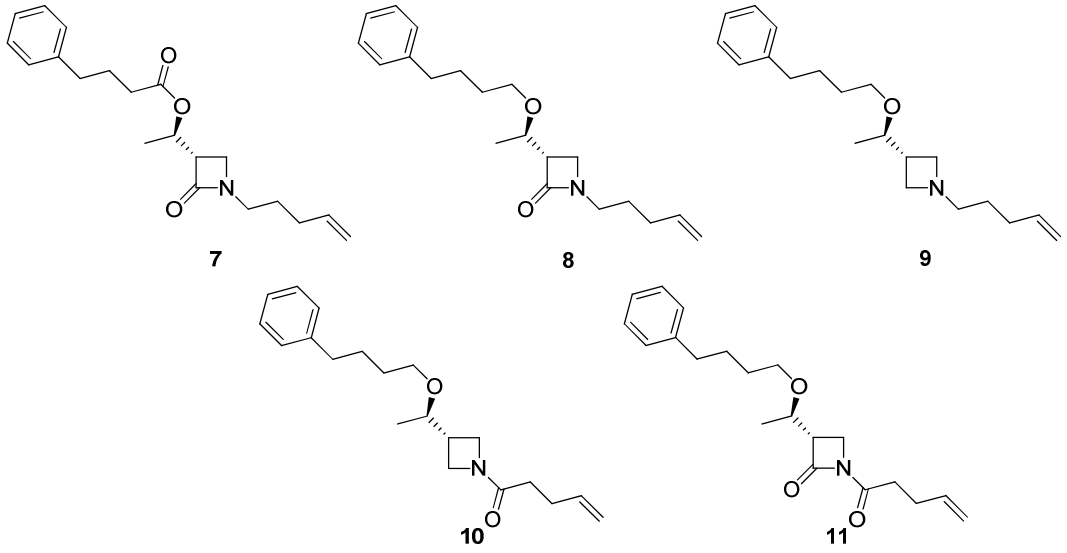

Figure 5. Analogues of lead compound **1** synthesized and evaluated in 2010 and 2011 to display the relative importance of carbonyl functions.

By this SAR study, we could conclude that the imide function (formed by the β-lactam carbonyl and the exocyclic carbonyl) is essential for the inhibition and the ester function of the lateral chain can be replaced by an ether function. More surprisingly, we also established that the exocyclic carbonyl of the imide function is more indispensable than the endocyclic carbonyl, *i.e.* the carbonyl of the β-lactam ring. Afterward, we followed the eventual hydrolysis processing of compound **11** (**8** in chapter 5), in presence of purified hFAAH and in presence of FAAH contained in mouse liver homogenate. These experiments drove to the evidence that our compounds are not hydrolyzed by hFAAH, *i.e.* they are not slow substrates of FAAH. However, our β-lactams were hydrolyzed by the other hydrolases contained in the liver extracts.

At this stage, we could conclude that our compounds act in a reversible manner like compound **5** (OL135, Figure 4C), *i.e.* formation of a reversible tetrahedral intermediate, or like compound **6** (Figure 4D), *i.e.* high affinity between the inhibitor and aminoacids of the catalytic site. Then, we undertook the synthesis of two compounds which present a good leaving-group (**12** (**4b** in chapter 6) and **13** (**4e** in chapter 6), Figure 6) fixed on the crucial exocyclic carbonyl.[11] We designed these compounds in view of switching from a reversible mechanism to an irreversible one, if a covalent interaction should occur between the active serine and the exocyclic carbonyl of the inhibitor. Indeed, the corresponding tetrahedral intermediate should evolve irreversibly towards the acyl-enzyme intermediate.

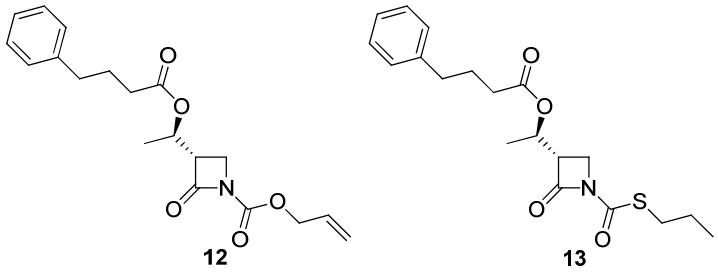

Figure 6. Bioisosteres of lead compound **1** synthesized and evaluated in 2012

From washout experiments, we noted compounds **12** and **13** still behave like reversible inhibitors. The insertion of a good leaving-group did not lead to an irreversible inhibition which suggests that no nucleophilic attack occurs on the inhibitors. This long march of thought drove us to the strong assumption that our compounds act in a non-covalent mode of

inhibition. Like compound **6**, favorable interactions with some residues from the catalytic site must stabilize the inhibitor inside the active pocket.
Such a mode of action was totally unpredictable at the beginning of our thesis. Hence, our β-lactams keep a nice touch of originality in the world of serine hydrolases inhibition. For the first time, we have demonstrated that the mode of action of β-lactamic inhibitors is totally different versus classical serine enzymes featuring the Ser-His-Asp triad, than versus FAAH characterized by the unique Ser-Ser-Lys triad. The non-covalent interaction between β-lactamic inhibitors and FAAH could not be demonstrated by the in-house MS methods because the inhibitor-enzyme complex is not stable enough to be visible by this technique. The only relevant technique should be the X-ray diffraction analysis of a co-crystal between the inhibitor and hFAAH. But this is another story… for another thesis.

To summarize, during our thesis, we completely explored the *in vitro* characterization of novel β-lactamic inhibitors. We determined the optimal lipophilic groups and the optimal length of the two side-chains, giving the lead compound **1** (chapter 4). Then, we clarified the reversible mechanism of inhibition (chapters 5 and 6) and checked that improvements of LogP values and PSA did not correlate with a loss of activity, but on the contrary with the preservation of nanomolar activities (chapter 6). This last information is particularly essential for further *in vivo* experiments. Indeed, it is not uncommon to observe excellent activities *in vitro* and not *in vivo*, because of a low solubility or a low permeability through cellular membranes.

It would appear now very interesting to continue the characterization of β-lactamic FAAH inhibitors at the cellular level and, why not, at the whole organism (mouse or rat) level. That would allow to check, on the one hand, whether they increase the level of anandamide in cells or tissues, and on the other hand, whether they provoke physiological cannabimimetic effects, for instance anti-inflammatory effects, in specific models of inflammation.

Concerning the design of new compounds, we propose to explore more deeply the imide pharmacophore for the discovery of novel potent inhibitors of hFAAH. Since the β-lactam motif itself is not essential, the model of endocyclic/exocyclic carbonyl groups fixed on a nitrogen atom could be built by using larger cycles than the four-membered one, as illustrated in Figure 7, where R represents a lipophilic chain fitting into the hFAAH active site.

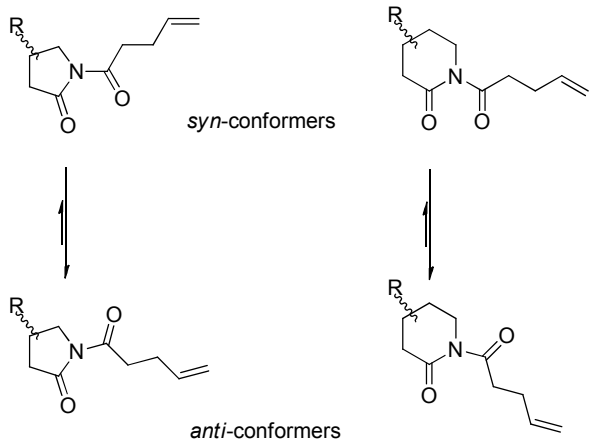

Figure 7. Monocyclic analogues of the imide pharmacophores for FAAH inhibition

Thanks to the free rotation around the N-$CO_{(exo)}$ bond, both *syn* and *anti* configurations of the imide carbonyls are accessible. From docking experiments, it appeared that the *syn* configuration should be the active one. However, this question could be addressed, for instance, by the synthesis and evaluation of bicyclic compounds where the *syn* conformation is imposed (Figure 8).

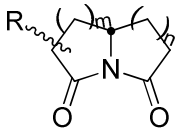

Figure 8. Bicyclic analogues of the imide pharmacophores for FAAH inhibition

Our thesis provides a playroom for imaginative medicinal chemists!

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11. Feledziak, M., Lambert, D. M. and Marchand-Brynaert, J. *submitted* **2012**.

VIII Annexes

VIII.1 Experimental section (supplementary data of articles)

Supporting information *J.Med.Chem 2009, 52 (22), 7054-7068*
Supporting information *J.Med.Chem 2011, 54 (19), 6812-6823*
Supporting information *Euro.J.Med.Chem 2012, submitted.*

VIII.2 Unpublished results (in collaboration with J. Caruano)

β-Lactams Derived from a Carbapenem Chiron are Selective Inhibitors of Human Fatty Acid Amide Hydrolase versus Human Monoacylglycerol Lipase

Marion Feledziak, Catherine Michaux, Allan Urbach, Geoffray Labar, Giulio G. Muccioli, Didier M. Lambert, and Jacqueline Marchand-Brynaert.

Supporting information

Table of contents

Synthesis of the compound **8**.

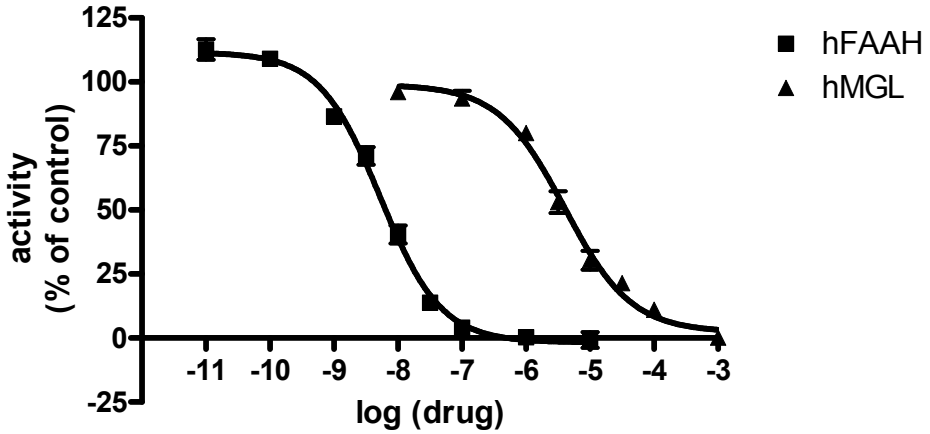
To a solution of azetidinone **7** (1eq.) in dimethylformamide (2.6 mL/mmol) at 20 °C, were added sodium thiophenolate (2 eq.) and diisopropylethylamine (1.2 eq.) under argon atmosphere. The mixture was stirred during 4 h, then diluted in diethyl ether and washed with brine, dried over $MgSO_4$, filtered and concentrated under vacuum. After purification by flash chromatography (cyclohexane/ethyl acetate), a white solid was obtained.
(3R,4R)-3-[1(R)-(*tert*-butyldimethylsilyloxy)-ethyl]-4-(phenylthio)-azetidin-2-one. Yield : 95 %. (4.4 g from 0.014 mol of **1**). R_f = 0.51 (cyclohexane/Ethyl acetate : 5/2). ^{1}H NMR (200 MHz, $CDCl_3$) : δ = 0.05 (s, 3H), 0.06 (s, 3H), 0.80 (s, 9H), 1.14 (d, 3H, *J* = 6.3 Hz), 2.95 (m, 2H), 4.15 (m, 1H), 4.98 (d, 1H, *J* = 2.3 Hz), 6.81 (br s, 1H), 7.12-7.49 (m, 5H). ^{13}C NMR (50 MHz, $CDCl_3$) : δ = -4.4, -3.6, 18.6, 22.9, 26.4, 56.9, 65.2, 66.2, 129.3, 130.0, 132.5, 134.4, 167.7. Registry Number : 158515-29-8.
To a solution of azobisisobutyronitrile (AIBN, 0.2 eq.) and tris(trimethylsilyl)silane hydride (TTMSS, 5 eq.) in refluxing toluene (7.8 mL/mmol), was added the previously obtained azetidinone (1 eq.) under argon atmosphere. The mixture was refluxed during 24 h and AIBN was added in three portions (3x0.2 eq.) each two hours. Toluene was removed under vacuum, and the oily residue was purified by flash chromatography (cyclohexane/ethyl acetate) and a white solid was obtained.
(3S)-3-[1(R)-(*tert*-butyldimethylsilyloxy)-ethyl]-azetidin-2-one. Yield : 84 %. (1.1 g from 5.92 mmol). R_f = 0.35 (cyclohexane/Ethyl acetate : 1/1)^{1}H NMR (250 MHz, $CDCl_3$) : δ = 0.066 (s, 3H), 0.074 (s, 3H), 0.83 (s, 9H), 1.15 (d, 3H, *J* = 5.9 Hz), 3.14-3.30 (m, 3H), 4.15 (m, 1H), 6.31 (br s, 1H). ^{13}C NMR (50 MHz, $CDCl_3$) : δ = -5.1, -4.4, 17.4, 22.4, 25.6, 37.7, 59.1, 65.3, 169.8. Registry Number : 109323-90-2.

Table 1. pI_{50} and Standard Deviation of each tested compound

entry	compound	R^1	n	R^2	m	R^3	pI_{50} *h*FAAH	pI_{50} *h*MGL
1	**11a**	OAc	2	Ph	-	-	3.65 ± 0.22	-
2	**11b**	OAc	3	Ph	-	-	3.74 ± 0.06	-
3	**11c**	OAc	2	Alk	-	-	3.27 ± 0.02	-
4	**13a**	OAc	2	Ph	2	Ph	5.66 ± 0.08	-
5	**13b**	OAc	2	Ph	3	Ph	5.95 ± 0.06	-
6	**13d**	OAc	2	Ph	1	biPh	6.08 ± 0.12	-
7	**14a**	OAc	3	Ph	2	Ph	5.31 ± 0.04	-
8	**14b**	OAc	3	Ph	3	Ph	5.34 ± 0.12	-
9	**14d**	OAc	3	Ph	1	biPh	6.15 ± 0.21	-
10	**15e**	OAc	2	Alk	2	Alk	5.70 ± 0.03	-
11	**12a**	H	2	Ph	-	-	3.39 ± 0.07	-
12	**12b**	H	3	Ph	-	-	nd	-
13	**12c**	H	4	Ph	-	-	nd	-
14	**12d**	H	2	Alk	-	-	5.10 ± 0.02	-
15	**12e**	H	3	Alk	-	-	nd	-
16	**16a**	H	2	Ph	2	Ph	6.80 ± 0.02	-
17	**16b**	H	2	Ph	3	Ph	7.31 ± 0.06	-
18	**16c**	H	2	Ph	4	Ph	7.04 ± 0.02	-
19	**16d**	H	2	Ph	1	biPh	7.30 ± 0.04	-
20	**17a**	H	3	Ph	2	Ph	7.25 ± 0.04	-
21	**17b**	H	3	Ph	3	Ph	7.53 ± 0.06	-
22	**17c**	H	3	Ph	4	Ph	7.34 ± 0.03	-
23	**17d**	H	3	Ph	1	biPh	7.50 ± 0.08	-
24	**18b**	H	4	Ph	3	Ph	6.35 ± 0.13	-
25	**18d**	H	4	Ph	1	biPh	6.63 ± 0.08	-
26	**19b**	H	2	Alk	3	Ph	8.27 ± 0.05	5.39 ± 0.07
27	**19d**	H	2	Alk	1	biPh	7.93 ± 0.02	5.73 ± 0.11
28	**19e**	H	2	Alk	2	Alk	7.01 ± 0.02	4.63 ± 0.10
29	**19f**	H	2	Alk	3	Alk	7.50 ± 0.07	5.73 ± 0.08
30	**20b**	H	3	Alk	3	Ph	8.01 ± 0.07	5.07 ± 0.09
31	**20d**	H	3	Alk	1	biPh	7.84 ± 0.06	4.83 ± 0.13
32	**24**	H	-	-	1	biPh	5.19 ± 0.04	-

Figure 1. Selected representatives 'Dose-response' curves, **19b** (a), **19d** (b), **19f** (c), **20b** (d) and **20d** (e).

a.



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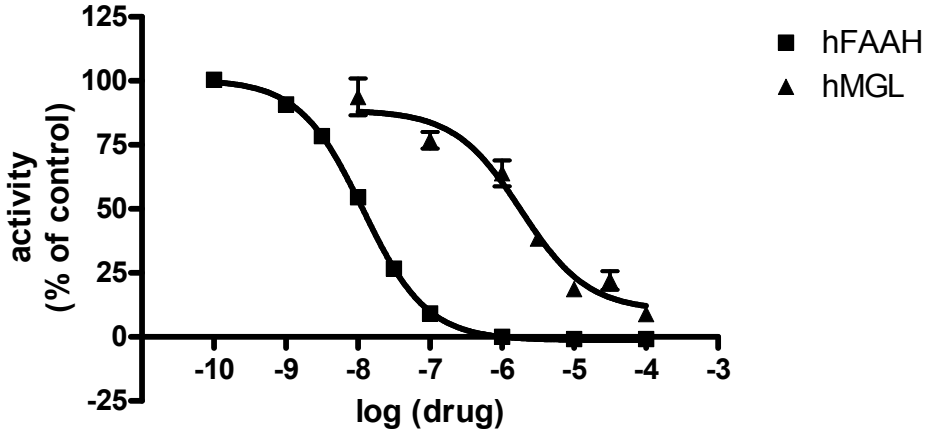
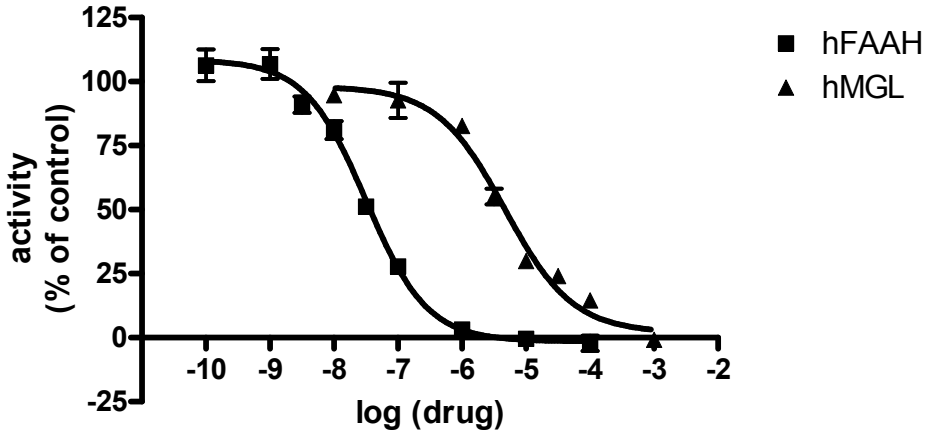


c.



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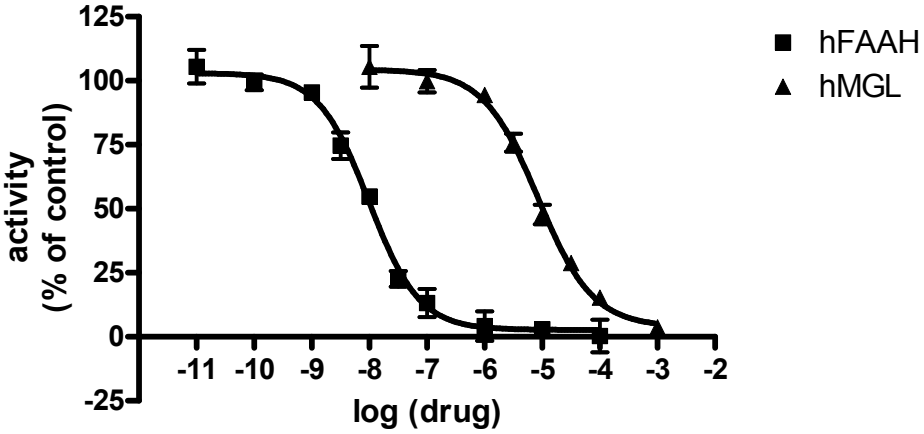
125
100
75
50
25
0
-25
activity
(% of control)
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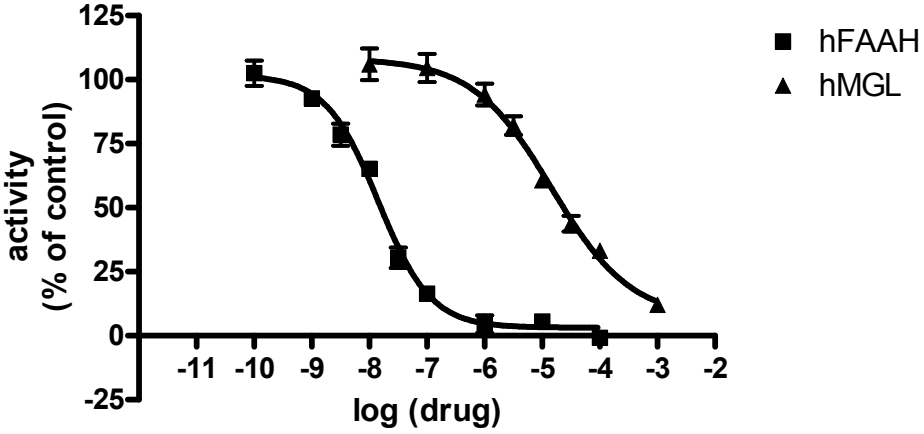
log (drug)
hFAAH
hMGL

e.


125
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activity
(% of control)
-11 -10 -9 -8 -7 -6 -5 -4 -3 -2
log (drug)
hFAAH
hMGL

Figure 2 : Docking of **19b** in mode I (Z configuration) showing aminoacids involved in hydrophobic contacts.

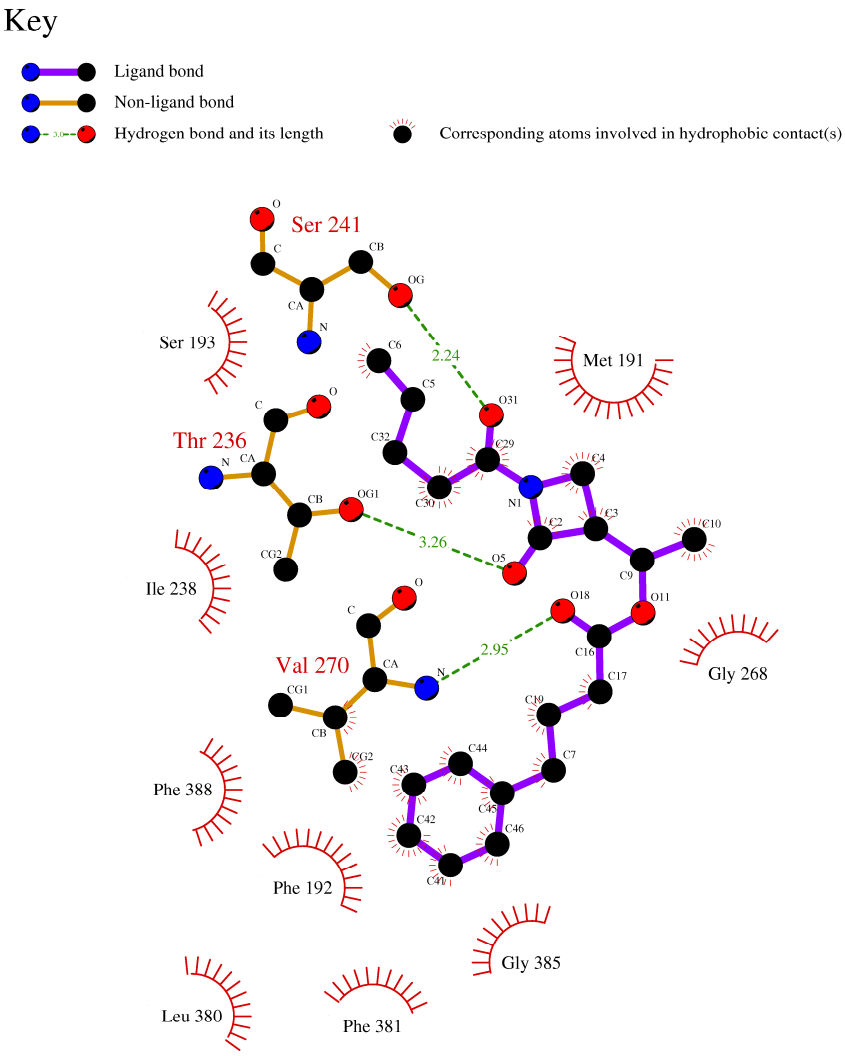


Figure 3 : Docking of **19d** in mode II (Z configuration) showing aminoacids involved in hydrophobic contacts.

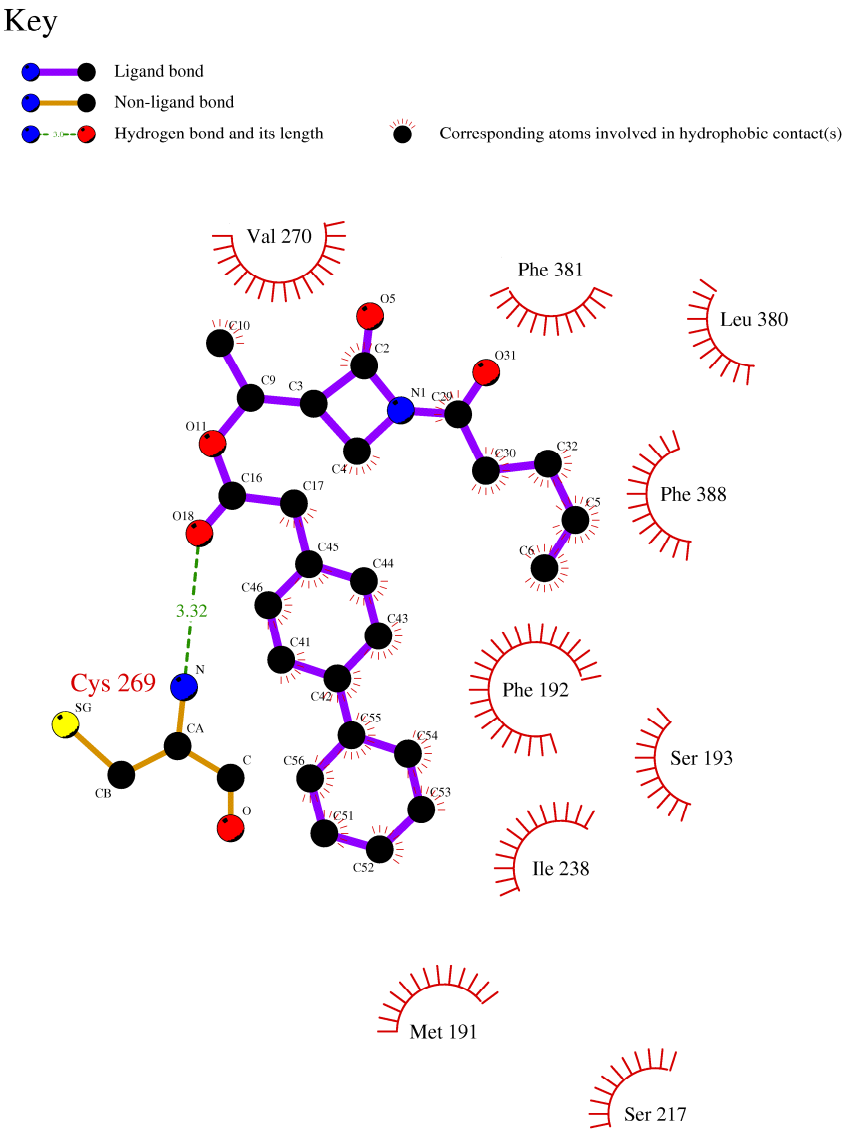
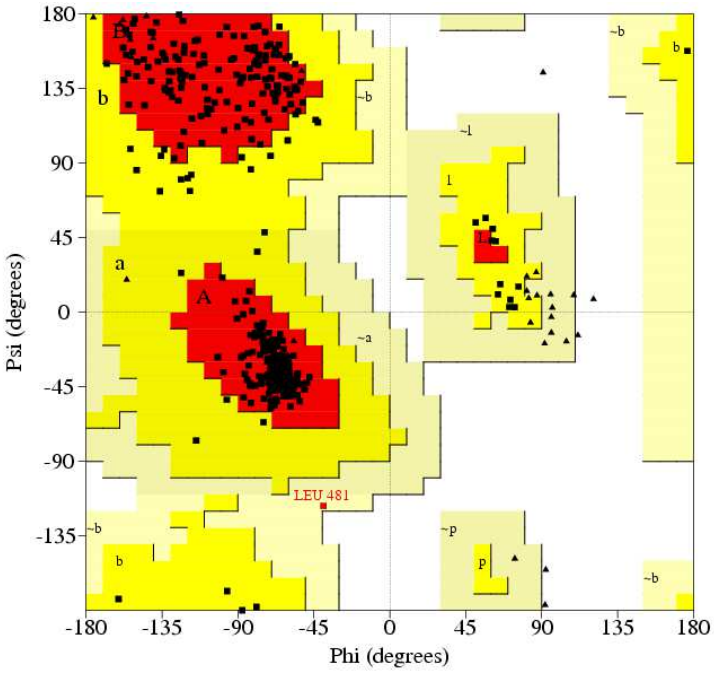


Figure 4 : Ramachandran plot of the modelled human FAAH.



SAR and LC/MS studies of β-Lactamic inhibitors of human Fatty Acid Amide Hydrolase (*h*FAAH). Evidence of a non-hydrolytic process

Marion Feledziak[†§], Giulio G. Muccioli[⊥], Didier M. Lambert[§], and Jacqueline Marchand-Brynaert[†].*

Supporting information

Table of contents

General procedure for *N*-alkylation (11 and 16). To a stirred solution of the starting azetidinone (**SM**) (1 equiv) in tetrahydrofuran (9.2 mL/mmol) at r.t., were added tetrabutylammonium hydrogen sulphate (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, and the inorganic precipitate was filtered off, washed with tetrahydrofuran and the filtrate was concentrated under vacuum. After purification by flash chromatography (cyclohexane/ethyl acetate), a colourless oil was obtained (**11** and **16**).

1-(4-Methoxybenzyl)-3(S)-[1(*R*)-(*tert*-butyldimethylsilyloxy)-ethyl]-azetidin-2-one (16). Yield: 79% (487 mg from 1.76 mmol of **SM**). $[\alpha]_D$ = -25.2 (c = 2.1). R_f = 0.43 (cyclohexane/ethyl acetate : 1/1). MS (ESI): *m/z*: 350.00 ($(M + H)^+$), 372.01 ($(M + Na)^+$), 721.42 ($(2M + Na)^+$). ^{1}H NMR (300 MHz, $CDCl_3$) : δ = 0.04 (s, 3H), 0.05 (s, 3H), 0.83 (s, 9H), 1.15 (d, 3H, *J* = 6.3 Hz), 3.04-3.19 (m, 3H), 3.77 (s, 3H), 4.14-4.23 (m, 1H), 4.21 (d, 1H, *J* = 14.9 Hz, AB system), 4.37 (d, 1H, *J* = 14.9 Hz, AB system), 6.86 (d, 2H, *J* = 8.6 Hz), 7.16 (d, 2H, *J* = 8.6 Hz). ^{13}C NMR (75 MHz, $CDCl_3$): δ = -4.8, -4.4, 14.2, 18.0, 22.7, 25.8, 40.7, 45.3, 55.3, 57.4, 65.3, 114.1, 127.8, 129.5, 159.1, 168.2. IR (cm^{-1}): υ = 2854-2955, 1747, 1612, 1514, 1464, 1402, 1246, 837. HRMS: $C_{19}H_{31}NO_3SiNa$: calculated: 372.1971, found: 372.1965.

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 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 colourless oil (**12**) or a white solid (**17** and **22**) was obtained.

1-(4-Methoxybenzyl)-3(S)-[1(*R*)-hydroxyethyl]-azetidin-2-one (17). Yield: 99% (133 mg from 0.57 mmol of **16**). $[\alpha]_D$ = -1.3 (c = 1.5). R_f = 0.28 (ethyl acetate/methanol : 99/1). MS (ESI): *m/z*: 236.18 ($(M + H)^+$), 258.17 ($(M + Na)^+$). ^{1}H NMR (300 MHz, $CDCl_3$) : δ = 1.24 (d, 3H, *J* = 6.4 Hz), 2.52 (broad s, 1H), 3.11-3.23 (m, 3H), 3.78 (s, 3H), 4.16 (m, 1H), 4.31 (d, 1H, *J* = 14.9 Hz, AB system), 4.34 (d, 1H, *J* = 14.9 Hz, AB system), 6.85 (d, 2H, *J* = 8.6 Hz), 7.16 (d, 2H, *J* = 8.6 Hz). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 21.4, 41.1, 45.4, 55.4, 57.0, 65.0, 114.2, 127.6, 129.5, 159.2, 168.5. IR (cm^{-1}): υ = 3416, 2903-2964, 1728, 1612, 1514, 1412, 1248. HRMS: $C_{13}H_{17}NO_3Na$: calculated: 258.1106, found: 258.1118.

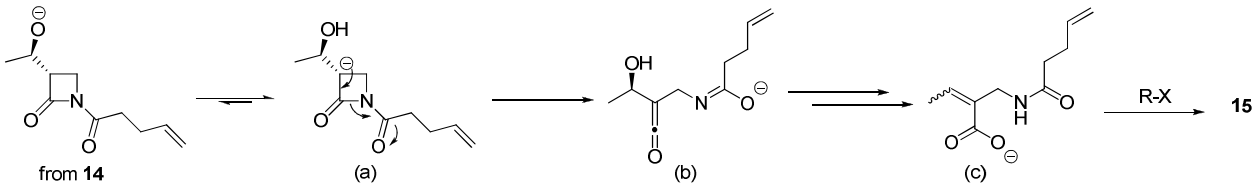
1-(4-Methoxyphenyl)-3(S)-[1(*R*)-hydroxyethyl]-azetidin-2-one (22). Yield: 99% (66 mg from 0.30 mmol of **21**). R_f = 0.44 (ethyl acetate). MS (ESI): *m/z*: 222.13 ($(M + H)^+$), 244.18 ($(M + Na)^+$). ^{1}H NMR (500 MHz, $CDCl_3$) : δ = 1.32 (d, 3H, *J* = 6.4 Hz), 2.45 (broad s, 1H), 3.33 (m, 1H), 3.60-3.65 (m, 2H), 3.77 (s, 3H), 4.26 (m, 1H), 6.84 (d, 2H, J = 9.0 Hz), 7.27 (d, 2H, J = 9.0 Hz). ^{13}C NMR (125 MHz, $CDCl_3$) : δ =21.6, 41.0, 55.6, 56.2, 65.2, 114.4, 117.7, 132.0, 156.2, 165.0. IR (cm^{-1}) : υ = 3423, 2926-2970, 1713, 1514, 1246. HRMS : $C_{12}H_{16}NO_3$: calculated : 222.11302, found : 222.11254.

1-(4-Methoxyphenyl)-3(S)-[1(*R*)-(*tert*-butyldimethylsilyloxy)-ethyl]-azetidin-2-one (21). To a stirred suspension of **SM** (1 equiv), dry K_2CO_3 (3 equiv) and CuI (5 % mol) in dry dioxane (0.9 mL/mmol of **SM**), were added freshly distilled *N,N*-dimethylethylenediamine (20 % mol) and bromoanisol (2 equiv) under argon atmosphere. The reaction mixture was refluxed for 24 h and then the inorganic precipitate was filtered off on silica gel, washed with ethyl acetate and the filtrate was concentrated under vacuum. After purification by flash chromatography (cyclohexane/ethyl acetate), a white solid was obtained. Yield : 61% (356

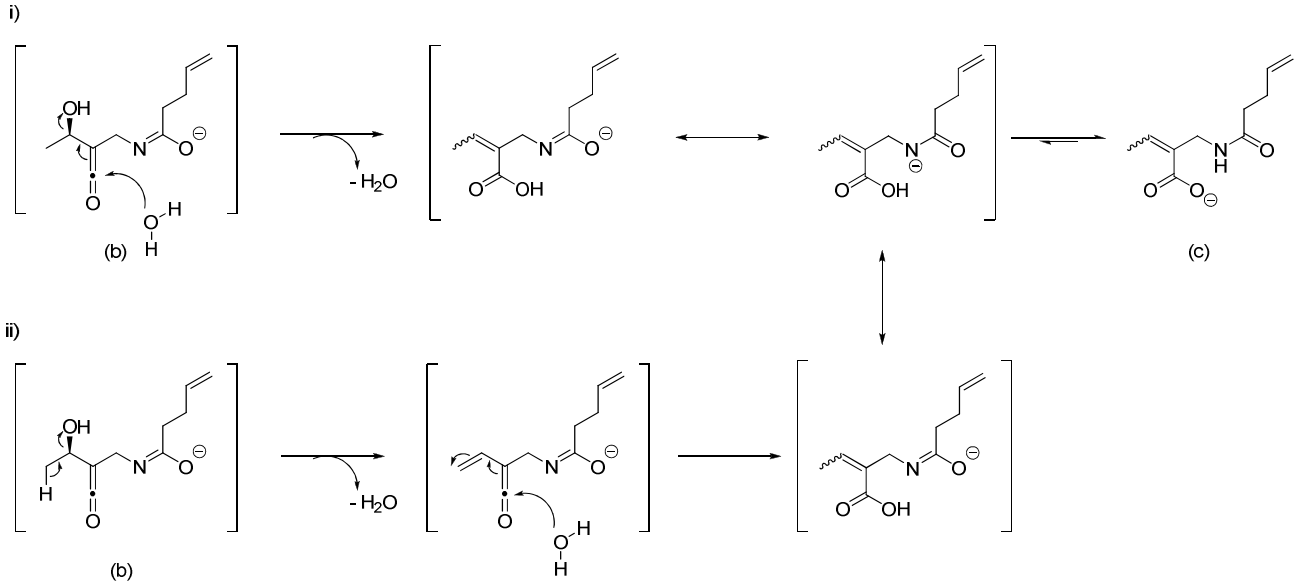
mg from 1.74 mmol of **SM**). R_f = 0.50 (cyclohexane/ethyl acetate : 1/1). MS (ESI) : *m/z* : 336.06 ($(M + H)^+$), 358.19 ($(M + Na)^+$). ^{1}H NMR (300 MHz, $CDCl_3$) : δ = 0.03 (s, 3H), 0.07 (s, 3H), 0.79 (s, 9H), 1.24 (d, 3H, *J* = 6.2 Hz), 3.26 (m, 1H), 3.55 (m, 1H), 3.63 (dd, 1H, *J* = 5.3 Hz, *J* = 2.7 Hz), 3.77 (s, 3H), 4.29 (m, 1H), 6.85 (d, 2H, J = 9.0 Hz), 7.28 (d, 2H, J = 9.0 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) : δ = -4.9, -4.2, 17.9, 22.7, 25.7, 40.4, 55.6, 56.7, 65.4, 114.4, 117.5, 132.3, 156.0, 158.8, 165.1. IR (cm^{-1}) : υ = 2854-2955, 1743, 1514, 1466, 1391, 1246. HRMS : $C_{18}H_{29}NO_3SiNa$: calculated : 358.1814, found : 358.1801.

4-Phenylbutyl-2-(pent-4-enamidomethyl)but-2-enoate (15). 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 was 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 colourless oil was obtained. Yield: 47% (30 mg from 0.19 mmol of **16)**. R_f = 0.22 (cyclohexane/ethyl acetate : 5/3). MS (ESI): *m/z*: 330.18 ($(M + H)^+$), 352.22 ($(M + Na)^+$). ^{1}H NMR (300 MHz, $CDCl_3$) : δ = 1.67-1.80 (m, 4H), 1.98 (d, 3H, *J* = 7.2 Hz), 2.21 (m, 2H), 2.35 (m, 2H), 2.65 (m, 2H), 4.10 (d, 2H, J = 6 Hz), 4.17 (m, 2H), 4.90-5.10 (m, 2H), 5.79 (m, 1H), 6.02 (broad s, 1H), 6.96 (q, 1H, J = 7.2 Hz), 7.15-7.32 (m, 5H). ^{13}C NMR (75 MHz, $CDCl_3$) : δ = 14.7, 27.9, 28.4, 29.6, 35.2, 35.6, 35.9, 64.7, 115.6, 126.1, 128.6, 129.8, 137.1, 141.5, 167.5, 171.8. IR (cm^{-1}) : υ = 2853-2924, 1705, 1651, 1452, 1288.

Discussion about rearrangement of hydroxyketene:

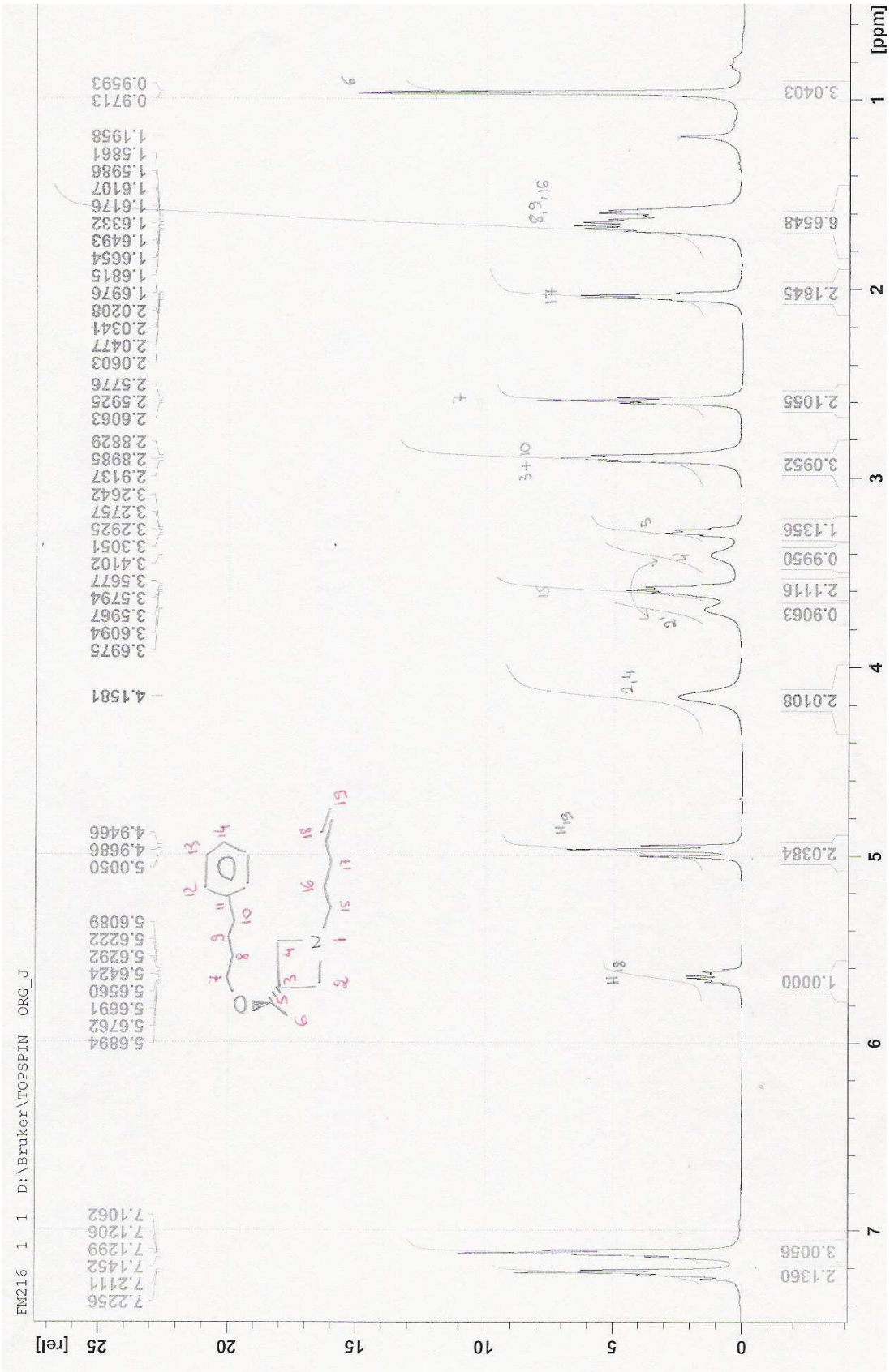


In our mind, there are two likely explanations concerning the rearrangement of the hydroxyketene (b): i) an adventitious and catalytic amount of water from the medium hydrolyzed the ketene function leading to the elimination of hydroxyle and to the formation of α,β-unsaturated carboxylate (c). ii) The dehydratation occurs at first, spontaneously, and the resulting water molecule hydrolyzed the ketene into α,β-unsaturated carboxylate (c). In both cases, the carboxylate form, obtained by proton transfer, reacted with bromide to lead to the corresponding ester **15**.

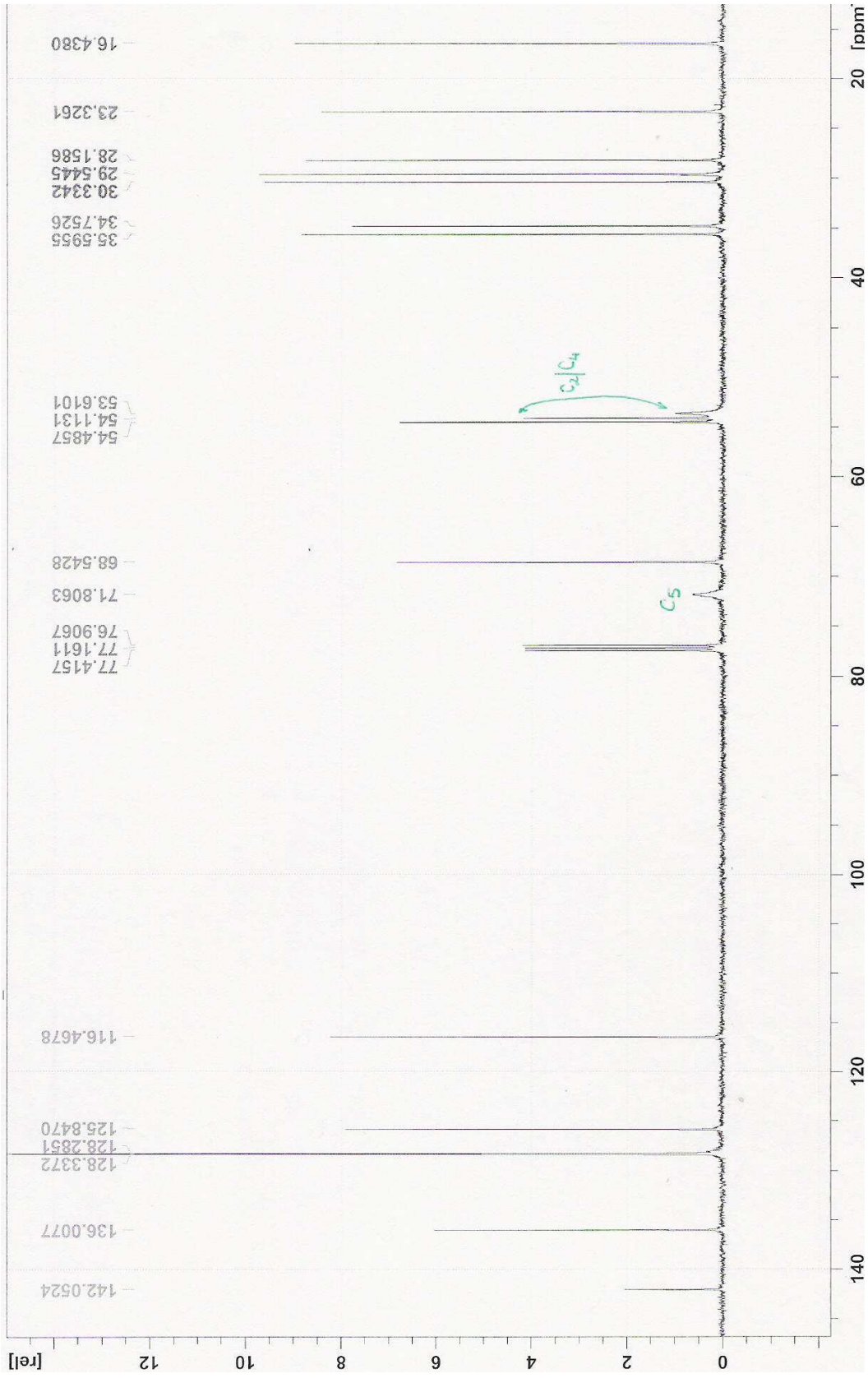


Direct hydrolysis of **14** and/or the derived alkoxide (OH deprotonation) was not considered because we worked under controlled anhydrous conditions.

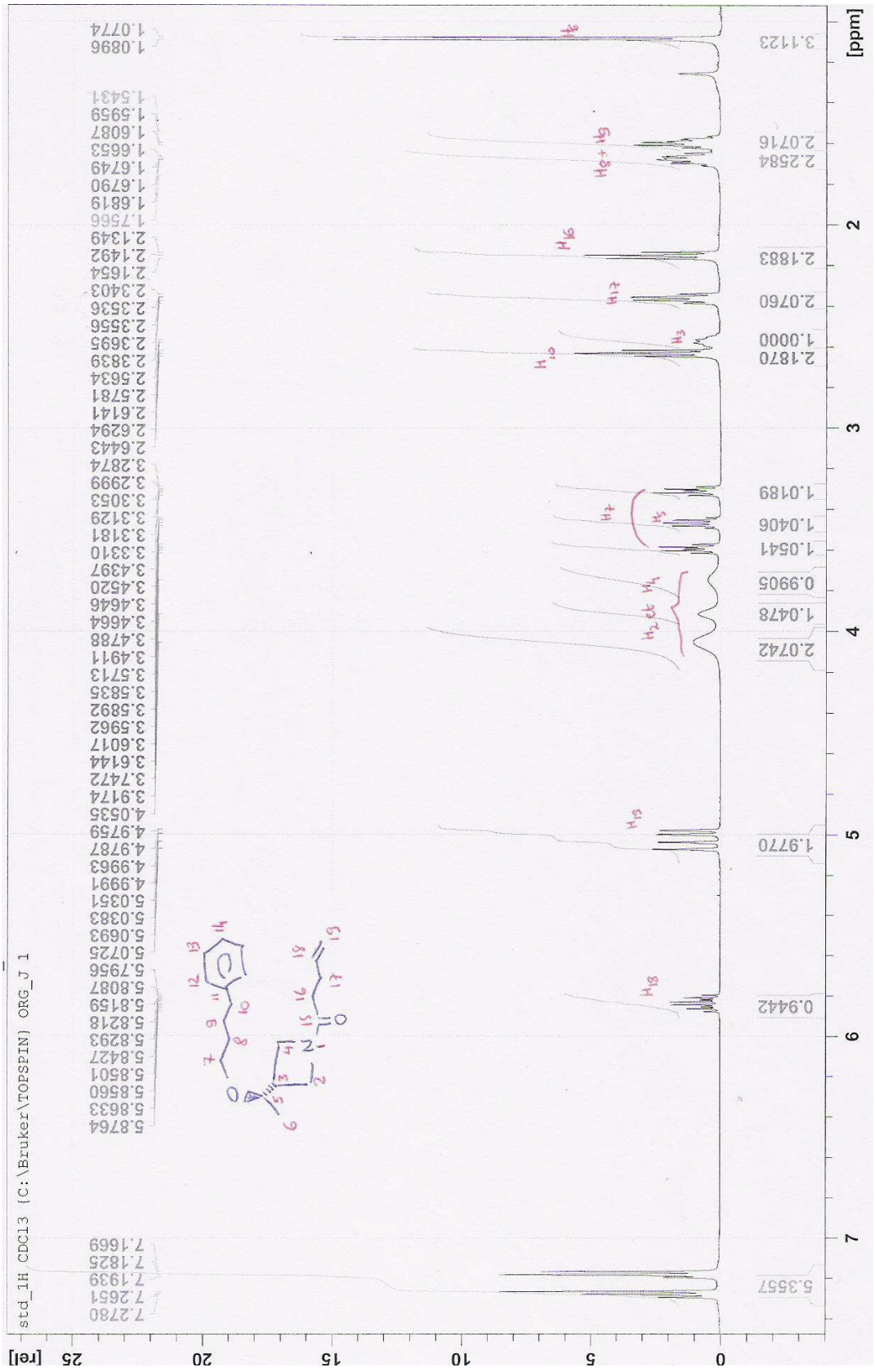
^{1}H NMR spectra of compound 7 in $CDCl_3$ (500 MHz, 25 °C).



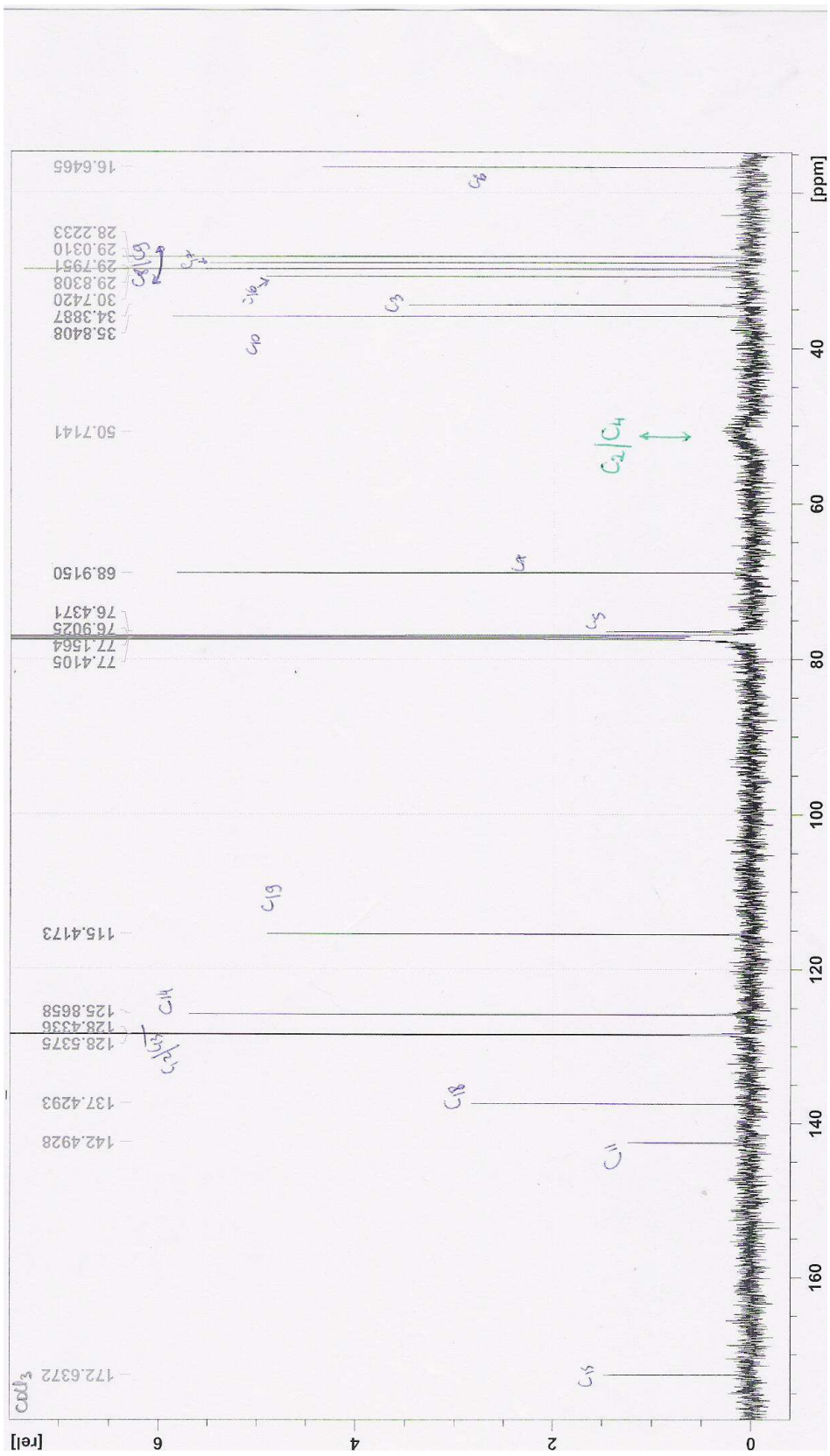
^{13}C NMR spectra of compound 7 in $CDCl_3$ (125 MHz, 25 °C).



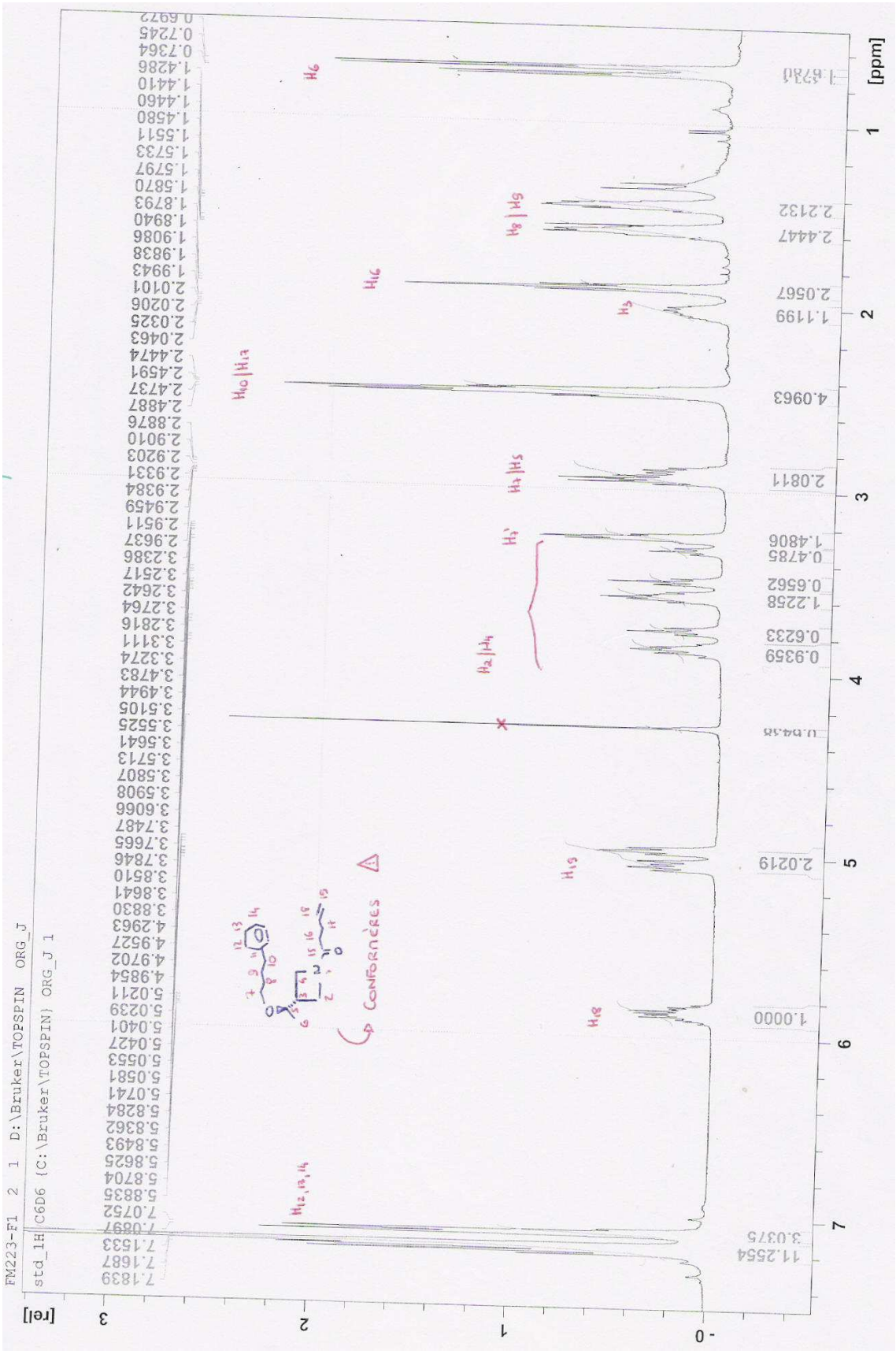
^{1}H NMR spectra of compound 8 in $CDCl_3$ (500 MHz, 25 °C).



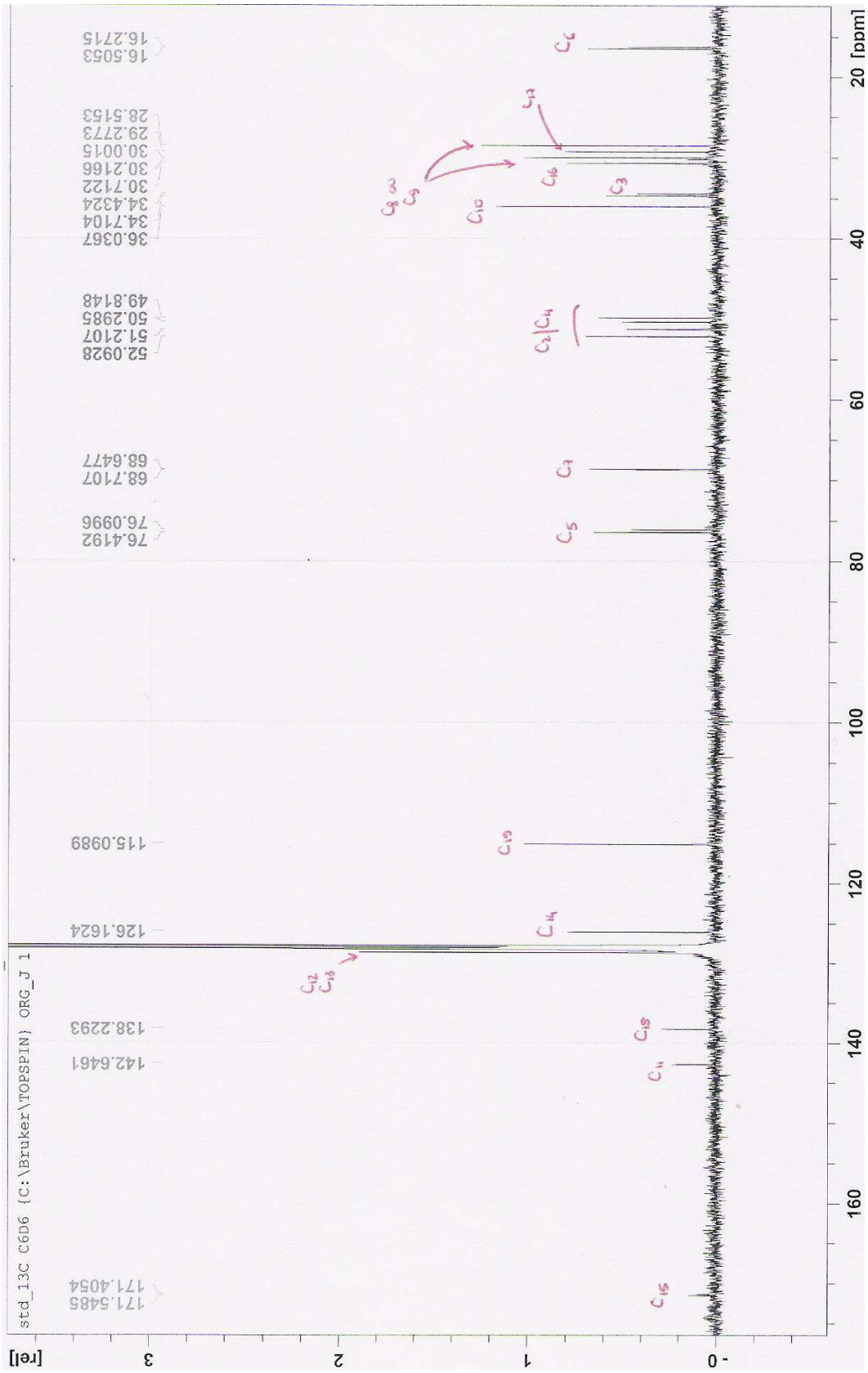
^{13}C NMR spectra of compound 8 in $CDCl_3$ (125 MHz, 25 °C).



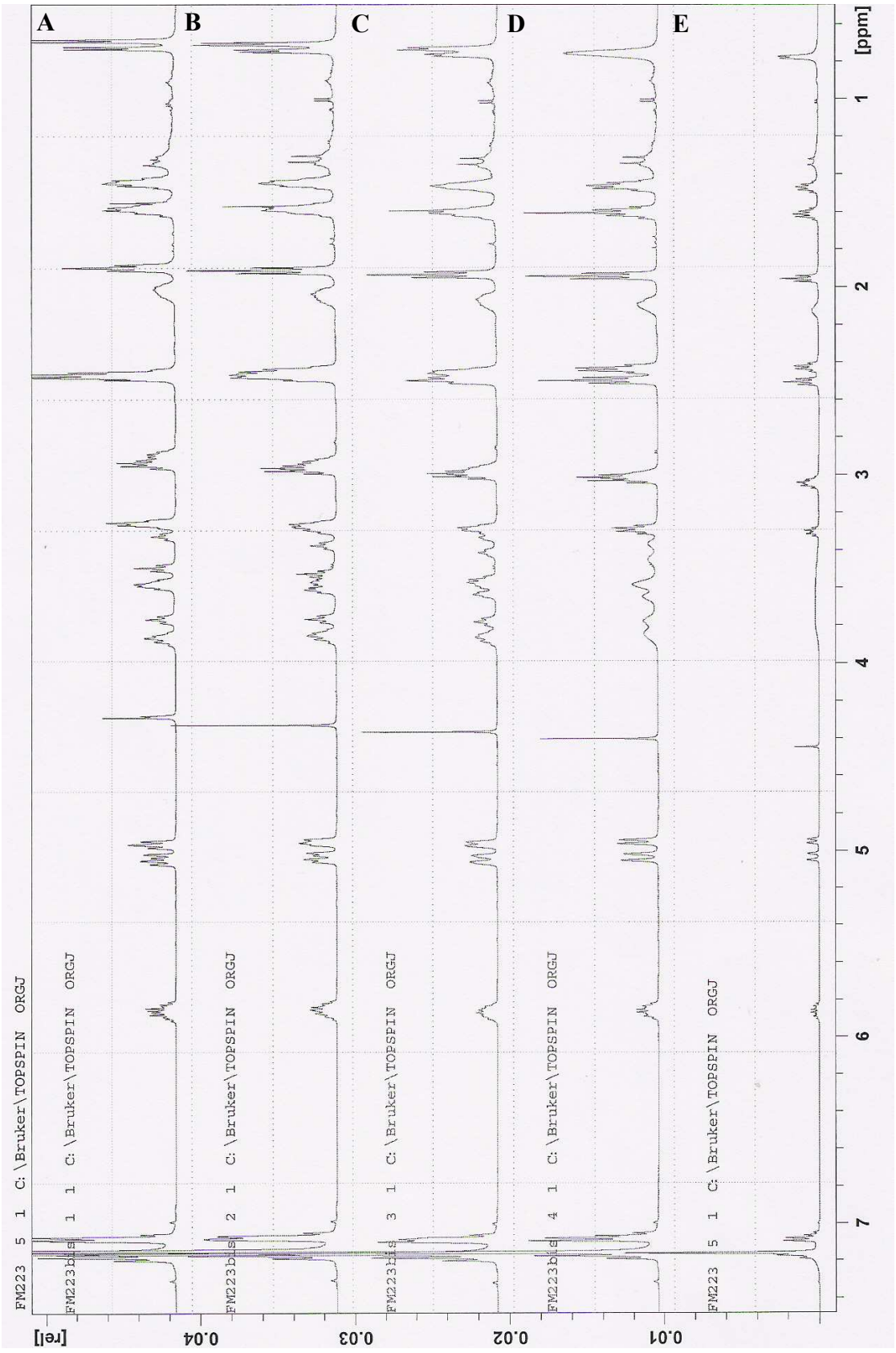
^{1}H NMR spectra of compound 8 in C_6D_6 (500 MHz, 25 °C).



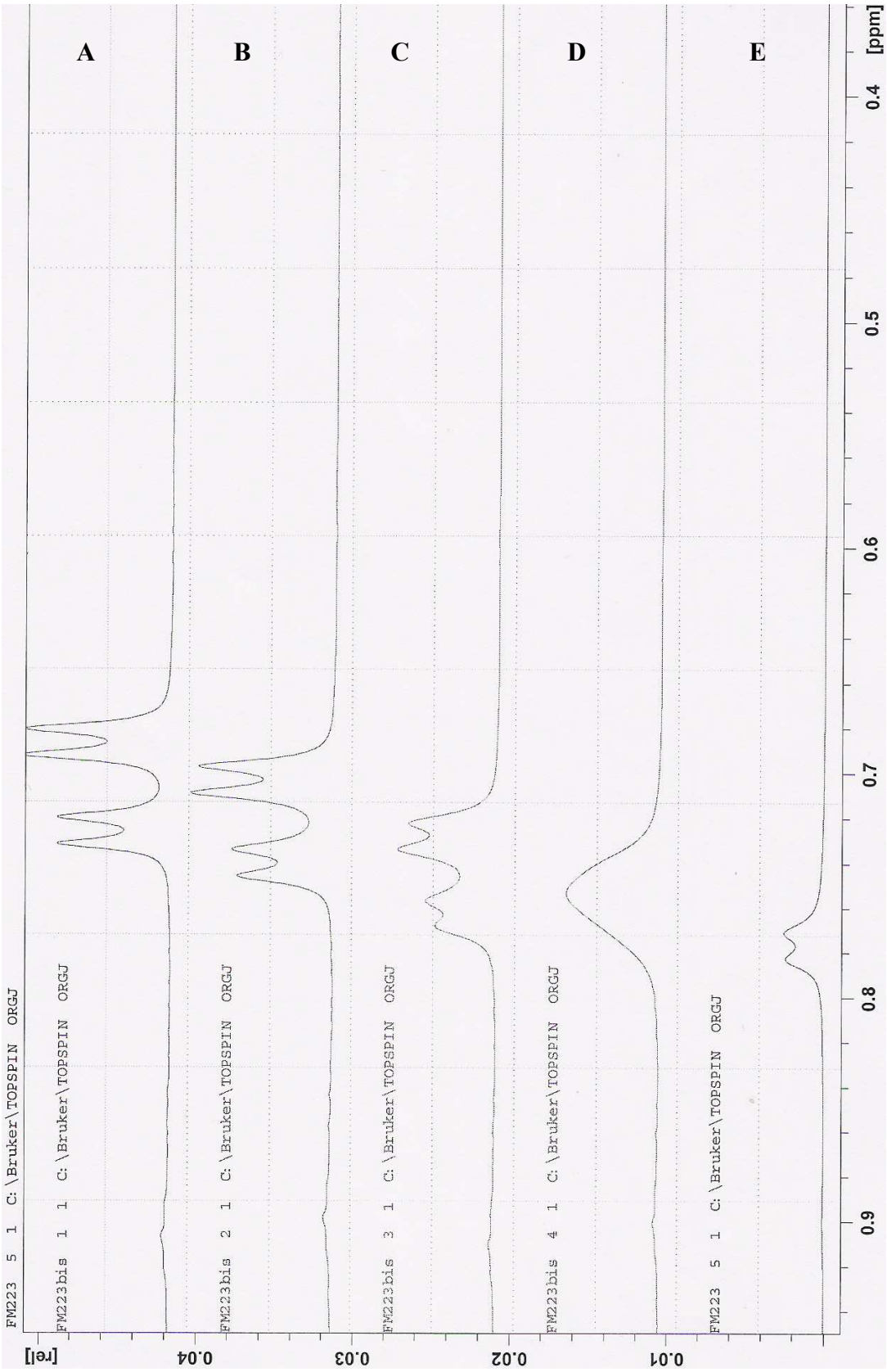
^{13}C NMR spectra of compound 8 in C_6D_6 (125 MHz, 25 °C).



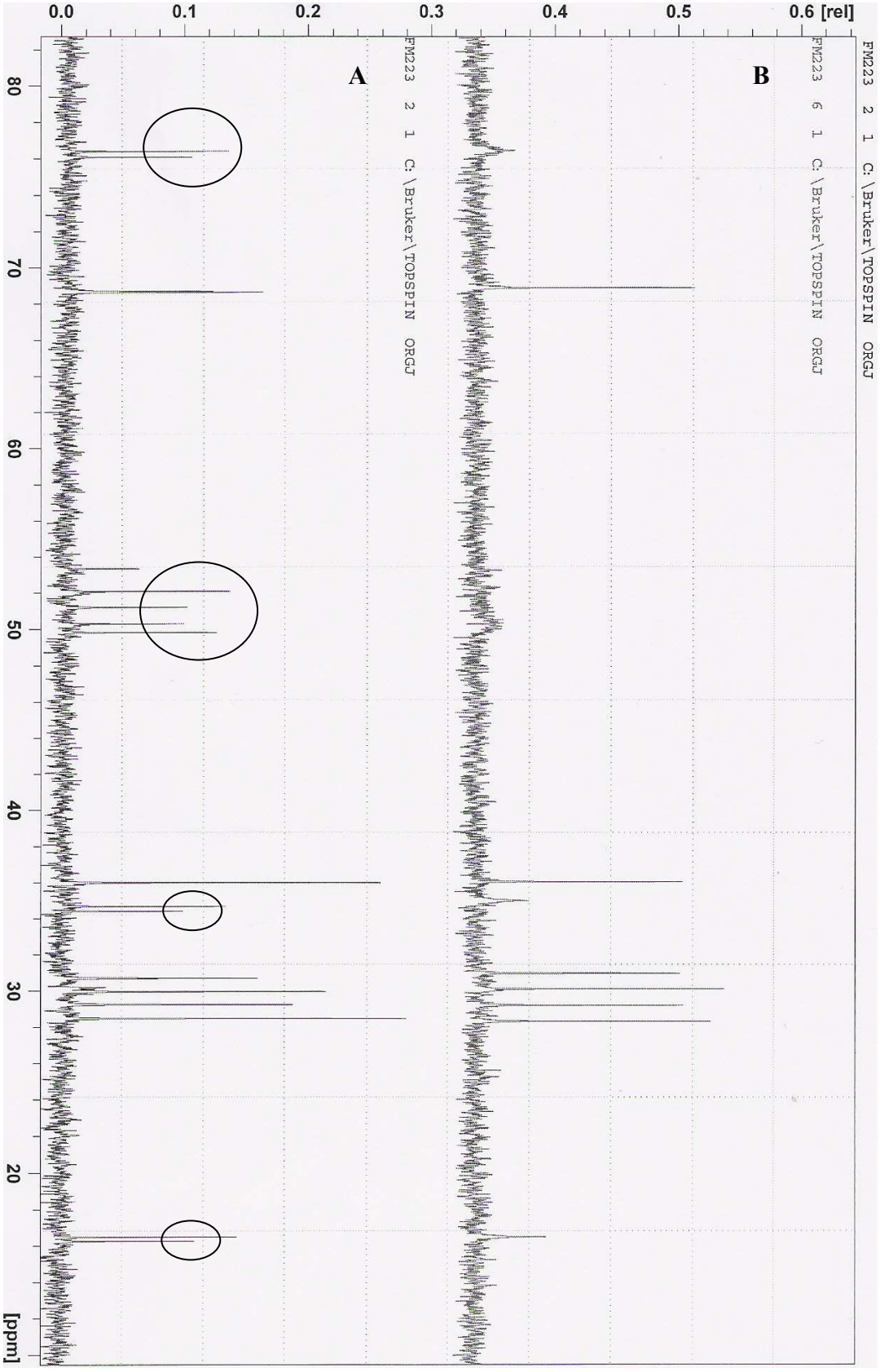
^{1}H NMR spectra of compound 8 in C_6D_6 (500 MHz). A) at 25 °C, **B**) at 37 °C, **C**) at 45 °C, **D**) at 60 °C and **E**) at 75 °C.



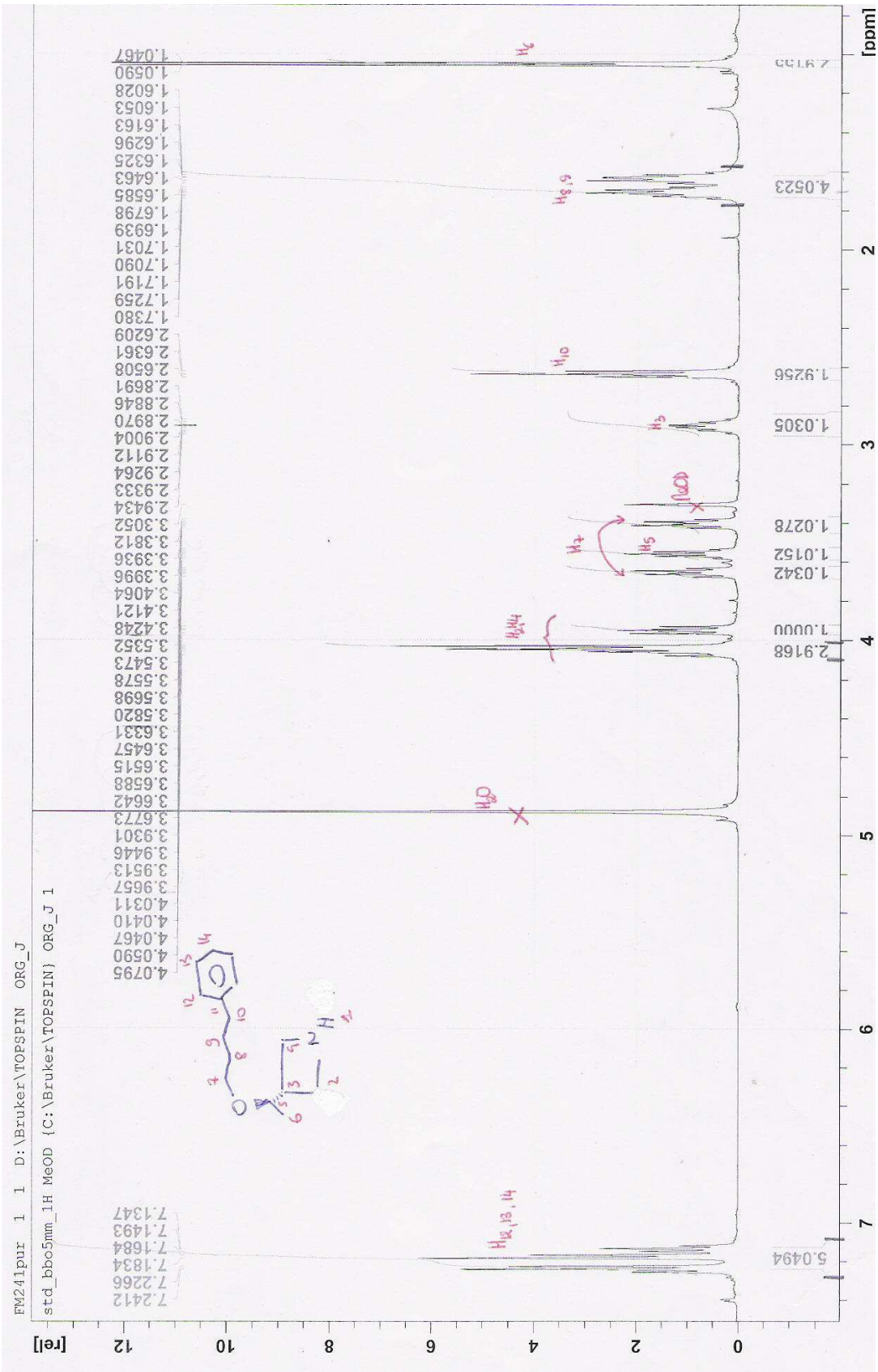
^{1}H NMR spectra of compound 8 in C_6D_6 (500 MHz). A) at 25 °C, **B**) at 37 °C, **C**) at 45 °C, **D**) at 60 °C and **E**) at 75 °C.



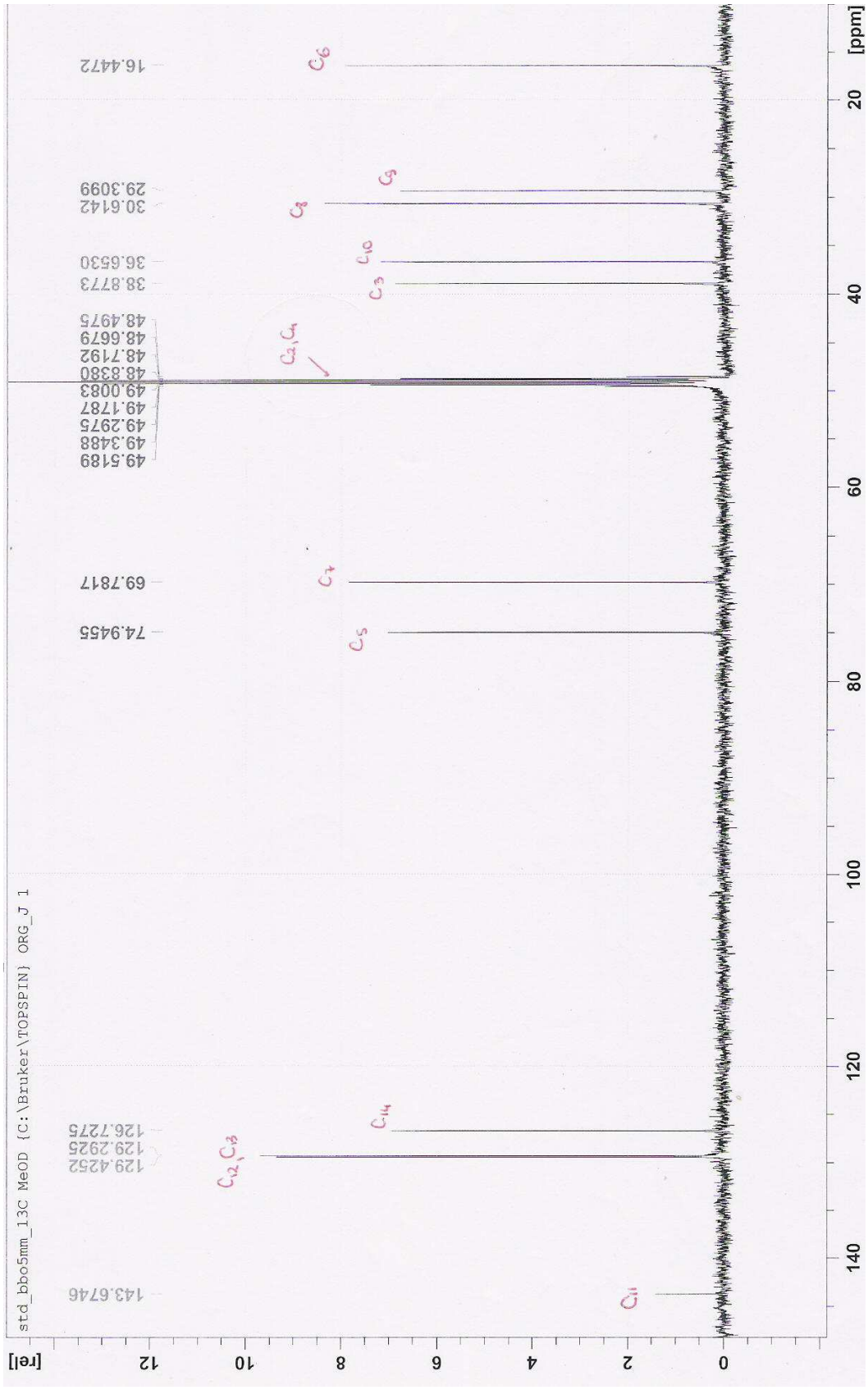
^{13}C NMR spectra of compound 8 in C_6D_6 (125 MHz). **A**) at 25 °C and **B**) at 75 °C. Surrounded peaks which correspond to *β*-lactam ring carbons or are close to it, are split into two at 25 °C (**A**) and disappear at 75 °C (**B**).



[1]H NMR spectra of compound 20 in MeOD (500 MHz).



^{13}C NMR spectra of compound 20 in MeOD (125 MHz).



^{13}C NMR spectra of compound 20 in C_6D_6 (125 MHz).

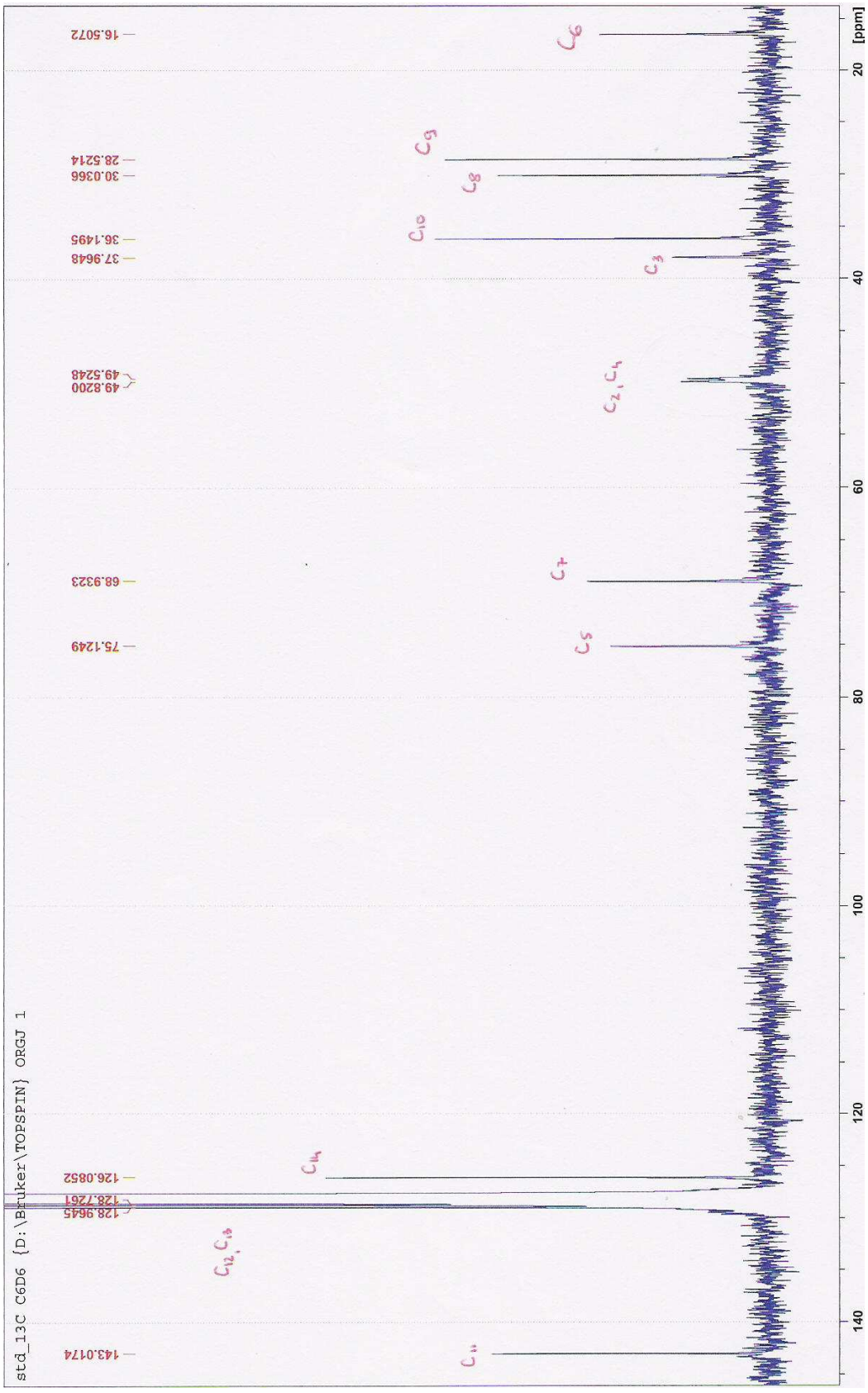


Figure 1. HPLC-MS analysis of **4** hydrolysis by liver homogenate.
A) appearance of ion m/z=198.11302 compared to denatured liver.
B) appearance of ion m/z=262.14432 compared to denatured liver.

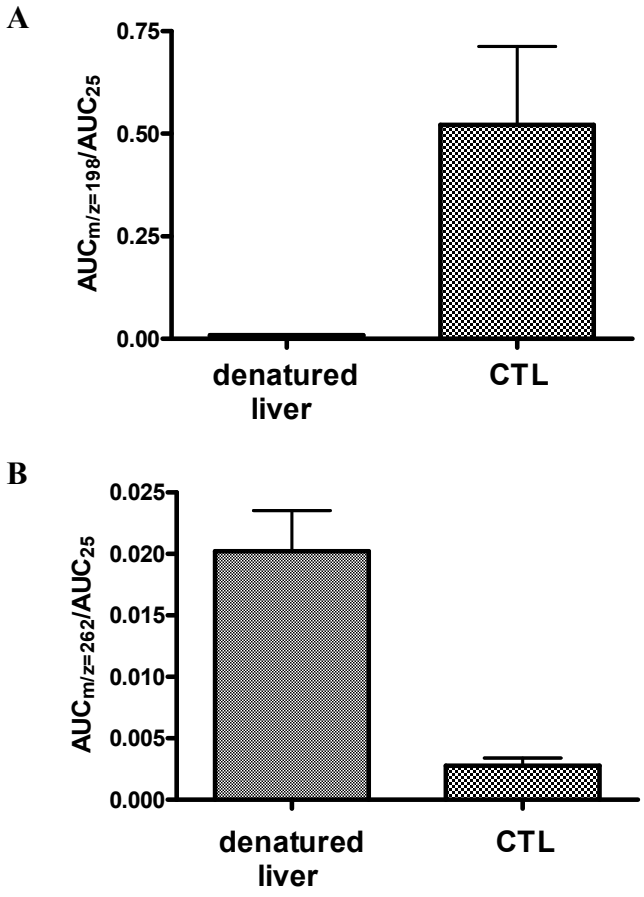
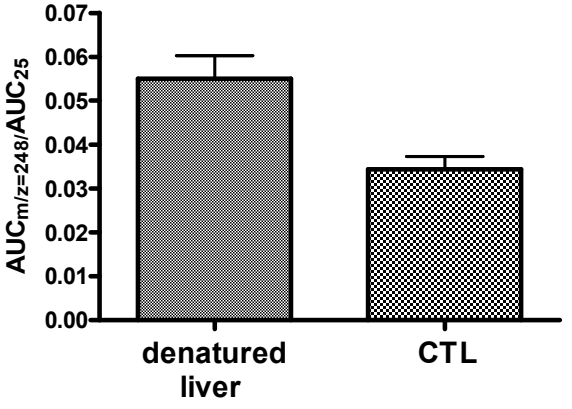


Figure 2. HPLC-MS analysis of **9** hydrolysis by liver homogenate.
Appearance of ion m/z=248.16505 compared to denatured liver.



An unprecedented reversible mode of action of β-lactams for the inhibition of human Fatty Acid Amide Hydrolase (*h*FAAH)

Marion Feledziak, Didier M. Lambert, and Jacqueline Marchand-Brynaert.

Supporting information

Table of contents

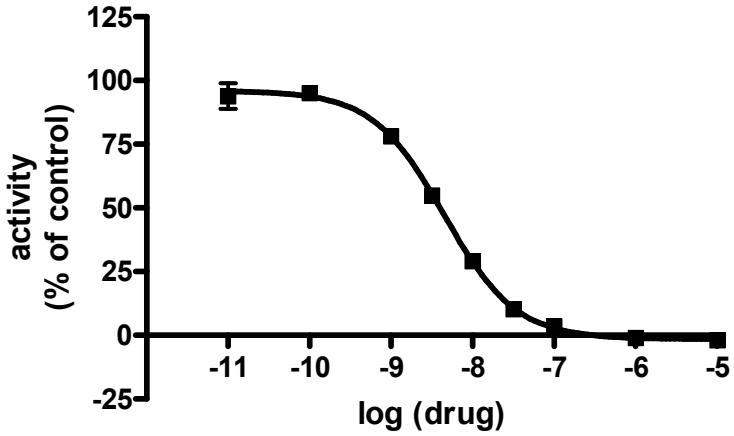
Synthesis of compound 11.

To a solution of **10a** (1 equiv) in dry DCM (9 mL/mmol), under argon atmosphere, were added triethylamine (1.1 equiv) and phenethylisocyanate (1.1 equiv). The reaction mixture was stirred over night at room temperature and diluted with DCM. The solution was extracted with water three times and the aqueous layers were extracted with DCM. The organic layers were combined, dried over $MgSO_4$, filtered and concentrated under vacuum. After purification by flash chromatography (DCM/AcOEt), a colourless oil was obtained.

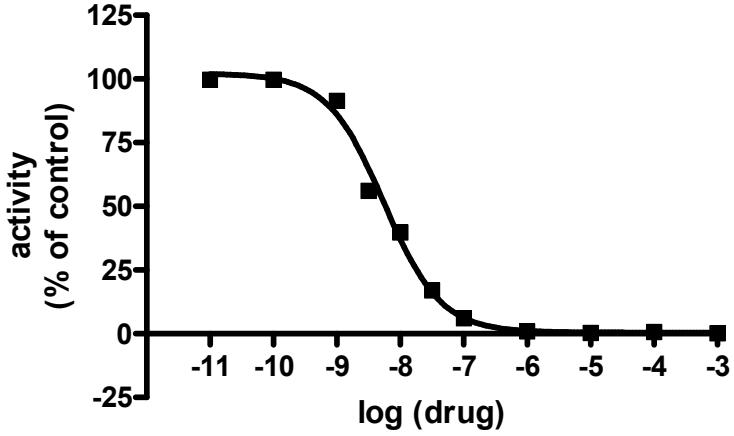
(11): Purification by flash chromatography (DCM/AcOEt 1:1) gave **11** (mg, %) as a colourless oil: *R*f =0.21 (DCM/AcOEt, 1:1); ^{1}H NMR (300MHz, $CDCl_3$): δ=1.89 (d, J =7.0 Hz, 3H), 2.21-2.30 (m, 2H), 2.32-2.41 (m, 2H), 2.84-2.91 (m, 2H), 3.51-3.62 (m, 2H), 4.12 (d, J =6.4 Hz, 2H), 4.97-5.12 (m, 2H), 5.72-5.90 (m, 1H), 6.22 (br s, 1H), 6.50-6.61 (m, 1H), 6.90 (br s, 1H), 7.20-7.35 ppm (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$): δ=14.1, 29.5, 35.3, 35.6, 35.7, 41.1, 115.8, 126.5, 128.7, 128.8, 133.4, 134.8, 136.9, 139.1, 167.9, 172.8 ppm.

Selected representatives 'Dose-response' curves, **4b** (a), **4c** (b), **4d** (c) and **4e** (d).

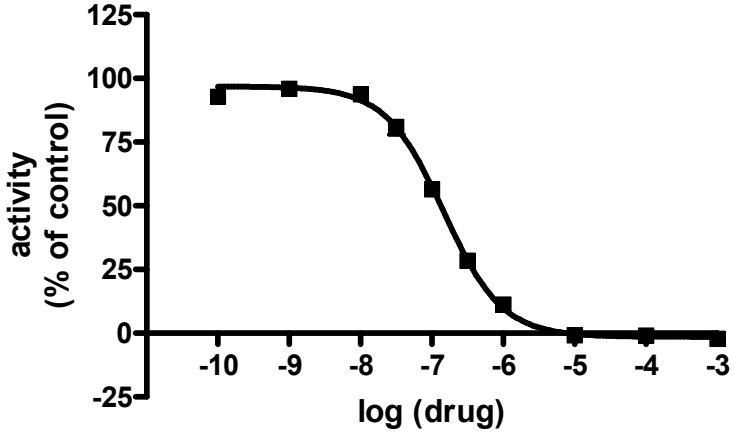
a.



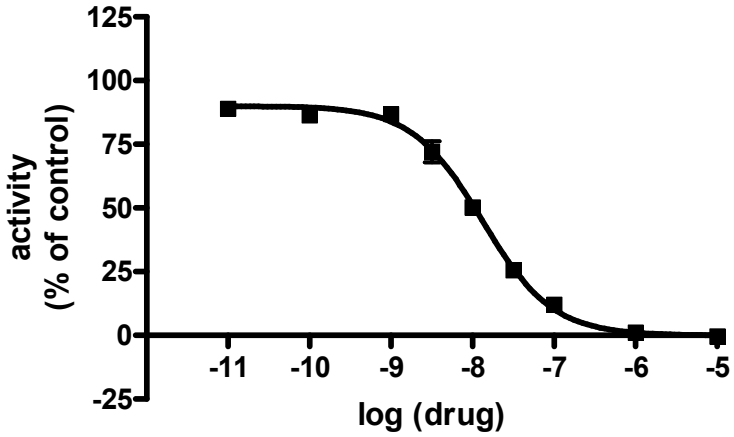
b.



c.



d.



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During six months, Joséphine Caruano, a student in Master II at the University of Grenoble (France), was welcomed to do a training in our laboratory. Throughout this experience, she learnt the chemistry of β-lactams and the pharmacology of FAAH and MAGL. To this end, J. Caruano synthesized a novel family of β-lactamic compounds, inspired from the work of this thesis. In order to possibly optimize FAAH inhibition and/or MAGL inhibition, a SAR study was performed with all of her compounds. The results of this training are presented herein.

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Université catholique de Louvain
Institute of Condensed Matter and Nanoscience (LLN)
Louvain Drug Research Institute (LEW)
Medicinal chemistry groups

"Synthèse et évaluation pharmacologique d'une nouvelle famille de β-lactames comme inhibiteurs de la FAAH et de la MAGL"

Joséphine Caruano

2012

Promoters:
Jacqueline Marchand-Brynaert
Marion Feledziak

Introduction

The aim of the project was to evaluate the importance of the position of the lipophilic arm connected at C3-C5-O position in our lead structure (**1**, Figure 1)[1]. Thus, J. Caruano synthesized a series of compounds where this ester chain was connected at the C4-C5-O position (**2a-g**, Figure 1). The N-acyl chain was conserved from the lead compound (pentenoyl chain) and the O-acyl chain was modulated with various lipophilic groups (R = Ph, BiPh, 3-ind, OBn, 2-pyr and 4-pyr) conserving the optimal length of the chain.

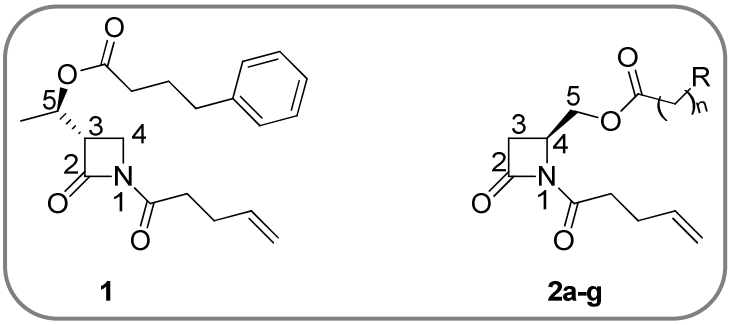


Figure 1. From our lead compound to novel potential β-lactamic inhibitors of FAAH

The design of such compounds was facilitated by the fact that our laboratory has the chiral precursor **3** at disposal for the synthesis of **2a-g** (Figure 2). This precursor was prepared by S. Gérard during his thesis (2002), in view of synthesizing a set of inhibitors of human leucocyte elastase (HLE), another serine hydrolase.[2]

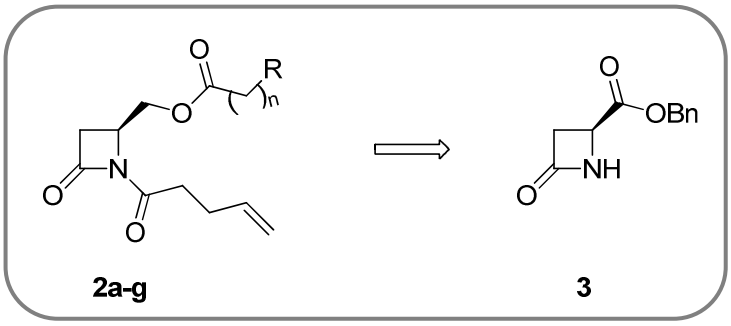


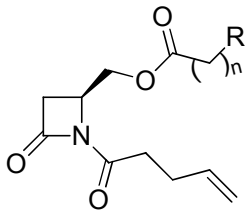
Figure 2. Synthesis of compounds **2a-g** from precursor **3**

Results and conclusion

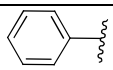
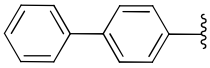
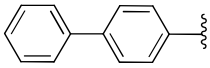
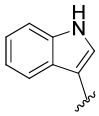
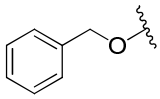
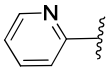
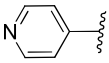
Thus, from the precursor **3** and using classical protocols of reduction and acylation, J. Caruano obtained seven compounds (**2a-g**) equipped with two lipophilic arms. All the

compounds were tested in FAAH inhibition and for some in MAGL inhibition. The results are resumed in table 1.

Table 1.



2a-g

compound	n	R	IC_{50} (hFAAH)[a]	IC_{50} (hMAGL)[a]
2a	3		5030	108100
2b	0		915	/
2c	1		7817	/
2d	2		8049	166100
2e	1		42520	/
2f	3		87060	/
2g	3		173000	/

[a] IC_{50} in μM

As depicted in the table 1, all the compounds exhibit a moderate activity against hFAAH and a very weak activity against hMAGL. This suggests that the ester chain at the C4 position does not fit the catalytic pocket as well as at the C3 position. In our previous publication, we docked the lead compound **1** in a model of hFAAH catalytic site. We showed that the ester chain was in interaction with a lipophilic pocket consisting in three phenylalanine residues (F192, F381 and F388, Figure 3). This lipophilic cavity is probably not reached by interconverting the ester chain. However, we can observe that selectivity for hFAAH towards hMAGL is conserved even though the activity against FAAH is low.

It appears interesting to perform docking studies to explain the loss of activity and to completely conclude this work.

This work will be submitted as a letter in Bioorganic and Medicinal Chemistry Letters.

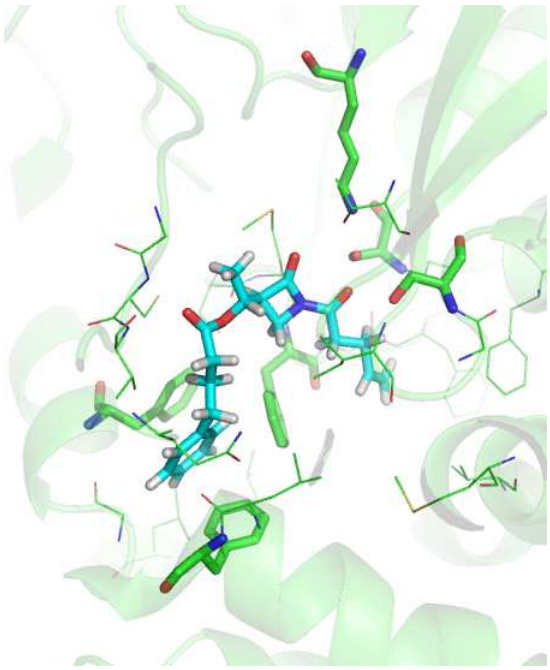


Figure 3. Proposed binding mode of **1** into the model of human FAAH

References

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2. Gérard, S., Galleni, M., Dive, G. and Marchand-Brynaert, J. Synthesis and evaluation of N1/C4-substituted β-lactams as PPE and HLE inhibitors. *Bioorg. Med. Chem.* **2004**, 12(1): 129-138.