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3-Alkenyl-2-azetidinones as fatty acid amide hydrolase inhibitors

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ABSTRACT

A series of novel 2-azetidinones (β-lactams) bearing short alkenyl chains at C3 and N1 have been prepared and evaluated in vitro as inhibitors of human FAAH. Compound **9c** (1-(4′-pentenoyl-3-(4′-pentenyl)-2-azetidinone)) featured an IC_{50} value of 4.5 μM and a good selectivity for FAAH versus MGL.

Cannabis possesses multiple and beneficial therapeutic properties, exploited for centuries in the traditional Chinese and Indian medicine (analgesic, sedative, febrifuge, antidepressant, ...). Nowadays, cannabis extracts and derivatives are mainly known for their illicit uses as psychotropic agents inducing unacceptable adverse effects. However, Δ^9-tetrahydrocannabinol (Δ^9-THC), the main psychoactive component of cannabis (Fig. 1, structure **A**), is able to prevent nausea and vomiting, to stimulate appetite, to decrease intraocular pressure, to provoke bronchodilatation, amongst many other activities.[1] The cloning of two cannabinoid receptors mediating the pharmacological effects of Δ^9-THC, about 15 years ago,[2] stimulated the search of endogenous ligands of these receptors. At the present time, several endocannabinoids[3] have been disclosed: they are fatty acid amides and esters, such as arachidonoylethanolamide (AEA, anandamide) (Fig. 1, structure **B**) and 2-arachidonoylglycerol (2-AG) (Fig. 1, structure **C**).

The endocannabinoids which share pharmacological properties with the exogenous ligand Δ^9-THC offers new opportunities in therapeutic strategies avoiding undesirable side effects.[3] Nevertheless, the effective medical use of AEA and 2-AG is severely hampered by their rapid inactivation due to enzymic degradation. Two major enzymes are involved in catabolic regulation of these two lipid messengers, namely fatty acid amide hydrolase (FAAH)[4] and monoglyceride lipase (MGL),[5] respectively.

FAAH is a serine hydrolase which does not exert its activity through the Ser-His-Asp triad as commonly observed in this family, but through the unusual Ser-Ser-Lys triad: Ser 241 acts as the nucleophile and Lys 142 serves as general acid/base catalyst, while the role of Ser 217 is still unclear.[4a] FAAH inhibitors are expected to bring therapeutic advances in fields like the control of pain and anxiety, the treatment of inflammatory conditions, or sleep disorders.[6]

MGL (also named monoacylglycerol lipase) is also a serine hydrolase featuring the catalytic triad Ser122-Asp239-His269. Its study and the search of inhibitors, comparatively to FAAH, are quite recent in the literature.[7]

To date, two main classes of FAAH inhibitors with therapeutic potential have been disclosed: aromatic carbamates and ureas[8] that irreversibly acylate the active site serine and α-keto-oxazole

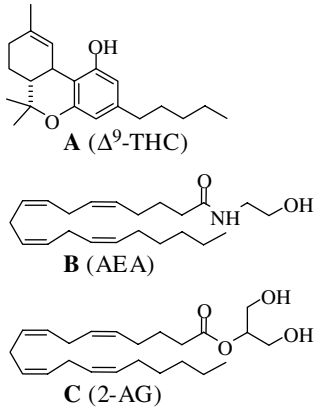

Figure 1. Cannabinoid derivatives.

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compounds[9] that form reversible hemiketals with the active site serine. Other heterocyclic structures have been investigated,[10] but surprisingly, 2-azetidinone derivatives (also named β-lactams) have never been reported as potential inhibitors of FAAH, though this family of highly reactive amides is well known to inhibit serine enzymes[11] of bacterial or mammalian origins. For instance, penicillin and cephalosporin antibiotics are inhibitors of D, D-peptidases of the bacterial cell wall (Fig. 2, structures **D** and **E**),[12] penam sulfones are inhibitors of β-lactamases, the bacterial defence enzymes (Fig. 2, structure **F**),[12] and monocyclic azetidinones are inhibitors of human leukocyte elastase (Fig. 2, structure **G**).[13]

In this paper, we describe the synthesis of a series of monocyclic 2-azetidinones equipped in positions C3/N1 with short alkenyl chains and their evaluation in vitro as potential inhibitors of FAAH and MGL human enzymes. Inhibitors featuring an electrophilic carbonyl as warhead to interact with the active serine Ser 241, such as α-keto-oxazoles, are usually substituted with two lipophilic side chains of various lengths. We selected relatively short alkenyl chains (limited to 5-carbons) to decorate the azetidinone scaffold, essentially for performing preliminary biochemical evaluations with compounds easily prepared and well soluble in the assay medium. The alkene motifs offer the possibility to further functionalise the molecules (chain lengthening by olefin metathesis reaction, for instance). Considering the published crystal structure of rat FAAH bound to methyl arachidonyl fluorosphosphonate (MAFP) as a model,[9d] we assumed that C3 or/and N1-substituted azetidinones could fit into the active site, with the appended groups located in the lipophilic pocket, depending on the initial positioning of the β-lactam core.

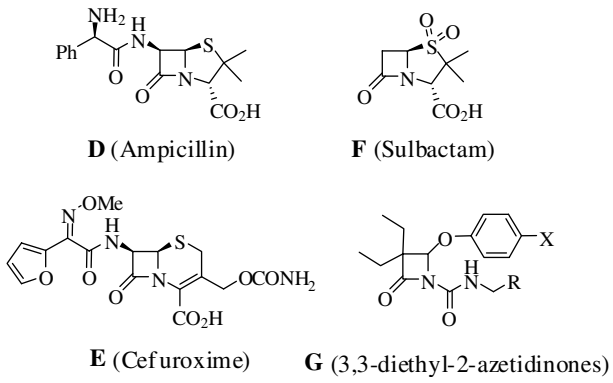
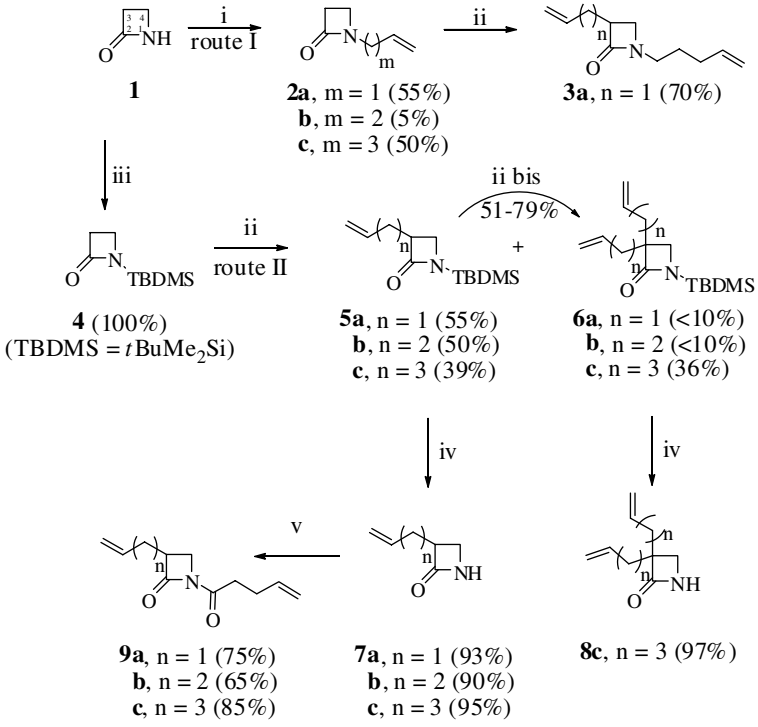


Figure 2. Azetidinone inhibitors of serine proteases.

The previous publications related to this kind of β-lactam functionalisation mainly concern the positions C4 and N1, in particular for the construction of 1,4-fused rings by olefin metathesis reaction, and utilize 4-acetoxy-2-azetidinone as starting material.[14] Our strategy towards 1,3-substituted 2-azetidinones makes use of the commercially available parent compound **1**, considering two synthetic routes: (I) N1 alkylation followed by C3 alkylation and (II) C3 alkylation followed by N1 functionalisation. As detailed below, only the second route allowed to reach the C3 structural variety required for preliminary biochemical evaluations. Scheme 1 summarises our synthetic results.

β-Lactam **1** was *N*-alkylated with allyl bromide (**a**), 1-butenyl bromide (**b**) and 1-pentenyl bromide (**c**) under solid–liquid phase transfer conditions. The method of Tarling[14a] (KOH, 18-crown-6, benzene, 20 °C, RBr) gave poor yields of *N*-substituted azetidinones **2**, while the method of Zbigniew[15] (Scheme 1, step i) furnished **2a** and **2c** in 55% and 50% yield, respectively, after chromatography. But only 5% yield of **2b** could be isolated because elimination occurred in the case of 1-butenyl bromide. Other conditions were tested without success (LiHMDS, THF, −50 °C; NaH, DMF, 0 °C). The enolate of **2c** was formed by deprotonation with LDA at low temperature;[16] quenching with allyl bromide led to 1-(4′-pentenyl)-3-(2′-propenyl)-2-azetidinone (**3a**) in good yield (route I, step



Scheme 1. Synthesis of 1,3-substituted azetidinones. Reagents and conditions: (i) alkenyl bromide, K_2CO_3, Bu_4NBr catal., CH_3CN, 80 °C, 15 h; (ii) LDA, THF, −78 °C, 0.5–1 h; then alkenyl bromide, −78 to 20 °C, 15 h; (iii) $^tBuMe_2SiCl$, DIEA, CH_2Cl_2, 20 °C, 24 h; (iv) CsF, MeOH, 20 °C, 2 h; (v) acid chloride, pyridine, CH_2Cl_2, 20 °C, 4 h.

ii). But no product could be obtained when treating the enolate of **2c** with 3-butenyl or 4-pentenyl bromides. We found that the C3 alkylation of β-lactam **1** (route II) was more conveniently performed after *N*-protection by silylation. *tert*-Butyldimethylsilyl derivative **4** (Scheme 1, step iii) was prepared as usual, then enolate was formed (LDA, THF, −78 °C) and reacted with the alkenyl bromides. In all cases, a mixture of mono- and bis-alkylated products **5** and **6** was recovered, in variable relative amounts, depending on the quantities of base (1 to 1.5 equivalents) and bromide (1.2 to 5 equivalents) used. Compounds **5** and **6** could be separated by column chromatography: the best yields of **5a**, **5b**, **5c** and **6c** were obtained with 1.1 equivalents of LDA/1.1 equivalents of bromide and 1.5 equivalents of LDA/5 equivalents of bromide, respectively (route II, step ii). Compounds **6a** and **6b** (step ii bis) were also prepared by a second alkylation of **5a** and **5b** in the same conditions (51% and 79% yields). From our experiments, it appeared that *N*-alkyl azetidinone (**2c**) behaves differently from *N*-silyl azetidinone (**4**). In the first case, the corresponding lithium enolate, in THF solution, is a poor nucleophile (reaction only with allyl bromide, the best electrophile) and a weak base (absence of bis-alkylation product resulting from the deprotonation of **3** by the enolate of **2**). In the second case, the lithium enolate of **4** is more reactive (quenching with all the tested electrophiles) and also more basic (formation of bis-alkylation product in the presence of only one equivalent of LDA). In fact, amide resonance is strongly disfavoured by a *N*-silyl substituent and azetidinone **4** thus looks like a cyclobutanone. TBDMS deprotection was performed quantitatively by treatment with cesium fluoride[16] (Scheme 1, step iv). Disappointingly, attempts to *N*-alkylate **7c** under the phase-transfer conditions of route I were unsuccessful. Therefore, we decided to introduce a N1 side chain via the reaction with an acid chloride as powerful electrophile. All mono-alkenyl azetidinones **7a**, **b** and **c** were easily *N*-acylated with 4-pentenoyl chloride in the presence of pyridine (Scheme 1, step v) to furnish compounds **9a**, **b** and **c** in good yields after chromatographic purification. Azetidinones **2**–**9** were characterised by IR, ^{1}H NMR, ^{13}C NMR and HR-Mass spectroscopies. Table 1 summarises the ^{13}C NMR data: typically the azetidinone C2 carbonyl gave a signal at 167–169 ppm for *N*-alkyl derivatives (**2**–**3**), at 173–178 ppm for *N*-silyl derivatives (**4**–**6**), at 171–174 ppm for N–H derivatives (**7**–**8**) and at 167–168 ppm for *N*-acyl derivatives (**9**). In the ^{1}H NMR spectra, H3 proton of monosubstituted derivatives (**3**, **5**, **7** and **9**) gave a multiplet at 3.20–3.30 δ; the two H4 protons appeared as two doublets of doublets with a geminal coupling constant of 5.6–6.6 Hz and the vicinal cis and trans coupling constants of 6.0–6.6 Hz and 1.6–3.0 Hz, respectively. The chemical shifts of H4-*cis* and H4-*trans* were 3.20–3.30 δ and 2.85–2.95 δ for compounds **3**, **5** and **7**. The *N*-acyl substituent of **9** induced a deshielding of about 0.3 δ (H4-*cis* at 3.64-3.67 δ and H4-*trans* at 3.24–3.28 δ). The 3,3′-bis-substituted azetidinones (**6**, **8**) showed a singlet at 2.97–3.04 δ for the two H4 protons. (see Supplementary data for complete characterisation).[17]

In conclusion, by using 1-TBDMS-2-azetidinone (**4**) as starting material (route II) instead of 2-azetidinone (**1**, route I), we were able to prepare C3-substituted azetidinones bearing alkenyl chains of various lengths (see Supplementary data for organic synthesis complete description). The compounds **5**–**9**, which issued from this synthetic approach were tested against the target enzymes.

The novel azetidinones have been evaluated in a competitive hydrolytic assay of human FAAH (overexpressed in *Escherichia coli*) and human MGL (overexpressed in *E. coli*) versus their [^{3}H]-radiolabelled natural substrates AEA and 2-OG, respectively.[18,19] The results reported in Table 2 show that all compounds **5**–**9** interact with FAAH and MGL and that **9b** and **9c** behave as good FAAH inhibitors. Except for **5a** and **5b**, azetidinones were more active against FAAH than against MGL. Inhibition effect of the C3 geminally substituted product was less efficient in comparison to that of the C3/N1-disubstituted product (compare **6c** with **5c**, and **8c** with **7c**; see Table 2, entries 3–4 and 7–8). The best inhibitors of FAAH were the 3-(4′-pentenyl)-2-azetidinones (**5c**, **7c** and **9c**; see Table 2, entries 3, 7 and 11) versus their lower homologs (**5a**, **b**, **7a**, **b** and **9a**, **b**, respectively). Moreover, the presence of a N1 acyl side chain greatly enhanced the inhibitory activity (series **9** compared to **7** and **5**). IC_{50} values were determined for the most active compounds. In particular 3-(4′-pentenyl)-1-(4′-pentenoyl)-2-aze-

Table 2
Biochemical evaluation

Entry	Compound	FAAH %inhibition[a]	IC_{50} (μM)	MGL %inhibition[a]	IC_{50} (μM)	Selectivity
1	**5a**[b]	43.6 ± 4.4		77.5 ± 5.2		
2	**5b**[b]	49.5 ± 1.3		69.9 ± 8.9		
3	**5c**[b]	76.9 ± 2.4		54.9 ± 4.7		
4	**6c**	25.2 ± 6.2		10.3 ± 11.8		
5	**7a**[b]	56.6 ± 6.0		15.6 ± 8.7		
6	**7b**[b]	48.0 ± 1.7		8.1 ± 14.7		
7	**7c**[b]	80.9 ± 3.4		54.9 ± 6.0		
8	**8c**	80.5 ± 3.8	259 ± 28	36.3 ± 9.1		
9	**9a**[b]	96.5 ± 0.2	61.8 ± 2.9	46.4 ± 3.5		
10	**9b**[b]	102.9 ± 5.7	21.9 ± 0.5	58.1 ± 7.3	817 ± 151	37
11	**9c**[b]	100.7 ± 2.5	4.5 ± 0.2	62.1 ± 7.0	657 ± 89	146

[a] Inhibition of enzymic activity (% of control) at 10^{-3} M; $n = 3$.
[b] Compound tested as racemic mixture.

Table 1
^{13}C NMR data (125 MHz, $CDCl_3$, ppm)

Compound	C2	C3	C4	3-side chain	1-side chain
2a	167.3	38.7	36.7	/	44.6; 118.2; 131.7
2c	167.4	38.9	36.5	/	26.8; 31.1; 41.4; 115.4; 137.2
3a	169.6	48.4	44.5	32.6; 116.9; 134.3	26.9; 31.1; 41.0; 115.4; 137.2
4	172.7	39.0	36.7	/	−6.1; 18.6; 26.0
5a	175.0	50.5	42.4	32.8; 116.7; 134.3	−6.2; −6.3; 18.3; 25.8
5b	175.7	51.1	43.4	28.5; 31.0; 115.1; 137.5	−6.2; −6.3; 18.3; 25.9
5c	175.9	51.6	43.3	26.1; 28.7; 33.4; 114.7; 138.2	−6.2; −6.3; 18.4; 25.9
6a	176.9	58.9	45.5	37.8; 118.3; 133.4	−6.1; 18.3; 25.9
6b	177.7	59.6	47.6	28.6; 32.5; 114.8; 138.1	−6.0; 18.6; 26.3
6c	178.2	59.9	47.4	23.6; 32.7; 33.9; 114.6; 138.2	−6.2; 18.2; 25.9
7a	171.4	50.2	41.0	32.4; 116.9; 134.1	/
7b	172.1	50.5	41.8	27.8; 30.9; 115.1; 137.2	/
7c	172.2	51.2	41.7	26.1; 28.1; 33.3; 114.7; 138.0	/
8c	174.5	59.8	46.1	23.5; 32.1; 33.8; 114.7; 138.1	/
9a	167.2	47.6	41.9	32.1; 117.9; 132.8	27.8; 35.6; 115.6; 136.3; 170.3
9b	167.8	47.8	42.7	27.6; 30.9; 115.5; 136.3	27.8; 35.5; 115.9; 136.6; 170.3
9c	167.9	48.6	42.8	26.1; 28.0; 33.3; 115.2; 137.6	28.1; 35.8; 115.6; 136.4; 170.4

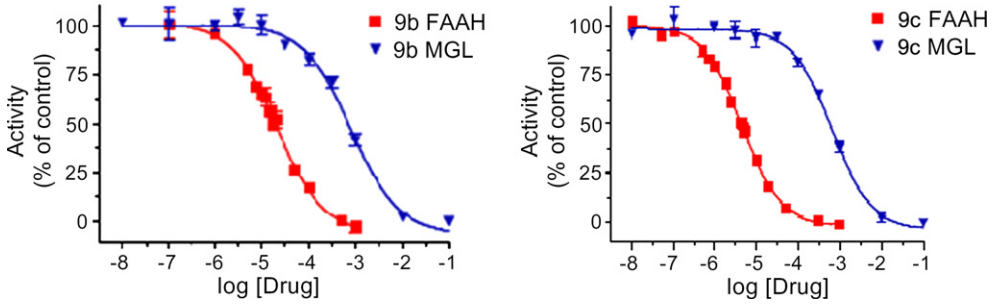


Figure 3. Azetidinone inhibitors of FAAH and MGL, **9b** and **9c**.

tidinone (**9c**) featured an IC_{50} of 4.5 μM and a good selectivity for FAAH versus MGL; the lower homolog **9b** was slightly less active and less selective (Fig. 3) (see Supplementary data for graphical representation of inhibition activity of all tested compounds).

The potential activity of selected lipophilic β-lactams against representative serine enzymes has been further assayed. Compounds **9b** and **9c** were found to be inactive against Porcin Pancreatic Elastase (PPE), while compound **9a** totally inhibited PPE at 100 μM concentration[20,21] (see Supplementary data for graphical representation of PPE inhibition by **9a** at 100, 50 and 25 μM). Compounds **5a** and **7a**, the N1 TBDMS-protected and N1-unsubstituted precursors of **9a**, respectively, were not inhibitors of PPE. Lastly, the activity of compound **9a** was evaluated against four bacterial enzymes, Penicillins-Binding Proteins (PBPs), namely R39, PBP2a, PBP2x and PBP5.[22] A weak inhibition could be observed for R39 only, a low molecular weight DD-carboxypeptidase.[23]

Thus compound **9c** (3-(4′-pentenyl)-1-(4′-pentenoyl)-2-azetidinone) emerged from this screening as a promising 'hit' for the discovery of novel FAAH inhibitors.[24] This β-lactam derivative is active at the micromolar level and selective for FAAH versus other serine enzymes. Studies are in progress to improve the activity and to determine the mode of action at the atomic level.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.05.081.

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17. Representative protocols: synthesis of 3-(4′-pentenyl)-1-(4′-pentenoyl)-2-azetidinone (**9c**).
C-Alkylation of 1-(*tert*-butyldimethylsilyl)-2-azetidinone: a solution of LDA (1.15 equiv) in THF (20 mL) was added dropwise, at −78 °C, to a solution of **4** (registry number: 117505-49-4) (1 g, 5.4 mmol) in THF (25 mL, 0.215 M solution) under argon atmosphere. After 30 min of stirring at −78 °C, 5-bromopentene (1.15 equiv) was added. The mixture was allowed to slowly reach room temperature. After 15 h at 20 °C, aqueous NH_4Cl (10%, 50 mL) was added. The mixture was extracted with CH_2Cl_2 (2× 50 mL) and the organic layer was washed with water (3× 30 mL), dried over $MgSO_4$ and concentrated under vacuum. Flash chromatography on silica gel (cyclohexane–EtOAc, 5:1) yielded 3-(4′-pentenyl)-1-(*tert*-butyldimethylsilyl)-2-azetidinone (**5c**) as a pale yellow oil (500 mg, 37%) and 3,3′-bis-(4′-pentenyl)-1-(*tert*-butyldimethylsilyl)-2-azetidone (**6c**) as a yellow oil (400 mg, 23%).

Characterisation of **5c**: R_f = 0.62; IR (film) ν 2858–2930, 1747, 1641, 1320, 1195, 839 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 0.20 (s, 3H), 0.22 (s, 3H), 0.94 (s, 9H), 1.48 (m, 2H), 1.60 (m, 1H), 1.78 (m, 1H), 2.06 (td, J = 6.7, 7.2 Hz, 2H), 2.87 (dd, J = 2.8, 6.0 Hz, 1H), 3.24 (m, 1H), 3.30 (dd, J = 6.0, 6.0, 1H), 4.95 (dd t, J = 1.7, 1.7, 10.5 Hz, 1H), 5.00 (dd t, J = 1.7, 1.7, 17.1 Hz, 1H), 5.78 (dd t, J = 6.7, 10.5, 17.1 Hz, 1H); ^{13}C NMR: see Table 1; HRMS (CI) calcd for $C_{14}H_{27}NOSi$: 254.1940, found: 254.1935. Characterisation of **6c**: R_f = 0.84; IR (film) ν 2850–2930, 1738, 1640, 1325, 1194, 837 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 0.20 (s, 6H), 0.93 (s, 9H), 1.39 (m, 2H), 1.48 (m, 2H), 1.58 (t, J = 7.7 Hz, 4H), 2.05 (m, 4H), 2.97 (s, 2H), 4.95 (dd, J = 1.7, 10.5 Hz, 2H), 5.00 (dd, J = 1.7, 17.1 Hz, 2H), 5.78 (dd t, J = 6.7, 10.5, 17.1 Hz, 2H); ^{13}C NMR: see Table 1; MS (CI) m/e 322.2 ($C_{19}H_{35}NOSi$, M+1).

Deprotection of N1: azetidinone **5c** (230 mg, 0.91 mmol) dissolved in methanol (15 mL) was treated with CsF (2 equiv) at 20 °C during 2 h. After solvent evaporation, the residue was dissolved in EtOAc (25 mL) and washed with brine (2× 30 mL). Drying over $MgSO_4$ and concentration under vacuum gave crude 3-(4′-pentenyl)-2-azetidinone (**7c**) as a yellow oil(120 mg, 95% yield). Characterisation of **7c**: R_f (cyclohexane–EtOAc, 5:2) = 0.26; ^1H NMR (250 MHz, $CDCl_3$) δ *1.2–1.8 (m, 4H), 1.99–2.05 (m, 2H), 2.93 (dd, J* = 1.6, 5.6 Hz, 1H), 3.10 (m, 1H), 3.33 (dd, J = 5.6, 6.4 Hz, 1H), 4.90 (dd, J = 1.6, 9.9 Hz, 1H), 4.95 (dd, J = 1.6, 17.0 Hz, 1H), 5.68 (dd t, J = 6.7, 9.9, 17.0 Hz, 1H), 6.53 (br s, NH); ^{13}C NMR: see Table 1; MS (CI) m/e 140.1 ($C_8H_{14}NO$, M+1).

Acylation of N1: a solution of azetidinone **7c** (370 mg, 2.66 mmol) and pyridine (1.5 equiv) in CH_2Cl_2 (15 mL) was treated with 4-pentenoyl chloride (2 equiv) at 20 °C, under argon atmosphere, for 4 h. After dilution with CH_2Cl_2 (40 mL), the mixture was washed successively with 3.3 N HCl (30 mL), 10% $NHCO_3$ (30 mL) and brine (30 mL). The organic layer was dried over $MgSO_4$, concentrated under vacuum and flash chromatographed on silica gel (cylcohexane–EtOAc, 5:2) to yield the title compound **9c** as a colourless oil (490 mg, 85%). Characterisation of **9c**: R_f = 0.6; IR (film) ν 2859-2936, 1790, 1702, 1640, 1316 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.50 (m, 2H), 1.60–1.85 (m, 2H), 2.07 (td, J = 6.6, 7.5 Hz, 2H), 2.40 (td, J = 6.6, 7.5 Hz, 2H), 2.78 (t, J = 7.5 Hz, 2H), 3.24 (m, 2H), 3.67 (dd, J = 6.6, 6.6 Hz, 1H), 4.94-5.04 (m, 4H), 5.75 (dd t, J = 6.6, 10.5, 17.1 Hz, 1H), 5.83 (dd t, J = 6.6, 10.5, 17.1 Hz, 1H); ^{13}C NMR: see Table 1; HRMS (ESI) calcd for $C_{13}H_{19}NO_2$+Na: 244.1313, found 244.1314.

18. Evaluation against FAAH and MGL. Human FAAH[19a] and human MGL[19b] were obtained as previously reported and used to screen the compounds at 1 mM and to obtain the IC_{50} values. Briefly, FAAH (in 165 μL of Tris–HCl, pH 7.4) was added on ice to glass tubes containing either drugs or DMSO (10 μL). Hydrolysis was initiated by adding 25 μL of [^{3}H]-AEA (50,000 dpm, 2 μM final concentration) in Tris–HCl containing 0.1% BSA and tubes were incubated for 10 min at 37 °C. Reactions were stopped by rapidly placing the tubes on ice and adding 400 μL of ice-cold MeOH–$CHCl_3$ (1:1 v/v). Following centrifugation (850g, 5 min, 4 °C) the [^{3}H]-ethanolamine in the aqueous layer was recovered (200 μL) and counted by liquid scintillation. Blanks were prepared (buffer instead of FAAH) and the values systematically subtracted. MGL activity was assayed in Tris–HCl, pH 8, using a similar protocol and using [^{3}H]-oleoylglycerol (50,000 dpm, 10 μM final concentration) as substrate.
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20. Evaluation against PPE has been performed according to Ref. 21. To 2 mL of the solution of substrate (*N*-succinyl-L-alanyl-L-alanyl-L-alanyl-*p*-nitroanilide, 300 μM in 100 mM Tris buffer, pH 7.5) were added 20 μL of the solution of tested compound (**9a–c**; 10^{-2}, 5×10^{-3}, 2.5×10^{-3}, 10^{-4} M in *N*-methylpyrrolidone) and 67 μL of the solution of enzyme (6 μM in 50 mM acetate buffer, pH 5; [E] = 200 nM). The appearance of *p*-nitroaniline, due to substrate hydrolysis, was measured (with a Varian Cary 3 Bio spectrophotometer) at 410 nm as a function of time. Curves recorded for **9a** at concentrations of 100, 50 and 25 μM are given as Supplementary data. Porcin Pancreatic Elastase (PPE) was purchased from Sigma (solution at 0.35 mg/mL).
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23. Evaluation against PBPs has been realized at the University of Liège, Centre d'Ingénierie des Protéines (CIP), Belgium. The inhibition studies were performed at 30 °C in 50 mM sodium phosphate buffer, pH 7 and 5% dimethylformamide. For the PBP2a and PBP5, 0.5 M NaCl was added. The β-lactam **9a** (100 μM) was mixed with the protein (0.8–2.5 μM) and incubated for 16 h. The residual free PBP was then counter-labelled with 25 μM fluorescein-labelled ampicillin for 45 min. After, the reaction was stopped by addition of SDS–PAGE loading buffer and incubation at 100 °C for 4 min. The reaction mixture was subjected to SDS–PAGE. The fluorescent complexes were visualised using a Molecular Imager FX and quantified with the Quantity One software (Biorad). In each assay, background fluorescence was subtracted. Residual activities, expressed as percentages of the initial activity, were (%): PBP2a: 87 ± 7; PBP5: 104 ± 4; PBP2x: 108 ± 4; R39: 73 ± 3 (experiments done in triplicate).
24. As stated by H. Wang et al. in a recent paper discussing the selection of 420 compounds from more than a half million compounds (by using virtual screening with a pharmacophore model of CB1 receptor antagonists) and then the in vivo evaluation of this selected library, compounds with IC_{50} or K_i lower than 10 μM are generally considered as 'hits'. Wang, H.; Duffy, R. A.; Boykow, G. C.; Chackalamannil, S.; Madison, V. S. *J. Med. Chem.* **2008**, *51*, 2439.