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Published in:
European Journal of Medicinal Chemistry

DOI:
10.1016/j.ejmech.2009.01.007

Published: 01/01/2009

Document Version
Peer-reviewed accepted author manuscript, also known as Final accepted manuscript or Post-print

Author's accepted manuscript, published in European Journal of Medicinal Chemistry 44 (2009) 2994–3008

The synthesis and biological evaluation of *para*-substituted phenolic *N*-alkyl carbamates as endocannabinoid hydrolyzing enzyme inhibitors

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ARTICLE INFO

Keywords:
Endocannabinoid
N-Arachidonoylethanolamine
2-Arachidonoylglycerol
Fatty acid amide hydrolase
Monoglyceride lipase

ABSTRACT

A series of *para*-substituted phenolic *N*-alkyl carbamates were evaluated for their FAAH and MGL inhibitory activities. The compounds were generally selective for FAAH, with IC_{50} values in the nM range, whereas inhibition of MGL required concentrations three orders of magnitude higher. The most potent compounds, dodecylcarbamic acid 4-(4,5-dihydrothiazol-2-yl)phenyl (**12**) and 4-(1,2,3-thiadiazol-4-yl)phenyl (**26**) esters, inhibited FAAH and MGL with IC_{50} values at the low-nanomolar (IC_{50}s; 0.0063 and 0.012 μM) and the low-micromolar ranges (IC_{50}s; 2.1 and 1.0 μM), respectively. Compound **26** also inhibited both FAAH-dependent AEA uptake and AEA hydrolysis (IC_{50}; 0.082 μM) by intact RBL2H3 cells, and could also reduce 2-AG hydrolysis by these cells at concentrations ≥0.030 μM.

1. Introduction

The endocannabinoids constitute an important class of endogenous lipids that serve as chemical messengers both in the brain and the periphery. The two main endocannabinoids, *N*-arachidonoylethanolamine (anandamide, AEA, **1**) [1] and 2-arachidonoylglycerol (2-AG, **2**) [2,3], bind to and activate both cannabinoid receptors, CB_1 [4,5] and CB_2 [6], that bind also exogenous cannabinoids like Δ^9-tetrahydrocannabinol (Δ^9-THC, **3**) (Fig. 1). The activation of CB_1 and CB_2 receptors by cannabinoids induces several beneficial therapeutic effects such as pain relief and reduction of inflammation [7]. However, the cannabinergic effects of AEA and 2-AG remain weak in vivo owing to their rapid inactivation by the hydrolytic enzymes fatty acid amide hydrolase (FAAH, FAAH-1) [8] and monoglyceride lipase (MGL) [9], respectively.

FAAH is the main metabolizing enzyme responsible for the AEA degradation. AEA is also hydrolyzed by other enzymes such as FAAH-2, a second FAAH isoform [10], and *N*-acylethanolamine-hydrolyzing acid amidase (NAAA) [11]. FAAH has a broad substrate selectivity, and it has been well characterized both pharmacologically and biochemically. Therefore, several FAAH inhibitors have been developed (see for review Ref. [12]), including various fatty acid derivatives [13–17], and non-lipid inhibitors such as α-keto heterocycles [18–20], carbamate derivatives [21–25], (thio)-hydantoins [26] and most recently, selective piperidine/piperazine ureas [27,28]. Probably, the most well-studied FAAH inhibitors are the carbamate-based 3′-carbamoylbiphenyl-3-yl ester (**4**, URB597) (Fig. 2) [21,29] and the α-keto heterocycle-based 7-phenyl-1-(5-(pyridin-2-yl)oxazol-2-yl)heptan-1-one (OL-135) [19], which have gained considerable promise for treating anxiety, inflammation, and pain [7,19,29–31].

Besides being the main metabolizing enzyme for AEA, FAAH is also able to hydrolyze 2-AG [32]. However, the main enzyme responsible for the inactivation of 2-AG in the brain has been suggested to be MGL (EC 3.1.1.23) [9,33,37], although other hydrolytic activities may also contribute to the metabolism [34–37]. MGL is a serine hydrolase, which hydrolyzes medium- and long-chain fatty acid esters such as 2-AG and its analogue 2-oleoylglycerol (2-OG) [9,38].

In contrast to FAAH, there is neither a 3D structure available for MGL nor the exact information of amino acid residues in the vicinity of the active site of the enzyme. Therefore, the design of potent and selective MGL inhibitors has remained a challenging

Abbreviations: AEA, *N*-arachidonoylethanolamine, anandamide; 2-AG, 2-arachidonoylglycerol; FAAH, fatty acid amide hydrolase; MGL, monoglyceride lipase.

doi:10.1016/j.ejmech.2009.01.007

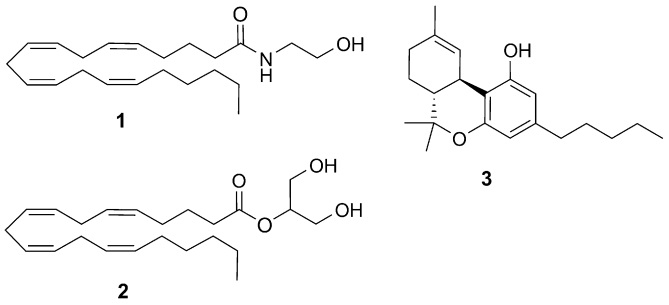


Fig. 1. Structures of *N*-arachidonoylethanolamine (AEA, **1**), 2-arachidonoylglycerol (2-AG, **2**), and Δ^9-tetrahydrocannabinol (Δ^9-THC, **3**).

task so far. However, very recently, during revision of this manuscript, Long et al. published a potent and selective MGL inhibitor called JZL184, which was shown to raise brain 2-AG in mice by eight-fold without altering AEA [39]. The other known potent inhibitors of this enzyme are compounds like hexadecylsulfonyl-fluoride (HDSF), methyl arachidonylfluorophosphonate (MAFP), and diisopropyl fluorophosphate [9,34,38] that are highly unselective and not useful for in vivo studies. The same is true for bis(dimethylthio)mercury, the contaminant responsible for the MGL inhibitory effects originally ascribed to 6-methyl-2-[(4-methylphenyl)amino]-4*H*-3,1-benzoxazin-4-one (URB754) [40]. Additionally, the *N*-biphenyl-3-cyclohexylcarbamic acid cyclohexyl ester (**5**, URB602) (Fig. 2) was originally suggested to be a selective MGL inhibitor [41], although its selectivity data has recently been questioned [42,43]. Sulfhydryl-reactive compounds such as *p*-chloromercuribenzoic acid, *N*-ethylmaleimide (NEM) [9,44,45] and disulfiram [46] inhibit MGL activity, indicating the presence of an essential sulfhydryl group at the active site of the enzyme. In a series of sulfhydryl-specific maleimide derivatives, *N*-arachidonylmaleimide (NAM, **6**) (Fig. 2) was found to be a potent inhibitor of MGL, inhibiting 2-AG hydrolysis in rat cerebellar membranes with an IC_{50} value of 140 nM [45], a result confirmed using human recombinant MGL (hrMGL), and 2-OG as substrate [46]. Despite containing a highly reactive maleimide group, NAM has been shown to exhibit rather high selectivity for MGL relative to other brain serine hydrolases [37]. Other approaches have also yielded compounds that inhibit MGL, but with potencies in the micromolar range [47]. For current knowledge of MGL and the inhibitors developed so far, see reviews [12,48,49].

The carbamate compound SPB01403 (**7**) (Fig. 3), found in virtual screening as reported in our previous study, could inhibit both

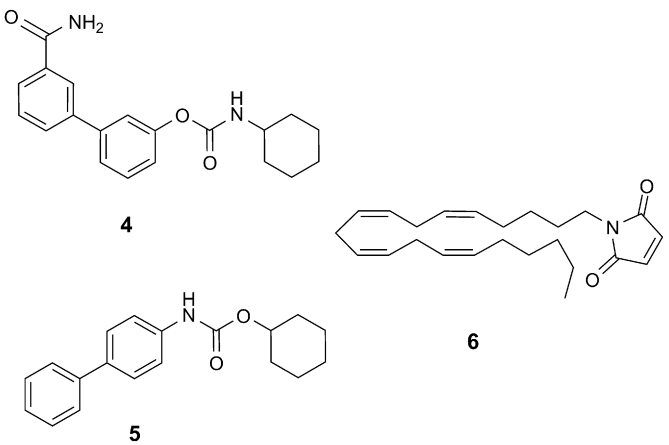


Fig. 2. Structures of URB597 (**4**), URB602 (**5**), and *N*-arachidonylmaleimide (NAM, **6**).

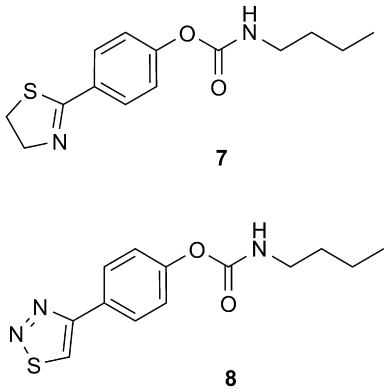
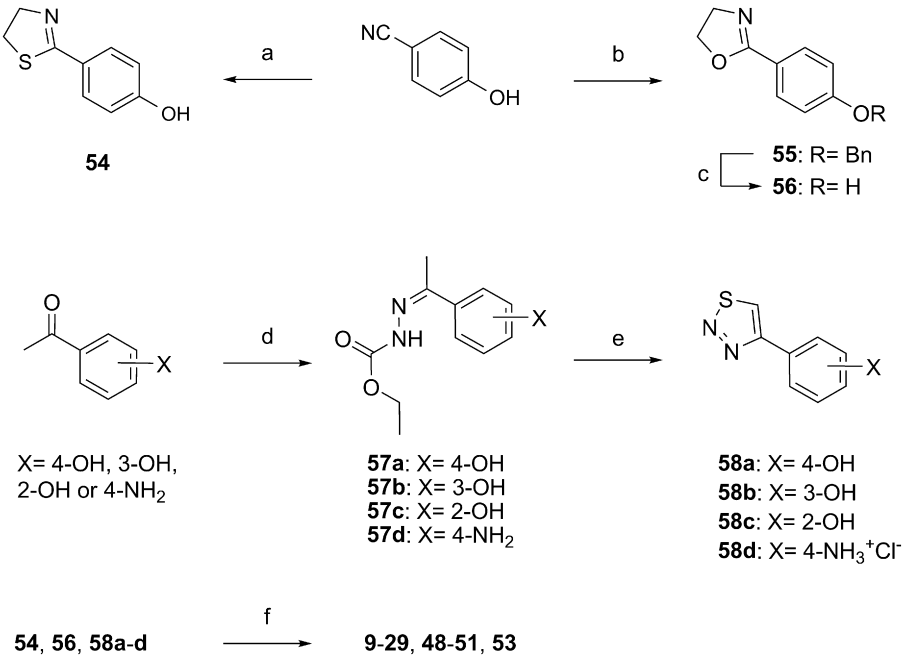


Fig. 3. The lead structures SPB01403 (**7**) and SEW01169 (**8**).

FAAH (IC_{50}; 0.52 μM) and MGL (IC_{50}; 30 μM) [50]. This observation raises the possibility that *O*-phenyl carbamate-based compounds can be optimized to give compounds with potent effects toward both FAAH and MGL, and that these compounds can be used in vivo to investigate the therapeutic potential of a concomitant blockade of hydrolysis of both AEA and 2-AG. Consequently, the structurally similar compound, SEW01169 (**8**) (Fig. 3), was purchased and tested for its FAAH and MGL inhibitory activity, and it was found to be slightly more potent FAAH inhibitor (IC_{50}; 0.16 μM) than **7**, albeit less potent inhibitor against 2-AG hydrolysis with remaining MGL activity 54% at 100 μM compound concentration in rat cerebellar membranes. Compound **8** was also tested against human recombinant MGL (hrMGL), and it was found to inhibit 2-OG hydrolysis with an IC_{50} value of 43 μM. Herein, we present the synthesis and biological evaluation of a series of phenolic *N*-alkyl carbamates derived from **7** and **8**, and their structure–activity relationships (SAR) for the inhibition of endocannabinoid hydrolyzing enzymes, FAAH and MGL.

2. Chemistry

The synthesis of compounds is outlined in Schemes 1 and 2. The majority of the carbamates were obtained by treating various phenols with suitable alkyl isocyanates in the presence of triethylamine; the *O*-phenyl carbamates **9**–**29**, **30** [51], **31**–**33**, **35** [53,54], **37**–**39** [55], **40** [52,55], **41**, **42** [53,56], **43** [57], **44**, **46**, and **48**–**51** were prepared from the corresponding phenols, and in the case of *N*-phenyl carbamate **52**, from cyclohexanol by a route described in Schemes 1 and 2. On the contrary, the *O*-phenyl carbamates **34** [52,55,58–60] and **36** [61] were prepared from 4-nitrophenyl chloroformate and the appropriate amine, yielding the desired products in 32–99% yield, with shorter reaction time than in the isocyanate method. The *O*-phenyl *N*-*n*-hexylcarbamates **45** and **47** were prepared from the corresponding methyl benzoates (Scheme 2). 4-(4,5-Dihydrothiazol-2-yl)phenol **54** [62] for the preparation of the carbamates **9**–**20** was prepared by simply heating the mixture of cysteamine and 4-cyanophenol (Scheme 1). Unfortunately this method was not suitable for the preparation of 4-(4,5-dihydrooxazol-2-yl)phenol **56** for the synthesis of the carbamates **21**–**24**, thus it was obtained by the reaction described by Witte and Seeliger [63] (Scheme 1). For this purpose the hydroxyl group of 4-cyanophenol was protected as the benzyl ether (**55**) [64]. (1,2,3-Thiadiazol-4-yl)phenols **58a**–**c** [65,66] for the preparation of the carbamates **25**–**29** and **48**–**51** [67] were obtained from phenyl ketones by the Hurd–Mori reaction via hydrazones **57a**–**c** [65,66] (Scheme 1). The same method was applied in the preparation of 4-(1,2,3-thiadiazol-4-yl)aniline hydrochloride (**58d**) for the synthesis of the urea compound **53** (Scheme 1).



Scheme 1. Synthesis of compounds **9**–**29**, **48**–**51** and **53**–**58a**–**d**. Reagents and conditions: (a) 2-aminoethanethiol, 100 °C, 88%; (b) i) BnBr, K_2CO_3, KI, acetone, reflux, 98%, ii) 2-aminoethanol, PhCl, reflux, 50%; (c) H_2, Pd/C, ethanol, rt, 75%; (d) ethyl carbazate, *p*-TsOH, toluene/ethanol, Dean–Stark, 69–100%; (e) $SOCl_2$, rt, 43–85%; (f) RNCO, Et_3N, toluene, rt-reflux, 20–100%; wherein R = Et, *n*-Pr, *n*-Hex, dodecyl, *t*-Bu, *c*-pentyl, *c*-Hex, Bn, 2-MeBn, 4-MeBn, 4-MeOBn, phenethyl.

3. Biological testing

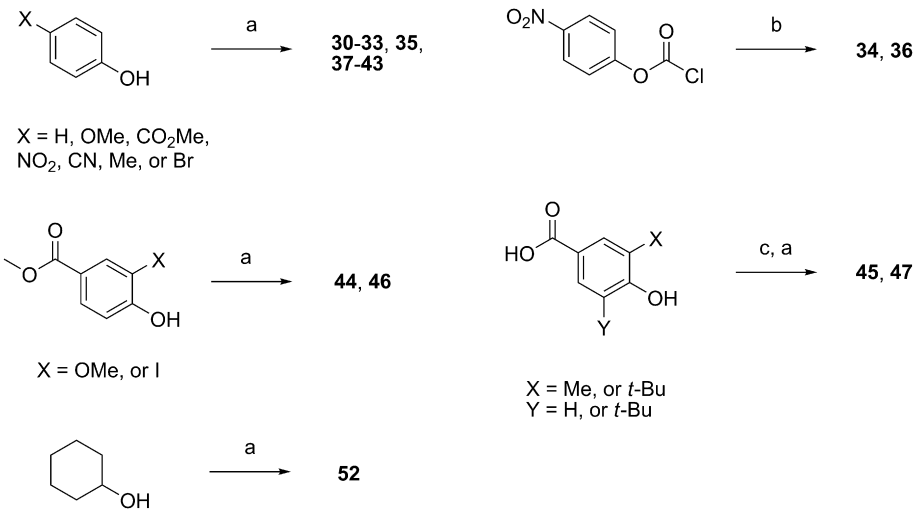
The inhibitory potencies against AEA hydrolysis (by FAAH) of all the prepared compounds **9**–**53**, and the leads **7** and **8** were determined in rat brain homogenate [50]. The compounds **7**–**10**, **13**–**26**, **28**, **31**–**33**, **36**, **42**, **43**, **49**, **51**, **52**, and **53** were tested for their ability to inhibit 2-AG hydrolysis (by MGL) in rat cerebellar membranes [34], while the MGL inhibitory potencies of the compounds **8**, **11**, **12**, **26**, **27**, **29**–**31**, **34**, **35**, **37**–**41**, **44**–**48**, **50**, and **51** were evaluated in hrMGL (Cayman Chemical), using tritium-labelled oleoylglycerol as substrate. In order to demonstrate the comparability of the two MGL methods used, the compounds **8**, **26**, **31** and **51** were tested in both assays. Moreover, inhibition of FAAH and MGL by reference compounds, FAAH inhibitor URB597 (**4**) and previously reported selective MGL inhibitor URB602 (**5**), were evaluated. The results of reference compounds showed good correlation with the ones reported in the literature [29,42,43].

4. Results and discussion

4.1. Structural optimization of the lead SPB01403 (7)

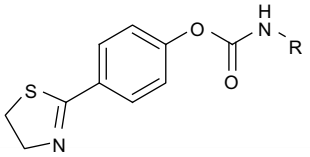
A series of *N*-alkyl derivatives of the lead SPB01403 (**7**) with dihydrothiazoline (**9**–**20**, Table 1) or dihydrooxazoline moiety (**21**–**24**, Table 2) at *para*-position in the phenyl ring were prepared and tested for their FAAH and MGL inhibitory activities.

Among the dihydrothiazoline moiety containing open chain *N*-alkyl carbamates (**7**, **9**–**12**) the chain length was the main factor



Scheme 2. Synthesis of compounds **30**–**47** and **52**. Reagents and conditions: (a) RNCO, Et_3N, toluene, rt-reflux, 50–100%; wherein R = *n*-Bu, *n*-Hex, *c*-Hex, dodecyl; (b) RNH_2, pyridine, CH_2Cl_2, reflux, 32–99%; wherein R = *n*-Bu, *c*-Hex; (c) methanol, H_2SO_4, reflux, 100%.

Table 1
IC_{50} values for the inhibition of MGL and FAAH by dihydrothiazoline derivatives **7**, and **9**–**20**.



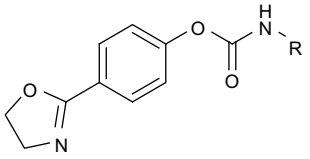
Compound	R	IC_{50} (95% CI)[a]	
		FAAH [μM]	MGL [μM]
7	*n*-Bu	0.52 (0.39–0.70)[b]	31 (25–39)[b]
9	Et	15 (10–22)	102 (80–131)[c]
10	*n*-Pr	1.7 (1.3–2.3)	46 (39–56)[d]
11	*n*-Hex	0.0082 (0.0068–0.0099)	8.4 (6.8–10)[e]
12	Dodecyl	0.0063 (0.0045–0.0087)	2.1 (1.7–2.6)[e]
13	*t*-Bu	7.0 (5.0–10.0)	No inhibition at 100 μM
14	*c*-Pentyl	0.13 (0.11–0.15)	11 (9–12)[d]
15[f]	*c*-Hex	0.10 (0.095–0.10)	27 (21–35)[c]
16	Bn	0.29 (0.25–0.33)	42 (35–49)[c]
17	2-MeBn	0.062 (0.056–0.060)	46 (30–52)[c]
18	4-MeBn	0.28 (0.12–0.40)	94 (85–105)[c]
19	4-MeOBn	0.32 (0.25–0.34)	46 (38–55)[g]
20	CH_2Ph	0.038 (0.035–0.042)	28 (25–31)

[a] IC_{50} values represent the mean from three independent experiments performed in duplicate with 95% confidence intervals shown in parenthesis. Enzyme activity was completely abolished at the highest tested concentration unless otherwise stated.
[b] See Ref. [50].
[c] Remaining enzyme activity was 12–19% at 1 mM.
[d] Remaining enzyme activity was 6–7% at 1 mM.
[e] Inhibition of hrMGL.
[f] Compound contained 5% of unidentified impurity by ^1H NMR.
[g] Remaining enzyme activity was 21–26% at 1 mM.

affecting the inhibition potencies toward both enzymes; with short *N*-ethyl group (**9**) the IC_{50} values against FAAH was 15 μM, whereas with longer *N*-*n*-hexyl (**11**) and *N*-dodecyl (**12**) groups the FAAH IC_{50} values were 0.0082 μM and 0.0063 μM, respectively. Moreover, compounds **11** and **12** inhibited hrMGL mediated 2-OG hydrolysis with IC_{50} values of 8.4 μM and 2.1 μM, respectively.

Replacement of the open chain *N*-alkyl groups with bulky *N*-*tert*-butyl group (**13**) reduced the inhibitory potencies toward FAAH and MGL (IC_{50}s; 7.0 μM and >100 μM, respectively). This is probably due to steric hindrance near the carbamate group, thus reducing crucial interactions within the active sites of the enzymes.

Table 2
IC_{50} values for the inhibition of MGL and FAAH by dihydrooxazoline derivatives **21**–**24**.



Compound	R	IC_{50} (95% CI)[a]	
		FAAH [μM]	MGL [μM]
21	*n*-Bu	0.12 (0.11–0.13)	34 (27–42)
22	*c*-Pentyl	0.10 (0.082–0.11)	11 (9–14)[b]
23	2-MeBn	0.034 (0.031–0.038)	35 (32–38)[c]
24	4-MeBn	0.18 (0.14–0.24)	125 (111–141)[d]

[a] IC_{50} values represent the mean from three independent experiments performed in duplicate with 95% confidence intervals shown in parenthesis. Enzyme activity was completely abolished at the highest tested concentration unless otherwise stated.
[b] Remaining enzyme activity was 21–26% at 1 mM.
[c] Remaining enzyme activity at 1 mM was 12–19%.
[d] Remaining enzyme activity was 6–7% at 1 mM.

The differences in the FAAH and MGL inhibition potencies among the other dihydrothiazoline derivatives with different *N*-alkyl groups (**14**–**20**) were mainly not notable. However, with cycloalkyl and benzylic groups (**14**–**19**) complete MGL inhibition was not observed, even at the highest concentrations tested (1 mM). This remaining enzyme activity can be explained by an additional enzyme activity distinct from MGL or by low aqueous solubility of these compounds. An interesting observation concerning the FAAH inhibition was that the *N*-2-methylbenzyl derivative (**17**) (IC_{50}; 0.062 μM) was more potent FAAH inhibitor than the other derivatives with cyclic or benzylic *N*-alkyl moiety (**14**–**16**, **18**, **19**) (IC_{50}s; 0.10–0.32 μM).

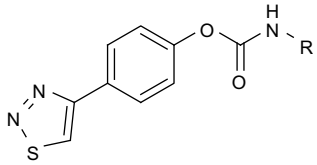
With respect to dihydrooxazoline derivatives **21**–**24** (Table 2), no clear difference in the inhibition potencies toward either FAAH or MGL was seen when comparing the results to those of the corresponding dihydrothiazoline derivatives **7**, **14**, **17**, and **18**. However, like in the series of dihydrothiazoline substituted *N*-cycloalkyl or *N*-benzylic derivatives, the *N*-2-methylbenzyl derivative (**23**) (IC_{50}; 0.034 μM) displayed better inhibitory potency against FAAH than the other *N*-alkyl derivatives in the dihydrooxazoline series (IC_{50}s; 0.10–0.18 μM).

4.2. Structural optimization of the lead SEW01169 (**8**)

After establishing the optimal *N*-alkyl groups for the inhibition of FAAH and MGL within the dihydrothiazol-2-yl and dihydrooxazol-2-yl substituted *O*-phenyl carbamates, a series of SEW01169 (**8**) derivatives with various and selected *N*-alkyl groups were prepared (Table 3).

Not surprisingly, in this series of 1,2,3-thiadiazol-4-yl substituted derivatives (**8**, **25**–**29**), the length and the shape of the *N*-alkyl group had a similar effect on the inhibitory potencies against both enzymes, FAAH and MGL, as it had in the previous series. Thus, by replacing the *N*-*n*-butyl chain of **8** by *N*-*n*-hexyl (**25**) the inhibitory potencies toward FAAH and MGL were improved (IC_{50}s; 0.019 and 11 μM, respectively). Furthermore, the most potent inhibitor in this series with *N*-dodecyl moiety (**26**) inhibited FAAH with an IC_{50} value of 0.012 μM. Moreover, hydrolysis of 2-AG in rat brain membranes as well as hrMGL mediated 2-OG hydrolysis

Table 3
IC_{50} values for the inhibition of MGL and FAAH by 1,2,3-thiadiazoline derivatives **8**, and **25**–**29**.



Compound	R	IC_{50} (95% CI)[a] or percentage of inhibition[b]	
		FAAH [μM]	MGL [μM]
8	*n*-Bu	0.16 (0.13–0.20)	46% at 100 μM 43 (27–66)[c,d]
25	*n*-Hex	0.019 (0.015–0.024)	11 (9.8–13)
26	Dodecyl	0.012 (0.011–0.014)	1.9 (1.7–2.1) 1.0 (0.8–1.4)[c]
27	*c*-Pentyl	0.020 (0.014–0.029)	44% at 100 μM[c]
28	*c*-Hex	0.021 (0.018–0.025)	28% at 100 μM
29	Bn	0.044 (0.034–0.056)	24 (15–39)[c,d]

[a] IC_{50} values represent the mean from three independent experiments performed in duplicate with 95% confidence intervals shown in parenthesis. Enzyme activity was completely abolished at the highest tested concentration unless otherwise stated.
[b] The percentage of inhibition is represented as the mean from two independent experiments performed in duplicates.
[c] Inhibition of hrMGL.
[d] Remaining enzyme activity 22–26% at 1 mM.

were inhibited by **26** with IC_{50} values of 1.9 μM and 1.0 μM, respectively. When comparing these results to those observed with the corresponding dihydrothiazoline derivative **12**, we can conclude that the heterocyclic moiety in these two compounds has a minor effect on their FAAH and MGL inhibitory capacities.

However, the compounds with *N*-cyclopentyl (**27**), *N*-cyclohexyl (**28**) and *N*-benzyl (**29**) moieties were more potent FAAH inhibitors than the corresponding dihydrothiazoline derivatives **14**–**16**, indicating that FAAH inhibitory activity could be affected by the type of heterocycle. This effect was not seen for MGL.

4.3. *The effect of* para*-substituents with different electronic properties*

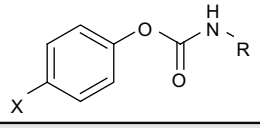
The catalytic site serine –OH of FAAH [68] and MGL is expected to have a strong interaction with the reactive carbonyls of the ligands. Therefore, we decided to study whether the electronic properties of the *para*-substituent in *O*-phenyl carbamates have an effect on the FAAH and MGL inhibitory activities. The presumption that electron-withdrawing groups in *para*-position on the phenyl ring would increase the electrophilicity of the carbonyl carbon prompted us to prepare a series of *N*-alkyl carbamates with various electron-withdrawing and electron-donating substituents (Table 4). Methoxy carbonyl (**30**–**33**), nitro (**34**–**36**), cyano (**37** and **38**), and bromine (**39**) were selected as electron-withdrawing (EWG), and methoxy (**40**) and methyl (**41**) as electron-donating groups (EDG). The neutral effect was evaluated with the unsubstituted *O*-phenyl carbamates **42** and **43**.

As evident from the results presented in Table 4, the EWG and EDG had opposite effects on the inhibitory potencies against MGL. EWG (methoxycarbonyl, nitro, and cyano) substituted compounds **30**–**39** maintained the potencies against MGL similar to those of the corresponding *N*-alkyl derivatives presented in Tables 1–3, whereas EDG (methoxy and methyl) substituted compounds **40** and **41** had clearly diminished inhibition potencies. However, differences in inhibitory potencies against FAAH were hardly notable when comparing the results of EWG-substituted *N*-*n*-butyl derivatives **30**, **34**, and **37** with that of corresponding methoxy-substituted **40**. On the contrary, within *N*-*n*-hexyl derivatives EWG-substituted **31**, **35**, **38**, and **39** were more potent than methyl-substituted **41**.

As observed in the series of the derivatives of **7** and **8**, the FAAH and MGL inhibitory potencies were enhanced by increasing the length of the *N*-alkyl group. The same was true in this series; for example, the *N*-dodecyl derivative with electron-withdrawing methoxy carbonyl group (**33**) was found to be as potent toward FAAH and MGL (IC_{50}s; 0.025 μM and 2.8 μM, respectively) as **12** and **26**. Moreover, to our surprise, the nonsubstituted compounds **42** and **43** were quite potent FAAH inhibitors (IC_{50}s; 0.091–0.092 μM) in comparison with the EWG-substituted **31**, **33**, **35**, **38** and **39**, while Tarzia et al. have reported that phenylcarbamic acid cyclohexyl ester lacks inhibitory activity against FAAH [21]. This observed increase in FAAH inhibition potencies might be due to more flexible and lipophilic nature of *N*-*n*-hexyl and *N*-dodecyl groups. The similar observation can be seen when comparing the results of *N*-cyclohexyl (**32**, **36**) and/or *N*-*n*-butyl derivatives (**30**, **34**, **37**, and **40**) to those of other *N*-*n*-hexyl (**31**, **35**, **38**, **39**, and **41**) and *N*-dodecyl derivatives (**33**).

Altogether, these results indicate that FAAH inhibition potencies might be more dependent on the *N*-alkyl moiety than the electronic properties of the *para*-substituent, while MGL inhibition potencies can be enhanced by EWG at *para*-position in the phenyl ring as well.

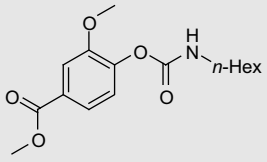
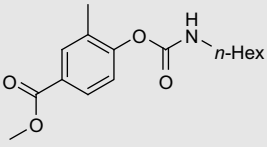
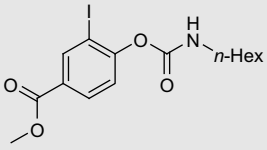
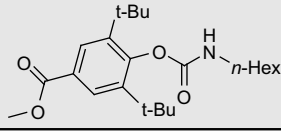
Table 4
IC_{50} values for the inhibition of MGL and FAAH by various EWG- and EDG-substituted *O*-phenyl carbamates **30**–**43**.



Compound	X	R	IC_{50} (95% CI)[a] or percentage of inhibition[b]	
			FAAH [μM]	MGL [μM]
30	CO_2Me	*n*-Bu	35% at 0.1 μM	19 (12–29)[c]
31	CO_2Me	*n*-Hex	0.031 (0.023–0.041)	35 (30–41.5) 19 (16–24)[c]
32	CO_2Me	*c*-Hex	41% at 0.1 μM	14 (12–18)
33	CO_2Me	Dodecyl	0.025 (0.022–0.030)	2.8 (2.5–3.2)
34	NO_2	*n*-Bu	16% at 0.1 μM	24 (18–33)[c]
35	NO_2	*n*-Hex	0.027 (0.021–0.035)	18 (12–27)[c]
36	NO_2	*c*-Hex	25% at 0.1 μM	19 (17–21)
37	CN	*n*-Bu	25% at 0.1 μM	30 (20–45)[c]
38	CN	*n*-Hex	0.013 (0.012–0.014)	12 (9.0–17)[c]
39	Br	*n*-Hex	0.018 (0.016–0.020)	17 (11–26)[c,d]
40	OMe	*n*-Bu	5% at 0.1 μM	No inhibition at 100 μM[c]
41	Me	*n*-Hex	27% at 0.1 μM	No inhibition at 100 μM[c]
42	H	*n*-Hex	0.091 (0.075–0.110)	41% at 100 μM
43	H	Dodecyl	0.092 (0.076–0.112)	36% at 100 μM

[a] IC_{50} values represent the mean from three independent experiments performed in duplicate with 95% confidence intervals shown in parenthesis. Enzyme activity was completely abolished at the highest tested concentration unless otherwise stated.
[b] The percentage of inhibition is represented as the mean from two independent experiments performed in duplicates.
[c] Inhibition of hrMGL.
[d] Remaining enzyme activity was 16% at 1 mM.

Table 5
Effect of the *ortho*-substituent in the phenyl ring on MGL and FAAH inhibition; IC_{50} values for the inhibition of MGL and FAAH by compounds **44**–**47**.

Compound	Structure	IC_{50} (95% CI)[a] or percentage of inhibition[b]	
		FAAH [μM]	MGL [μM]
44		0.0055 (0.0049–0.0062)	4.2 (2.7–6.2)[c]
45		0.055 (0.038–0.079)	22 (18–27)[c]
46		0.024 (0.021–0.025)	14 (11–16)[c]
47		4% at 0.1 μM	18% at 100 μM[c]

[a] IC_{50} values represent the mean from three independent experiments performed in duplicate with 95% confidence intervals shown in parenthesis. Enzyme activity was completely abolished at the highest tested concentration unless otherwise stated.
[b] The percentage of inhibition is represented as the mean from two independent experiments performed in duplicates.
[c] Inhibition of hrMGL.

In addition, a few derivatives of **31** with EDG (methoxy, **44**; methyl, **45**; *tert*-butyl, **47**) or EWG (iodine, **46**) at the *ortho*-position in the phenyl ring were prepared to study the effects of steric and electronic properties of *ortho*-substituents to MGL and FAAH inhibitory activity. As evident from Table 5, the *ortho*-methoxy substituent (**44**) improved the inhibitory potency toward both FAAH and hrMGL when compared to the results obtained with the compound **31** without any *ortho*-substituent. This might be due to favourable interactions between the oxygen atom in the methoxy group and the active site residues of the enzymes. Me- or I-substitution (**45** and **46**) did not have a significant effect on the affinity toward either FAAH or MGL. However, a bulky *tert*-butyl substituent (**47**) diminished the affinity toward these enzymes notably, which could be due to steric hindrance.

4.4. *Effect of the* meta- *and* ortho-*substitution in the phenyl ring*

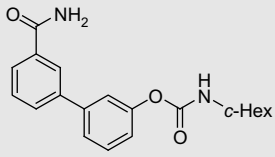
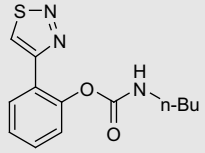
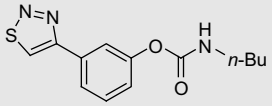
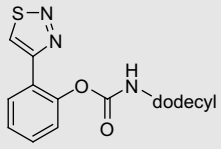
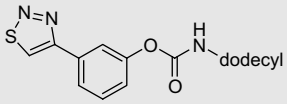
To support our previous results of the influence of the substituent's position in the phenyl ring on the FAAH and MGL inhibitory activities (i.e. *para*-substitution is more favourable to the MGL inhibitory activities) [22], *meta*- and *ortho*-analogues of **8** and its most potent derivative with *N*-dodecyl group (**26**) were prepared and tested (Table 6).

In our previous studies, *meta*-substituted carbamate URB597 (**4**) was found to inhibit AEA hydrolysis with an IC_{50} value of 0.0038 μM [50], which is comparable to the previously reported IC_{50} value of 0.0046 μM [29], and only 30% inhibition of 2-AG hydrolysis in rat brain membranes was evident at 1 mM [34].

Compound **48**, the *ortho*-substituted analogue of **8**, did not inhibit either of the enzymes; only 27% inhibition of MGL was observed at 100 μM, and no inhibition of FAAH could be detected at 0.1 μM. On the contrary, the *meta*-substituted analogue **49** inhibited FAAH with an IC_{50} value of 0.0069 μM. However, only 13% inhibition of MGL by **49** was observed at 100 μM concentration, which is similar to the results obtained with the *ortho*-substituted **48** and the *para*-substituted **8**.

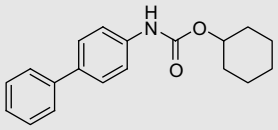
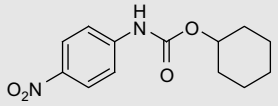
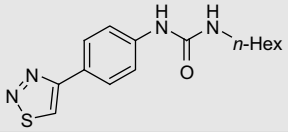
The *meta*-substituted compound **51** with a more lipophilic *N*-dodecyl group inhibited MGL with an IC_{50} value of 8.0 μM, being slightly less potent than the *para*-substituted analogue **26** (IC_{50}; 1.0 μM). Moreover, **51** inhibited FAAH with an IC_{50} value of

Table 6
Comparison of the *meta*- and *ortho*-substitution in the phenyl ring on MGL and FAAH inhibition; IC_{50} values for the inhibition of MGL and FAAH by **4** (URB597), and compounds **48**–**51**.

Compound	Structure	IC_{50} (95% CI)[a] or percentage of inhibition[b]	
		FAAH [μM]	MGL [μM]
4		0.0038 (0.0029–0.0050)[c]	30% at 1 mM[d]
48		No inhibition at 0.1 μM	27% at 100 μM[e]
49		0.0069 (0.0054–0.0088)	13% at 100 μM
50		0.050 (0.042–0.058)	18 (12–26)[e,f]
51		0.00024 (0.00020–0.00030)	57% at 30 μM[g] 8.0 (5.9–11.0)[e,h]

[a] IC_{50} values represent the mean from three independent experiments performed in duplicate with 95% confidence intervals shown in parenthesis. Enzyme activity was completely abolished at the highest tested concentration unless otherwise stated.
[b] The percentage of inhibition is represented as the mean from two independent experiments performed in duplicate.
[c] See Ref. [50].
[d] See Ref. [34].
[e] Inhibition of hrMGL.
[f] Remaining enzyme activity was 26% at 1 mM.
[g] Compound started to precipitate at concentrations over 30 μM.
[h] Remaining enzyme activity was 13% at 1 mM.

Table 7
Effect of the *O*-phenyl carbamate moiety on MGL and FAAH inhibition; IC_{50} values for the inhibition of MGL and FAAH by the reference compound **5** (URB602), and compounds **52** and **53**.

Compound	Structure	IC_{50} (95% CI)[a] or percentage of inhibition[b]	
		FAAH [μM]	MGL [μM]
5		108 (86–136)	30% at 100 μM[c]
52		9% at 0.1 μM	No inhibition at 100 μM
53		9% at 0.1 μM	No inhibition at 100 μM

[a] IC_{50} values represent the mean from three independent experiments performed in duplicate with 95% confidence intervals shown in parenthesis. Enzyme activity was completely abolished at the highest tested concentration unless otherwise stated.
[b] The percentage of inhibition is represented as the mean from two independent experiments performed in duplicate.
[c] Inhibition of hrMGL.

0.00024 μM, being more potent against FAAH than **26**. The *ortho*-substituted compound **50** with *N*-dodecyl group inhibited both FAAH and MGL, although with slightly higher IC_{50} values than *para*- and *meta*-substituted analogues **26** and **51**.

These data support the previous findings that *para*-substitution is more favourable to MGL inhibition, whereas *meta*-substitution is favourable for FAAH [22].

4.5. The effectiveness of O-phenyl carbamate structure on MGL and FAAH inhibition

To investigate the effectiveness of the *O*-phenyl carbamate structure on MGL and FAAH inhibition, two *N*-phenyl carbamates (URB602 (**5**) and **52**) and a urea compound **53** were tested. URB602

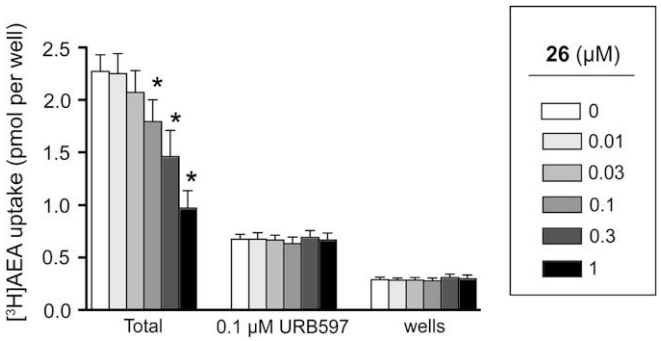


Fig. 5. Effect of **26** upon the uptake of 0.10 μM [^{3}H]AEA by either RBL2H3 cells in the absence or presence of URB597 (**4**) or by wells alone. Data are means and s.e.m., $n = 4$. $^*p < 0.05$ vs corresponding control, Dunnett's multiple comparison test following significant one-way ANOVA for repeated measures.

has been reported to be a selective inhibitor of MGL [41], although in our previous study it was shown to be totally inactive against 2-AG hydrolysis in rat brain membranes at 1 mM [42]. Moreover, in the present study 30% inhibition of hrMGL was seen at 100 μM. URB602 has also been reported to inhibit AEA hydrolysis by rat brain membranes (IC_{50}; 17 μM) [43]. In the present study URB602 inhibited FAAH with an IC_{50} value of 108 μM. Consequently, compounds **52** and **53** lacking *O*-phenyl carbamate structure showed no inhibition activity toward MGL, and only 9% inhibition of FAAH at 0.1 μM was observed with these compounds. These results establish that the *O*-phenyl carbamate structure in the compounds investigated in the present study is essential for the inhibitory activities toward both enzymes, FAAH and MGL. The results are presented in Table 7.

4.6. Reversibility

Compound **26** was investigated in more detail with respect to reversibility of FAAH and MGL inhibition. Reversibility was determined by measuring the recovery of FAAH and MGL enzymatic activity after a rapid and large dilution (500-fold) of the enzyme–inhibitor complexes.

In the reversibility studies the compound **26** was found to inhibit both FAAH and hrMGL in an irreversible manner (Fig. 4a and b). Inhibition of FAAH by URB597 (**4**) was shown to be irreversible and by 1-(oxazolo[4,5-*b*]pyridin-2-yl)-1-oxo-dodecane (CAY10435) [18] inhibition was reversible (Fig. 4a), as has been described in the

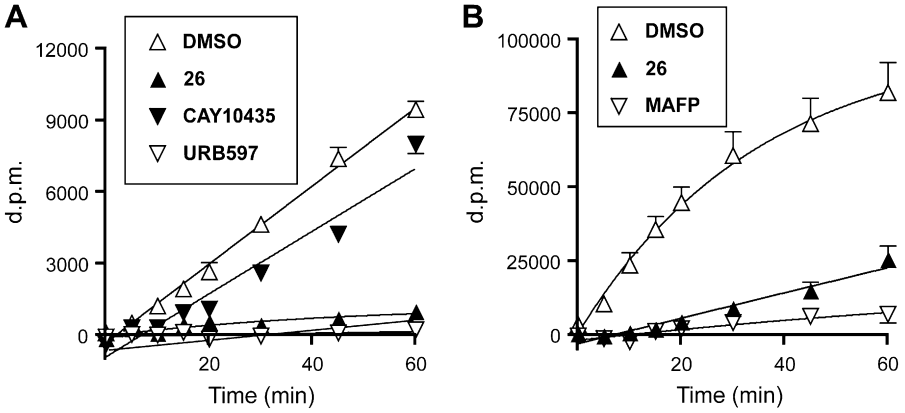


Fig. 4. (A) Reversibility of FAAH inactivation by **26** (240 nM), URB597 (76 nM) and CAY10435 (2.8 nM); (B) reversibility of hrMGL inactivation by **26** (36 μM) and MAFP (0.3 μM). Enzyme activity was measured at the indicated time-points after 500-fold dilution of the enzyme-inhibitor complex. For a fully reversible compound, the dilution should result in d.p.m. values approaching those seen with vehicle (DMSO) alone. The data represent the means and s.e.m. from three independent experiments performed in duplicate.

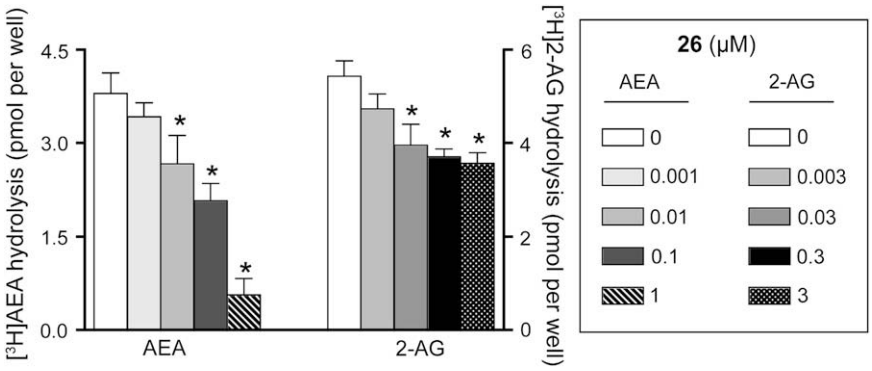


Fig. 6. Effect of **26** upon the hydrolysis of 0.10 μM [^{3}H]AEA and [^{3}H]2-AG by RBL2H3 cells. Data are means and s.e.m., $n = 5$ (AEA) or $n = 4$ (2-AG). *$p < 0.05$ vs corresponding control, Dunnett's multiple comparison test following significant one-way ANOVA for repeated measures. Expressing the data as % of control for AEA gave values of 92 ± 7, 70 ± 8, 55 ± 6 and 15 ± 7 for 0.001, 0.01, 0.1 and 1 μM **26**, respectively (means ± s.e.m.). These values give an IC_{50} value of 0.082 μM (constraining "top" and "bottom" values of the analysis to 100 and 0%, respectively).

literature [18,68]. Inhibition of hrMGL by MAFP was also shown to be irreversible (Fig. 4b).

4.7. The effects in intact cells

Compound **26** was investigated to its effects in intact cells. The effects of a compound upon FAAH activity in intact cells can be determined by measuring either the URB597 sensitive uptake of AEA or by following its hydrolysis [69]. In these assays **26** was found to be efficacious, and the IC_{50} value for the inhibition of the hydrolysis by intact RBL2H3 cells was 0.082 μM (Figs. 5 and 6). Compound **26** also reduced the hydrolysis of 2-AG by these cells at concentrations ≥0.03 μM (Fig. 6). However, this presumably reflects a FAAH component of 2-AG hydrolysis in these cells, since URB597 (**4**) also produced a partial inhibition of the 2-AG hydrolysis (data not shown).

5. Conclusions

In the present paper, we have developed a novel series of *para*-substituted phenolic *N*-alkyl carbamates as inhibitors of FAAH and MGL. The compounds inhibited FAAH with IC_{50} values in the nM range, whereas inhibition of MGL required concentrations three orders of magnitude higher. The FAAH inhibition potencies of the prepared *para*-substituted carbamates were highly affected by the shape and lipophilicity of the *N*-alkyl group; compounds with flexible and lipophilic *N*-*n*-hexyl and *N*-dodecyl groups were most potent inhibitors of FAAH.

Due the importance of FAAH as a potential target for the development of new therapeutical agents, these novel *para*-substituted carbamates present a useful extension to the previously reported *meta*-substituted carbamates as FAAH inhibitors [21–25], and may be useful for the development of new classes of FAAH inhibitors. Furthermore, this data may be valuable, together with that of the compounds reported earlier by us [22], in the derivation of 3D-QSAR models for the FAAH inhibition by *O*-phenyl carbamates.

Moreover, *O*-phenyl carbamates have been previously described extensively as potent FAAH inhibitors, but less is known about their ability to inhibit MGL. The present results indicate that in the compounds investigated in the present study the *O*-aryl carbamate structure, with an acyclic or a cyclic electron-withdrawing moiety at the *para*-position in the phenyl ring, and a lipophilic *N*-alkyl group are essential for MGL inhibitory activity. These novel compounds, albeit not being potent and selective, may be helpful in further design of compounds with more potent inhibitory activities against MGL.

6. Experimental protocols

6.1. Chemistry

Commercially available starting materials were used without further purification. SEW01169 and SPB01403 were purchased from Maybridge Chemicals LTD. URB597, URB602, CAY10435, and MAFP were purchased from Cayman Chemical. Solvents were distilled or dried with 4 Å molecular sieves prior to use. All dry reactions were performed under argon in flame- or oven-dried glassware. Analytical thin-layer chromatography was carried out on Merck silica gel F_{254} (60 Å, 40–63 μm, 230–400 mesh) precoated aluminium sheets and detected under UV-light. Purification of reaction products was carried out by flash chromatography (FC) on J.T. Bakers silica gel for chromatography (pore size 60 Å, particle size 50 nM). The ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance 500 or 400 spectrometers operating at 500.1/400 MHz for ^{1}H and 125.1/100 MHz for ^{13}C. Chemical shifts are reported in ppm on the δ scale from an internal standard of solvent ($CDCl_3$ 7.26 and 77.0 ppm; DMSO-d_6 2.50 and 39.51 ppm). Elemental analyses (CHN or CHNS) were recorded using a Thermo Quest CE Instrument EA 1110 CHNSO or Perkin Elmer 2400 CHN-elemental analyzers (Table 8). HRMS(ESI$^+$)-spectra were recorded on Waters LCT Premier spectrometer (Table 8). Melting points were determined in open capillaries using BÜCHI Melting Point B-545 or Stuart SMP and are uncorrected.

6.1.1. 4-(4,5-Dihydrothiazol-2-yl)phenol (**54**) [62]

A mixture of 4-cyanophenol (1.20 g, 10 mmol, 100 mol%) and 2-aminoethanethiol (1.20 g, 15 mmol, 150 mol%) was heated at 100 °C for 10 min and cooled to rt. The resulting yellow solid was suspended in water (8 mL) and the mixture was acidified with 1 M HCl (8 mL). Filtration and drying gave **54** (1.58 g, 88%) as a white solid: mp 201.8–203.2 °C; ^{1}H NMR (DMSO-d_6 400 MHz) δ 9.95 (s, 1H), 7.65–7.60 (m, 2H), 6.86–6.81 (m, 2H), 4.32 (t, $J = 8.2$ Hz, 2H), 3.38 (t, $J = 8.2$ Hz, 2H); ^{13}C NMR (DMSO-d_6) δ 165.6, 160.2, 129.9, 124.0, 115.4, 64.8, 33.0; HRMS calcd. for $C_9H_{10}NOS$ [M + H]: 180.0483, found: 180.0505.

6.1.2. 2-(4-Benzyloxyphenyl)-4,5-dihydrooxazole (**55**) [64]

To a refluxing solution of 4-cyanophenol (6.0 g, 50 mmol, 100 mol%), K_2CO_3 (13.8 g, 100 mmol, 200 mol%), and KI (4.15 g, 25 mmol, 50 mol%) in acetone (100 mL) was added BnBr (9.4 g, 55 mmol, 110 mol%) and the mixture was refluxed for 2 h. The

Table 8
Elemental and HRMS analysis data.

Compound	Formula	Calculated C, H, N	Found C, H, N
9	$C_{12}H_{14}N_2O_2S$	C, 57.58; H, 5.64; N, 11.19	C, 57.85; H, 5.48; N, 11.11
10	$C_{13}H_{16}N_2O_2S$	C, 59.07; H, 6.10; N, 10.60	C, 59.27; H, 6.00; N, 10.54
11	$C_{16}H_{22}N_2O_2S$	C, 62.72; H, 7.24; N, 9.14	C, 62.79; H, 7.25; N, 8.99
12	$C_{22}H_{34}N_2O_2S$	C, 67.65; H, 8.77; N, 7.17	C, 67.97; H, 9.05; N, 7.07
13	$C_{14}H_{18}N_2O_2S$	C, 60.41; H, 6.52; N, 10.06	C, 60.54; H, 6.39; N, 10.14
14	$C_{15}H_{18}N_2O_2S$	C, 62.04; H, 6.25; N, 9.65; S, 11.04 HRMS for $C_{15}H_{19}N_2O_2S$: 291.1167	C, 62.88; H, 6.42; N, 9.91; S, 10.78 Found: 291.1176
15	$C_{16}H_{20}N_2O_2S$	HRMS for $C_{16}H_{20}N_2O_2SNa$: 327.1143	Found: 327.1126
16	$C_{17}H_{16}N_2O_2S$	C, 65.36; H, 5.16; N, 8.97	C, 65.51; H, 5.42; N, 9.09
17	$C_{18}H_{18}N_2O_2S$	C, 66.23; H, 5.56; N, 8.58	C, 66.15; H, 5.63; N, 8.36
18	$C_{18}H_{18}N_2O_2S$	C, 66.23; H, 5.56; N, 8.58	C, 66.09; H, 5.65; N, 8.55
19	$C_{18}H_{18}N_2O_3S$	C, 63.14; H, 5.30; N, 8.18	C, 63.33; H, 5.14; N, 8.23
20	$C_{18}H_{18}N_2O_2S$	C, 66.23; H, 5.56; N, 8.58; S, 9.82	C, 65.93; H, 5.49; N, 8.70; S, 9.60
21	$C_{14}H_{18}N_2O_3$	C, 64.10; H, 6.92; N, 10.68	C, 64.01; H, 6.94; N, 10.71
22	$C_{15}H_{18}N_2O_3 \cdot H_2O$	C, 61.63; H, 6.90; N, 9.58	C, 61.53; H, 6.72; N, 9.75
23	$C_{18}H_{18}N_2O_3$	C, 69.66; H, 5.85; N, 9.03	C, 69.67; H, 5.71; N, 9.11
24	$C_{18}H_{18}N_2O_3$	C, 69.66; H, 5.85; N, 9.03	C, 69.67; H, 5.71; N, 9.11
25	$C_{15}H_{19}N_3O_2S$	C, 59.62; H, 6.54; N, 13.36	C, 60.02; H, 6.37; N, 13.74
26	$C_{21}H_{31}N_3O_2S$	C, 64.75; H, 8.02; N, 10.79	C, 64.36; H, 8.37; N, 10.58
27	$C_{14}H_{15}N_3O_2S$	C, 58.11; H, 5.23; N, 14.52	C, 57.86; H, 5.28; N, 14.23
28	$C_{15}H_{17}N_3O_2S$	C, 59.39; H, 5.65; N, 13.85	C, 59.00; H, 5.66; N, 13.86
29	$C_{16}H_{13}N_3O_2S$	C, 61.72; H, 4.21; N, 13.50	C, 61.41; H, 4.20; N, 13,24
30	$C_{13}H_{17}NO_4$	C, 62.14; H, 6.82; N, 5.57	C, 62.41; H, 6.93; N, 5.45
31	$C_{15}H_{21}NO_4$	C, 64.50; H, 7.58; N, 5.01	C, 64.39; H, 7.75; N, 5.06
32	$C_{15}H_{19}NO_4$	C, 64.97; H, 6.91; N, 5.05	C, 64.83; H, 7.10; H, 5.11
33	$C_{21}H_{33}NO_4$	C, 69.39; H, 9.15; N, 3.85	C, 69.66; H, 9.35; N, 3.90
34	$C_{11}H_{14}N_2O_4$	C, 55.46; H, 5.92; N, 11.76	C, 55.31; H, 6.00; N, 11.65
35	$C_{13}H_{18}N_2O_4$	C, 58.64; H, 6.81; N, 10.52	C, 59.05; H, 7.21; N, 10.10
36	$C_{13}H_{16}N_2O_4$	C, 59.08; H, 6.10; N, 10.60	C, 58.90; H, 6.21; N, 10.49
37	$C_{12}H_{14}N_2O_2$	C, 66.04; H, 6.47; N, 12.83	C, 65.99; H, 6.54; N, 12.70
38	$C_{14}H_{18}N_2O_2$	C, 68.27; H, 7.37; N, 11.37	C, 68.22; H, 7.47; N, 11.18
39	$C_{13}H_{18}BrNO_2$	C, 52.01; H, 6.04; N, 4.67	C, 52.08; H, 6.04; N, 5.04
40	$C_{12}H_{17}NO_3$	C, 64.55; H, 7.67; N, 6.27	C, 64.57; H, 7.79; N, 6.18
41	$C_{14}H_{21}NO_2$	C, 71.46; H, 9.00; N, 5.95	C, 71.35; H, 9,18; N, 6.02
42	$C_{13}H_{19}NO_2$	C, 70.56; H, 8.65; N, 6.33	C, 70.60; H, 8.78; N, 6.45
43	$C_{19}H_{31}NO_2$	C, 74.71; H, 10.23; N, 4.59	C, 74.81; H, 10.41; N, 4.70
44	$C_{16}H_{23}NO_5$	C, 62.12; H, 7.49; N, 4.53	C, 62.35; H, 7.65; N, 4.45
45	$C_{16}H_{23}NO_4$	C, 65.51; H, 7.90; N, 4.77	C, 65,23; H, 8.14; N, 5.17
46	$C_{15}H_{20}INO_4$	C, 44.46; H, 4.97; N, 3.46	C, 44,89; H, 5.12; N, 3.47
47	$C_{23}H_{37}NO_4$	C, 70.55; H, 9.53; N, 3.58	C, 70.37; H, 9.56; N, 3.61
48	$C_{13}H_{15}N_3O_2S$	C, 56.30; H, 5.45; N, 15.15	C, 56.06; H, 5.38; N, 14.89
49	$C_{13}H_{15}N_3O_2S$	C, 56.30; H, 5.45; N, 15.15	C, 55.87; H, 5.46; N, 15.14
50	$C_{21}H_{31}N_3O_2S$	C, 64.75; H, 8.02; N, 10.79	C, 64.48; H, 8.02; N, 10.73
51	$C_{21}H_{31}N_3O_2S$	C, 64.75; H, 8.02; N, 10.79	C, 64.67; H, 8.26; N, 10.75
52	$C_{13}H_{16}N_2O_4$	C, 59.08; H, 6.10; N, 10.60	C, 59.07; H, 6.51; N, 10.81
53	$C_{15}H_{20}N_4OS \cdot 1/14$ *n*-hexylamine	C, 60.07; H, 7.24; N, 17.40	C, 60.50; H, 7.42; N, 17.45

mixture was cooled to rt, poured into water (250 mL) and extracted with EtOAc (200 mL + 100 mL). The organic phase was washed with brine (150 mL), dried (Na_2SO_4), evaporated to dryness and the resulting residue crystallized from EtOAc/hexane giving in 4-benzyloxybenzonitrile as colourless needles (10.2 g, 97%).

$ZnCl_2$ (0.65 g, 4.8 mmol, 10 mol%) was melted by flame heating under high vacuum and cooled to rt under argon atmosphere. 4-Benzyloxy-benzonitrile (10.2 g, 48.7 mmol, 100 mol%) and chlorobenzene (180 mL) were added and the mixture was heated to reflux. Ethanolamine (14.5 mL, 245 mmol, 500 mol%) was added and the mixture was refluxed for 24 h. Another portion of ethanolamine (29 mL, 1000 mol%) was added and the mixture refluxed for additional 24 h and partitioned between water (500 mL) and EtOAc (500 mL). The organic phase was washed with brine (200 mL), dried (Na_2SO_4), filtered and evaporated to dryness. The residue was purified by FC (eluent 1–5% MeOH in CH_2Cl_2) and recrystallized (EtOAc:hexane) to give **55** (6.16 g, 50%) as white crystals: mp 136.7–137.5 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.90–7.80 (m, 2H), 7.43–7.30 (m, 5H) 7.00–6.98 (m, 2H), 5.11 (s, 2H), 4.46 (t, J = 9.5 Hz, 2H), 4.04 (t, J = 9.5 Hz, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 164.3, 161.1, 136.4, 129.8, 128.6, 128.1, 127.5, 120.5, 114.5, 70.0, 67.5, 54.8; HRMS calcd. for $C_{16}H_{16}NO_2$ [M + H]: 254.1181, found: 254.1169.

*6.1.3. 4-(4,5-Dihydrooxazol-2-yl)phenol (**56**) [70]*

Compound **55** (2.0 g, 7.9 mmol, 100 mol%) was dissolved in EtOH (50 mL), and the mixture was degassed with argon. 5% Pd/C (330 mg, 0.16 mmol, 2 mol%) was added and the mixture was degassed with hydrogen. The mixture was stirred under H_2 at rt for 90 min and filtered through a pad of celite and evaporated to dryness. Crystallization of the residue from EtOH gave **56** (0.96 g, 75%) as a white solid: mp 209.1–210.1 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.75–7.71 (m, 2H), 6.81–6.77 (m, 2H), 4.45–4.41 (t, J = 9.5 Hz, 2H), 3.98–3.93 (t, J = 9.5 Hz, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 165.8, 160.8, 129.7, 118.0, 114.8, 67.4, 53.4; HRMS calcd. for $C_9H_{10}NO_2$ [M + H]: 164.0712, found: 164.0738.

*6.1.4. General procedure for the preparation of compounds **57a**, **57b**, **57d**; N′-[1-(4-hydroxy-phenyl)-ethylidene]-hydrazinecarboxylic acid ethyl ester (**57a**) [65]*

A mixture of 4-hydroxyacetophenone (4.1 g, 30 mmol, 100 mol%), ethyl carbazate (3.3 g, 31.5 mmol, 105 mol%) and *p*-TsOH (280 mg, 1.5 mmol, 5 mol%) in dry toluene (100 mL) was refluxed for 2 h with a Dean–Stark trap. The solvent was removed under reduced pressure, and the crude residue was washed with diethyl ether to

give **57a** (6.85 g, 100%) as a pale yellow solid: ^{1}H NMR (DMSO-d_6, 500.1 MHz) δ 9.93 (s, 1H), 7.61 (d, J = 8.6 Hz, 2H), 6.80 (d, J = 8.6 Hz, 2H), 4.19 (q, J = 7.1 Hz, 2H), 2.18 (s, 3H), 1.28 (t, J = 7.1 Hz, 3H).

6.1.4.1. N′-*[1-(3-Hydroxy-phenyl)-ethylidene]-hydrazinecarboxylic acid ethyl ester (**57b**).* Pale brown solid (4.6 g, 69%): ^{1}H NMR (DMSO-d_6, 500.1 MHz) δ 10.08 (br s, 1H), 7.26–7.14 (m, 3H), 6.80 (ddd, J = 7.9, 2.4, 0.9 Hz, 1H), 4.20 (q, J = 7.1 Hz, 2H), 2.19 (s, 3H), 1.29 (t, J = 7.1 Hz, 3H); ^{13}C NMR (DMSO-d_6, 125.1 MHz) 158.1, 155.1, 140.6, 130.1, 126.4, 118.0, 116.9, 113.5, 61.4, 15.5, 14.8.

6.1.4.2. N′-*[1-(4-Amino-phenyl)-ethylidene]-hydrazinecarboxylic acid ethyl ester (**57d**).* Pale yellow solid (2.2 g, 100%). ^{1}H NMR (DMSO-d_6, 500.1 MHz) δ 9.82 (s, 1H), 7.48 (d, J = 8.6 Hz, 2H), 6.58 (d, J = 8.6 Hz, 2H), 5.55 (br s, 2H), 4.17 (q, J = 7.1 Hz, 2H), 2.13 (s, 3H), 1.28 (t, J = 7.1 Hz, 3H).

*6.1.5. General procedure for the preparation of compounds **58a**, **58b**, **58d**; 4-(1,2,3-thiadiazol-4-yl)phenol (**58a**) [65]*

Compound **57a** (4.5 g, 20.2 mmol, 100 mol%) was stirred overnight in thionyl chloride (50 mL, 3000 mol%) at rt. Excess thionyl chloride was removed under reduced pressure giving a crude product which was washed with hot $CHCl_3$ to afford **58a** (3.0 g, 85%) as a pale yellow solid: ^{1}H NMR (DMSO-d_6, 500.1 MHz) δ 9.42 (s, 1H), 7.99 (d, J = 8.6 Hz, 2H), 6.96 (d, J = 8.6 Hz, 2H).

*6.1.5.1. 3-(1,2,3-Thiadiazol-4-yl)phenol (**58b**).* Pale yellow solid (2.0 g, 71%): ^{1}H NMR (DMSO-d_6, 500.1 MHz) δ 9.76 (br s, 1H), 9.59 (s, 1H), 7.60–7.57 (m, 2H), 7.38 (dd, J = 7.9, 7.9 Hz, 1H); ^{13}C NMR (DMSO-d_6, 125.1 MHz) δ 162.9, 158.9, 134.0, 132.8, 131.3, 118.9, 117.3, 114.8.

*6.1.5.2. 4-(1,2,3-Thiadiazol-4-yl)phenylamine hydrochloride (**58d**).* Yellow solid (805 mg, 75%): ^{1}H NMR (DMSO-d_6, 500.1 MHz) δ 9.59 (s, 1H), 8.17 (d, J = 7.0, 2 H), 7.35 (d, J = 7.0, 2H); Anal. calcd. for ($C_8H_8N_3SCl$): C, 44.97; H, 3.77; N, 19.66; found: C, 44.84; H, 3.69; N, 19.41.

6.1.6. N′-*[1-(2-Hydroxy-phenyl)-ethylidene]-hydrazinecarboxylic acid ethyl ester (**57c**) [66]*

A mixture of 2-hydroxyacetophenone (3.6 ml, 4.1 g, 30 mmol, 100 mol%), ethyl carbazate (3.3 g, 31.5 mmol, 105 mol%) and *p*-TsOH (280 mg, 1.5 mmol, 5 mol%) in ethanol (100 mL) was refluxed for 22 h and kept overnight in the refrigerator. The precipitate was filtered off with suction and washed with diethyl ether to give **57c** (5.24 g, 78%) as light yellow needles: ^{1}H NMR (DMSO-d_6, 500.1 MHz) δ 12.94 (br s, 1H), 10.80 (br s, 1H), 7.57 (dd, J = 8.3, 1.7 Hz, 1H), 7.30–7.27 (m, 1H), 6.91–6.88 (m, 1H), 4.25 (q, J = 7.1 H, 2H), 1.31 (t, J = 7.1 Hz, 3H).

*6.1.7. 2-(1,2,3-Thiadiazol-4-yl)phenol (**58c**) [66]*

Compound **57c** (3.5 g, 15.7 mmol, 100 mol%) was stirred overnight in thionyl chloride (35 mL, 3000 mol%) at rt. Excess thionyl chloride was removed under reduced pressure. The crude product was filtered through a plug of silica (CH_2Cl_2) and then purified by FC (eluent CH_2Cl_2) to give **58c** (1.2 g, 43%) as a yellow solid:^{1}H NMR ($CDCl_3$, 500.1 MHz) δ 10.53 (br s, 1H), 8.80 (s, 1H), 7.65 (dd, J = 7.9, 1.7 Hz, 1H), 7.35 (ddd, J = 8.4, 7.1, 1.5 Hz, 1H).

*6.1.8. General procedure for the preparation of the compounds **9**–**33**, **35**, **37**–**44**, **46**, and **48**–**51**; dodecylcarbamic acid 4-(1,2,3-thiadiazol-4-yl)phenyl ester (**26**)*

To a mixture of **58a** (89 mg, 0.5 mmol, 100 mol%) and a catalytic amount of Et_3N in dry toluene (2 mL) was added dodecyl isocyanate (130 μL, 0.55 mmol, 100 mol%). The reaction mixture was gently refluxed for 2.5 h. After cooling, the solvent was evaporated. Purification by FC (eluent EtOAc/PE, 1:2) and recrystallization (EtOAc/hexane, 1:1) gave **26** (196 mg, 82%) as a white solid: mp 149.5–150.0 °C; ^{1}H NMR ($CDCl_3$, 500.1 MHz) δ 8.60 (s, 1H), 8.04 (d, J = 8.6 Hz, 2H), 7.29 (d, J = 8.6 Hz, 2H), 5.06 (s, 1H), 3.29 (m, 2H), 1.68–0.83 (m, 23H); ^{13}C NMR ($CDCl_3$, 125.1 MHz) δ 162.2, 154.2, 152.0, 129.7, 128.4, 127.8, 122.3, 41.3, 31.9, 29.8, 29.64, 29.62, 29.58, 29.54, 29.34, 29.26, 26.8, 22.7, 14.1; Anal. ($C_{21}H_{31}N_3O_2S$) C, H, N.

*6.1.8.1. Ethylcarbamic acid 4-(4,5-dihydrothiazol-2-yl)phenyl ester (**9**).* White solid (480 mg, 74%): mp 130.5–131.9 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.83–7.81 (m, 2H), 7.27–7.16 (m, 2H), 5.18 (s, 1H), 4.44 (t, J = 8.2 Hz, 2H), 3.39 (t, J = 8.2 Hz, 2H), 3.29 (q, J = 7.1 Hz, 2H), 1.21 (t, J = 7.2 Hz, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 167.5, 153.8, 153.2, 130.1, 129.5, 121.4, 65.2, 36.1, 33.8, 15.0; Anal. ($C_{12}H_{14}N_2O_2S$) C, H, N.

6.1.8.2. 2 n*-Propylcarbamic acid 4-(4,5-dihydrothiazol-2-yl)phenyl ester (**10**).* White solid (520 mg, 80%): mp 121.0–121.2 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.85–7.82 (m, 2H), 7.20–7.17 (m, 2H), 5.05 (s, 1H), 4.45 (t, J = 8.4 Hz, 2H), 3.42 (t, J = 8.4 Hz, 2H), 3.27 (q, J = 6.8 Hz, 2H), 1.62 (sext, J = 7.3 Hz, 2H), 0.99 (t, J = 7.5 Hz, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 167.4, 153.9, 153.3, 130.2, 129.5, 121.3, 65.2, 42.9, 33.8, 22.9, 11.1; Anal. ($C_{13}H_{16}N_2O_2S$) C, H, N.

6.1.8.3. n*-Hexylcarbamic acid 4-(4,5-dihydrothiazol-2-yl)phenyl ester (**11**).* Colourless crystals (290 mg, 88%): mp 87.6–88.7 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.86–7.80 (m, 2H), 7.21–7.15 (m, 2H), 5.11 (app t, 1H), 4.44 (t, J = 8.3 Hz, 2H), 3.41 (t, J = 8.3 Hz, 2H), 3.25 (q, J = 6.8 Hz, 2H), 1.56 (qui, J = 7.2 Hz, 2H), 1.40–1.25 (m, 6H), 0.90 (t, J = 6.9 Hz, 3H); ^{13}C NMR ($CDCl_3$, 400 MHz) δ 167.5, 153.9, 153.3, 130.1, 129.5, 121.4, 65.2, 41.3, 33.8, 31.4, 29.7, 26.4, 22.5, 14.0; Anal. ($C_{16}H_{22}N_2O_2S$) C, H, N.

*6.1.8.4. Dodecylcarbamic acid 4-(4,5-dihydrothiazol-2-yl)phenyl ester (**12**).* Colourless crystals (290 mg, 88%): mp 87.6–88.7 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.86–7.80 (m, 2H), 7.21–7.15 (m, 2H), 5.11 (app t, 1H), 4.44 (t, J = 8.3 Hz, 2H), 3.41 (t, J = 8.3 Hz, 2H), 3.25 (q, J = 6.8 Hz, 2H), 1.56 (qui, J = 7.2 Hz, 2H), 1.40–1.25 (m, 6H), 0.90 (t, J = 6.9 Hz, 3H); ^{13}C NMR ($CDCl_3$, 400 MHz) δ 167.5, 153.9, 153.3, 130.1, 129.5, 121.4, 65.2, 41.3, 33.8, 31.4, 29.7, 26.4, 22.5, 14.0; Anal. ($C_{22}H_{34}N_2O_2S$) C, H, N.

6.1.8.5. tert-*Butylcarbamic acid 4-(4,5-dihydrothiazol-2-yl)phenyl ester (**13**).* White solid (580 mg, 83%); mp 148.9–151.1 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.82–7.79 (m, 2H), 7.17–7.15 (m, 2H), 5.19 (s, 1H), 4.42 (t, J = 8.4 Hz, 2H), 3.42 (t, J = 8.3 Hz, 2H), 1.35 (s, 9H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 167.4, 153.2, 152.0, 130.0, 129.5, 121.9, 65.2, 50.8, 33.7, 28.7; Anal. ($C_{14}H_{18}N_2O_2S$) C, H, N.

*6.1.8.6. Cyclopentylcarbamic acid 4-(4,5-dihydrothiazol-2-yl)phenyl ester (**14**).* Pale yellow solid (670 mg, 92%): mp 170.5–173.0 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.87–7.79 (m, 2H), 7.21–7.16 (m, 2H), 5.06 (br d, J = 6.8 Hz, 1H), 4.44 (t, J = 8.3 Hz, 2H), 4.05 (sext, J = 6.9 Hz, 1H), 3.41 (t, J = 8.3 Hz, 2H), 2.02 (sext, J = 6.1 Hz, 2H), 1.77–1.56 (m, 4H), 1.54–1.43 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 167.9, 153.8, 153.7, 130.5, 129.9, 121.9, 65.6, 53.4, 34.2, 33.5, 23.9; Anal. ($C_{15}H_{18}N_2O_2S$) C, H, N.

*6.1.8.7. Cyclohexylcarbamic acid 4-(4,5-dihydrothiazol-2-yl)phenyl ester (**15**).* White solid (560 mg, 73%): mp 167.1–168.8 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.83–7.81 (m, 2H), 7.19–7.17 (m, 2H), 5.06 (s, 1H), 4.44 (t, J = 8.2 Hz, 2H), 3.41 (t, J = 8.4 Hz, 2H), 2.04–2.00 (m, 3H), 1.76–1.71 (m, 3H), 1.64–1.60 (m, 1H), 1.21–1.18 (m, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 168.8, 163.0, 156.7, 128.8, 125.5, 114.7, 65.6, 49.8, 39.1, 32.6, 25.2, 24.6; HRMS calcd. for $C_{16}H_{20}N_2O_2SNa$ [M + Na]: 327.1143, found: 327.1126.

*6.1.8.8. Benzylcarbamic acid 4-(4,5-dihydrothiazol-2-yl)phenyl ester (**16**).* White solid (400 mg, 79%): mp 129.8–131.4 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.82–7.77 (m, 2H), 7.35–7.20 (m, 5H), 7.17–7.08 (m, 2H), 5.88 (app t, 1H), 4.45–4.34 (m, 4H), 3.35 (t, $J = 8.3$ Hz, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 167.6, 154.0, 153.1, 137.8, 130.0, 129.4, 128.6, 127.5, 127.5, 121.3, 64.9, 45.0, 33.6; Anal. ($C_{17}H_{16}N_2O_2S$) C, H, N.

*6.1.8.9. (2-Methyl)-benzylcarbamic acid 4-(4,5-dihydrothiazol-2-yl)phenyl ester (**17**).* White solid (300 mg, 53%): mp 117.1–118.7 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.84–7.79 (m, 2H), 7.30–7.26 (m, 1H), 7.25–7.15 (m, 5H), 5.47–5.41 (m, 1H), 4.50–4.38 (m, 4H), 3.38 (t, $J = 8.3$ Hz, 2H), 2.35 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 167.4, 153.8, 153.2, 136.2, 135.4, 130.5, 130.2, 129.5, 128.3, 127.9, 126.2, 121.3, 65.1, 43.3, 33.8, 19.0; Anal. ($C_{18}H_{18}N_2O_2S$) C, H, N.

*6.1.8.10. (4-Methyl)-benzylcarbamic acid 4-(4,5-dihydrothiazol-2-yl)phenyl ester (**18**).* White solid (460 mg, 85%): mp 144.0–144.6 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.87–7.76 (m, 2H), 7.27–7.06 (m, 6H), 5.56 (br s, 1H), 4.48–4.33 (m, 4H), 3.39 (t, $J = 8.1$ Hz, 2H), 2.34 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 167.4, 154.0, 153.2, 137.4, 134.7, 130.2, 129.4, 129.3, 127.6, 121.4, 65.1, 45.0, 33.7, 21.0; Anal. ($C_{18}H_{18}N_2O_2S$) C, H, N.

*6.1.8.11. (4-Methoxy)-benzylcarbamic acid 4-(4,5-dihydrothiazol-2-yl)phenyl ester (**19**).* White solid (640 mg, 88%): mp 148.2–148.8 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.84–7.79 (m, 2H), 7.26–7.21 (m, 2H), 7.19–7.14 (m, 2H), 6.89–6.84 (m, 2H), 5.69–5.62 (m, 1H), 4.41 (t, $J = 8.3$ Hz, 2H), 4.34 (d, $J = 5.8$ Hz, 2H), 3.78 (s, 3H), 3.38 (t, $J = 8.3$ Hz, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 167.4, 159.0, 153.9, 153.1, 130.1, 129.9, 129.4, 129.0, 121.4, 114.0, 65.0, 55.2, 44.6, 33.7; Anal. ($C_{18}H_{18}N_2O_3S$) C, H, N.

*6.1.8.12. Phenethylcarbamic acid 4-(4,5-dihydrothiazol-2-yl)phenyl ester (**20**).* White solid (200 mg, 37%): mp 138.1–138.8 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.85–7.80 (m, 2H), 7.36–7.31 (m, 2H), 7.28–7.21 (m, 3H), 7.18–7.13 (m, 2H), 5.09 (app t, 1H), 4.44 (t, $J = 8.3$ Hz, 2H), 3.54 (dd, $J = 13.1$, 6.8 Hz, 2H), 3.41 (t, $J = 8.3$ Hz, 2H), 2.89 (t, $J = 7.0$ Hz, 2H); ($CDCl_3$, 100 MHz) δ 167.5, 153.9, 153.2, 138.4, 130.2, 129.5, 128.8, 128.7, 126.7, 121.4, 65.2, 42.3, 35.9, 33.8; Anal. ($C_{18}H_{18}N_2O_2S$) C, H, N, S.

6.1.8.13. n-*Butylcarbamic acid 4-(4,5-dihydrooxazol-2-yl)phenyl ester (**21**).* White solid (260 mg, 87%): mp 116–117 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.98–7.91 (m, 2H), 7.21–7.16 (m, 2H), 5.04 (br s, 1H), 4.43 (t, $J = 8.2$ Hz, 2H), 4.06 (t, $J = 8.2$ Hz, 2H), 3.28–3.26 (m, 2H), 1.60–1.52 (m, 2H), 1.45–1.35 (m, 2H), 0.96 (t, $J = 7.3$ Hz, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 164.0, 154.0, 153.5, 129.3, 124.5, 121.3, 67.6, 54.8, 40.9, 31.8, 19.8, 13.6; Anal. ($C_{14}H_{18}N_2O_3$) C, H, N.

*6.1.8.14. Cyclopentylcarbamic acid 4-(4,5-dihydrooxazol-2-yl)phenyl ester (**22**).* Pale yellow solid (300 mg, 90%): mp 81–82 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.00–7.89 (m, 2H), 7.23–7.14 (m, 2H), 5.10–5.01 (m, 1H), 4.43 (t, $J = 9.5$ Hz, 2H), 4.09–4.02 (m, 3H), 2.08–1.98 (m, 2H), 1.87–1.44 (m, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 164.0, 153.5, 153.4, 129.4, 124.5, 121.3, 67.6, 54.9, 53.0, 33.1, 23.5; Anal. ($C_{15}H_{18}N_2O_3 \cdot H_2O$) C, H, N.

*6.1.8.15. (2-Methyl)-benzylcarbamic acid 4-(4,5-dihydrooxazol-2-yl)phenyl ester (**23**).* White solid (190 mg, 50%): mp 125–126 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.94–7.90 (m, 2H), 7.30–7.25 (m, 1H), 7.23–7.09 (m, 5H), 5.62 (app t, $J = 5.2$ Hz, 1H), 4.44–4.36 (m, 4H), 4.00 (t, $J = 9.5$ Hz, 2H), 2.35 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 164.0, 153.9, 153.4, 136.2, 135.4, 130.5, 129.4, 128.2, 127.8, 126.2, 124.5, 121.3, 67.6, 54.7, 43.2, 18.9; Anal. ($C_{18}H_{18}N_2O_3$) C, H, N.

*6.1.8.16. (4-Methyl)-benzylcarbamic acid 4-(4,5-dihydrooxazol-2-yl)phenyl ester (**24**).* White solid (100 mg, 30%): mp 141–142.5 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.92–7.89 (m, 2H), 7.24–7.12 (m, 6H), 5.74 (br s, 1H), 4.45–4.38 (m, 4H), 4.04 (t, $J = 9.5$ Hz, 2H), 2.32 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 164.0, 154.0, 153.4, 137.4, 134.8, 129.4, 127.7, 124.7, 121.3, 67.6, 54.9, 45.0, 21.7; Anal. ($C_{18}H_{18}N_2O_3$) C, H, N.

6.1.8.17. n-*Hexylcarbamic acid 4-(1,2,3-thiadiazol-4-yl)phenyl ester (**25**).* White solid (44 mg, 28%): mp 146.4–148.2 °C; ^{1}H NMR ($CDCl_3$, 500.1 MHz) δ 8.61 (s, 1H), 8.04 (d, $J = 8.6$ Hz, 2H), 7.29 (d, $J = 8.6$ Hz, 2H), 5.06 (s, 1H), 3.31–3.27 (m, 2H), 1.62–1.58 (m, 2H), 1.41–1.33 (m, 6H), 0.91 (t, $J = 6.7$ Hz, 3H); ^{13}C NMR ($CDCl_3$, 125.1 MHz) δ 162.2, 154.2, 152.0, 129.7, 128.4, 127.8, 122.3, 41.3, 31.4, 29.8, 26.4, 22.5, 14.0; Anal. ($C_{15}H_{19}N_3O_2S$) C, H, N.

*6.1.8.18. Cyclopentylcarbamic acid 4-(1,2,3-thiadiazol-4-yl)phenyl ester (**27**).* White solid (262 mg, 90%): mp 189.7–190.3 °C; ^{1}H NMR ($CDCl_3$, 500.1 MHz) δ 8.60 (s, 1H), 8.03 (d, $J = 8.6$ Hz, 2H), 7.29 (d, $J = 8.6$ Hz, 2H), 5.03 (d, $J = 6.7$ Hz, 1H), 4.11–4.05 (m, 1H), 2.08–2.01 (m, 2H), 1.77–1.48 (m, 6H); ^{13}C NMR ($CDCl_3$, 125.1 MHz) δ 162.2, 153.6, 152.0, 129.7, 128.4, 127.8, 122.3, 53.1, 33.2, 23.5; Anal. ($C_{14}H_{15}N_3O_2S$) C, H, N.

*6.1.8.19. Cyclohexylcarbamic acid 4-(1,2,3-thiadiazol-4-yl)phenyl ester (**28**).* Light yellow solid (250 mg, 55%): mp 175.0–177.1 °C; ^{1}H NMR ($CDCl_3$, 500.1 MHz) δ 8.64 (s, 1H), 8.03 (d, $J = 8.6$ Hz, 2H), 7.29 (d, $J = 8.6$ Hz, 2H), 4.96 (d, $J = 7.5$ Hz, 1H), 3.63–3.55 (m, 1H), 2.05–2.02 (m, 2H), 1.78–1.74 (m, 2H), 1.44–1.35 (m, 2H), 1.65–1.17 (m, 6H); ^{13}C NMR ($CDCl_3$, 125.1 MHz) δ 162.2, 153.2, 152.0, 129.7, 128.3, 127.7, 122.3, 50.2, 33.2, 25.4, 24.7; Anal. ($C_{15}H_{17}N_3O_2S$) C, H, N.

*6.1.8.20. Benzylcarbamic acid 4-(1,2,3-thiadiazol-4-yl)phenyl ester (**29**).* White solid (300 mg, 96%): mp 164.5–165.2 °C; ^{1}H NMR ($CDCl_3$, 500.1 MHz) δ 8.61 (s, 1H), 8.05 (d, $J = 8.5$ Hz, 2H), 7.40–7.31 (m, 7 H), 5.40 (s, 1H), 4.49 (d, $J = 6.0$ Hz, 2H); ^{13}C NMR ($CDCl_3$, 125.1 MHz) δ 162.1, 154.3, 151.9, 137.8, 129.8, 128.8, 128.4, 128.0, 127.8, 127.7, 122.3, 45.4; Anal. ($C_{16}H_{13}N_3O_2S$) C, H, N.

*6.1.8.21. 4-(Butylcarbamoyloxy)benzoic acid methyl ester (**30**) [51].* White crystals (88 mg, 70%): mp 90.3–91.0 °C; ^{1}H NMR ($CDCl_3$, 500.1 MHz) δ 8.03 (d, $J = 8.5$ Hz, 2H), 7.20 (d, $J = 8.5$ Hz, 2H), 5.12 (s, 1H), 3.90 (s, 3H), 3.27–3.25 (m, 2H), 1.58–1.53 (m, 2H), 1.43–1.35 (m, 2H), 0.95 (t, $J = 7.3$ Hz, 3H); ^{13}C NMR ($CDCl_3$, 125.1 MHz) δ 166.4, 154.8, 153.7, 131.0, 126.8, 121.3, 52.1, 41.0, 31.8, 19.9, 13.7; Anal. ($C_{13}H_{17}NO_4$) C, H, N.

*6.1.8.22. 4-(Hexylcarbamoyloxy)benzoic acid methyl ester (**31**).* White crystals (30 mg, 22%): mp 87.0–87.7 °C; ^{1}H NMR ($CDCl_3$, 500.1 MHz) δ 8.04 (d, $J = 8.7$ Hz, 2H), 7.21 (d, $J = 8.7$ Hz, 2H), 5.04 (s, 1H), 3.91 (s, 3H), 3.29–3.25 (m, 2H), 1.60–1.56 (m, 2H), 1.38–1.33 (m, 6H), 0.90 (t, $J = 6.8$ Hz, 3H); ^{13}C NMR ($CDCl_3$, 125.1 MHz) δ 166.4, 154.8, 153.7, 131.0, 126.8, 121.3, 52.1, 41.3, 31.4, 29.7, 26.4, 22.5, 14.0; Anal. ($C_{15}H_{21}NO_4$) C, H, N.

*6.1.8.23. 4-(Cyclohexylcarbamoyloxy)benzoic acid methyl ester (**32**).* White solid (491 mg, 59%): mp 135.3–136.2 °C; ^{1}H NMR ($CDCl_3$, 500.1 MHz) δ 8.04 (d, $J = 8.7$ Hz, 2H), 7.21 (d, $J = 8.7$ Hz, 2H), 4.95 (d, $J = 7.4$ Hz, 1H), 3.90 (s, 3H), 3.61–3.53 (m, 1H), 2.03–2.00 (m, 2H), 1.77–1.73 (m, 2H), 1.65–1.16 (m, 6H); ^{13}C NMR ($CDCl_3$, 125.1 MHz) δ 166.4, 154.8, 152.8, 130.9, 126.7, 121.2, 52.0, 50.2, 33.1, 25.4, 24.7; Anal. ($C_{15}H_{19}NO_4$) C, H, N.

*6.1.8.24. 4-(Dodecylcarbamoyloxy)benzoic acid methyl ester (**33**).* White solid (168 mg, 92%): mp 93.4–94.4 °C; ^{1}H NMR

($CDCl_3$, 500.1 MHz) δ 8.04 (d, $J = 8.7$ Hz, 2H), 7.21 (d, $J = 8.7$ Hz, 2H), 5.03 (s, 1 H), 3.91 (s, 3H), 3.29–3.25 (m, 2H), 1.61–1.55 (m, 2H), 1.38–1.27 (m, 18H), 0.88 (t, $J = 6.5$ Hz, 3H); ^{13}C NMR ($CDCl_3$, 125.1 MHz) δ 154.6, 151.1, 131.0, 129.2, 125.1, 121.6, 41.3, 31.9, 29.8, 29.62, 29.61, 29.6, 29.5, 29.32, 29.25, 26.7, 22.7, 14.1; Anal. ($C_{21}H_{33}NO_4$) C, H, N.

6.1.8.25. n-*Hexylcarbamic acid 4-nitrophenyl ester (**35**) [54].* White solid (108 mg, 81%): mp 84.8–85.9 °C; ^{1}H NMR ($CDCl_3$, 500.1 MHz) δ 8.24 (d, $J = 9.2$ Hz, 2H), 7.31 (d, $J = 9.2$ Hz, 2H), 5.12 (s, 1H), 3.30–3.26 (m, 2H), 1.61–1.56 (m, 2H), 1.40–1.32 (m, 6H), 0.90 (t, $J = 6.8$ Hz, 3H); ^{13}C NMR ($CDCl_3$, 125.1 MHz) δ 156.0, 153.0, 144.7, 125.1, 121.9, 41.4, 31.4, 29.4, 26.4, 22.5, 14.0; Anal. ($C_{13}H_{18}N_2O_4$) C, H, N.

6.1.8.26. n-*Butylcarbamic acid 4-cyanophenyl ester (**37**).* White solid (286 mg, 66%): mp 105.2–106.9 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.64 (d, $J = 8.5$ Hz, 2H), 7.26 (d, $J = 8.5$ Hz, 2H), 5.26 (s, 1H), 3.36–3.22 (m, 2H), 1.45–1.33 (m, 2H), 0.95 (t, $J = 7.3$ Hz, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 154.5, 153.2, 133.4, 122.3, 118.4, 108.6, 40.9, 31.6, 19.8, 13.6; Anal. ($C_{12}H_{14}N_2O_2$) C, H, N.

6.1.8.27. n-*Hexylcarbamic acid 4-cyanophenyl ester (**38**).* White solid (393 mg, 80%): mp 94.4–95.2 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.69–7.61 (m, 2H), 7.31–7.23 (m, 2H), 5.24 (app. t, 1H), 3.35–3.21 (m, 2H), 1.62–1.52 (m, 2H), 1.41–1.26 (m, 6H), 0.90 (t, 3H, $J = 6.8$ Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 154.5, 153.2, 133.4, 122.3, 118.4, 108.6, 41.3, 31.3, 29.6, 26.3, 22.4, 13.9; Anal. ($C_{14}H_{18}N_2O_2$) C, H, N.

6.1.8.28. n-*Hexylcarbamic acid 4-bromophenyl ester (**39**) [53].* White solid (444 mg, 74%): mp 98.8–99.8 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.47–7.43 (m, 2H), 7.04–6.99 (m, 2H), 5.04 (br s, 1H), 3.28–3.21 (m, 2H), 1.60–1.51 (m, 2H), 1.40–1.25 (m, 6H), 0.90 (t, $J = 6.8$ Hz, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 154.1, 150.1, 132.2, 123.3, 118.1, 41.3, 31.4, 29.7, 26.4, 22.5, 14.0; Anal. ($C_{13}H_{18}BrNO_2$) C, H, N.

6.1.8.29. n-*Butylcarbamic acid 4-methoxyphenyl ester (**40**) [52,55].* White crystals (122 mg, 100%): mp 75.6–76.7 °C; ^{1}H NMR ($CDCl_3$, 500.1 MHz) δ 7.03 (d, $J = 8.9$ Hz, 2H), 6.86 (d, $J = 8.9$ Hz, 2H), 5.00 (s, 1H), 3.78 (s, 3H), 3.24–3.23 (m, 2H), 1.57–1.51 (m, 2H), 1.42–1.35 (m, 2H), 0.94 (t, $J = 7.3$ Hz, 3H); ^{13}C NMR ($CDCl_3$, 125.1 MHz) δ 156.8, 155.0, 144.6, 122.4, 114.3, 55.6, 40.9, 31.9, 19.9, 13.7; Anal. ($C_{12}H_{17}NO_3$) C, H, N.

6.1.8.30. n-*Hexylcarbamic acid* p-*tolyl ester (**41**).* White solid (375 mg, 80%): mp 72.7–73.7 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.17–7.11 (m, 2H), 7.02–6.97 (m, 2H), 5.00 (br s, 1H), 3.24 (q, $J = 6.7$ Hz, 2H), 2.32 (s, 3H), 1.60–1.50 (m, 2H), 1.40–1.27 (m, 6H), 0.90 (t, $J = 6.8$ Hz, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) 154.8, 148.8, 134.7, 129.7, 121.3, 41.2, 31.4, 29.8, 26.4, 22.5, 20.8, 14.0; Anal. ($C_{14}H_{21}NO_2$) C, H, N.

6.1.8.31. n-*Hexylcarbamic acid phenyl ester (**42**) [56].* White solid (95 mg, 86%): mp 37.6–38.8 °C; ^{1}H NMR ($CDCl_3$, 500.1 MHz) δ 7.35 (pt, $J = 7.8$ Hz, 2H), 7.18 (t, $J = 7.4$ Hz, 1H), 7.13 (d, $J = 7.8$ Hz, 2H), 4.98 (br s, 1H), 3.29–3.24 (m, 2H), 1.60–1.54 (m, 2H), 1.38 (m, 6H), 0.90 (t, $J = 6.8$ Hz, 3H); ^{13}C NMR ($CDCl_3$, 125.1 MHz) δ 154.6, 151.1, 129.2, 125.1, 121.6, 41.2, 31.4, 29.7, 26.4, 22.5, 14.0; Anal. ($C_{13}H_{19}NO_2$) C, H, N.

*6.1.8.32. Dodecylcarbamic acid phenyl ester (**43**) [57].* White solid (153 mg, 100%): mp 56.9–57.7 °C; ^{1}H NMR ($CDCl_3$, 500.1 MHz) δ 7.35 (pt, $J = 7.8$ Hz, 2H), 7.18 (t, $J = 7.4$ Hz, 1H), 7.13 (d, $J = 7.7$ Hz, 2H), 4.97 (s, 1H), 3.28–3.24 (m, 2H), 1.58–1.56 (m, 2H), 1.33–1.27 (m, 18H), 0.89 (t, $J = 6.8$ Hz, 3H); ^{13}C NMR ($CDCl_3$, 125.1 MHz) δ 154.6, 151.1, 129.2, 125.2, 121.6, 41.3, 31.9, 29.8, 29.63, 29.61, 29.6, 29.5, 29.3, 29.3, 26.7, 22.7, 14.1; Anal. ($C_{19}H_{31}NO_2$) C, H, N.

*6.1.8.33. 3-Methoxy-4-(hexylcarbamoyloxy)benzoic acid methyl ester (**44**).* White solid (91 mg, 59%): mp 86.9–88.6 °C; ^{1}H NMR ($CDCl_3$, 500.1 MHz) δ 7.66–7.63 (m, 2H), 7.16 (d, $J = 8.2$ Hz, 1H), 5.11 (s, 1H), 3.91 (s, 3H), 3.89 (s, 3H), 3.28–3.24 (m, 2H), 1.60–1.54 (m, 2H), 1.38–1.32 (m, 6H), 0.90 (t, $J = 6.6$ Hz, 3H); ^{13}C NMR ($CDCl_3$, 125.1 MHz) δ 166.5, 153.6, 151.5, 143.9, 128.1, 123.1, 122.6, 113.3, 56.1, 52.2, 41.4, 31.4, 29.7, 26.3, 22.5, 14.0; Anal. ($C_{16}H_{23}NO_5$) C, H, N.

*6.1.8.34. 3-Iodo-4-(hexylcarbamoyloxy)benzoic acid methyl ester (**46**).* White crystals (89 mg, 44%): mp 83.1–83.5 °C; ^{1}H NMR ($CDCl_3$, 500.1 MHz) δ 8.48 (d, $J = 2.6$ Hz, 1H), 8.01 (dd, $J = 8.4$, 2.6 Hz, 1H), 7.26 (d, $J = 8.4$ Hz, 1H), 5.19 (s, 1H), 3.91 (s, 3H), 3.31–3.27 (m, 2H), 1.63–1.57 (m, 2H), 1.40–1.31 (m, 6H), 0.90 (t, $J = 6.8$ Hz, 3H); ^{13}C NMR ($CDCl_3$, 125.1 MHz) δ 165.1, 154.7, 152.4, 140.7, 130.8, 128.8, 122.9, 90.3, 52.4, 41.5, 31.4, 29.7, 26.4, 22.6, 14.0; Anal. ($C_{15}H_{20}INO_4$) C, H, N.

6.1.8.35. n-*Butylcarbamic acid 2-(1,2,3-thiadiazol-4-yl)phenyl ester (**48**).* White solid (100 mg, 73%): mp 102.8–104.7 °C; ^{1}H NMR ($CDCl_3$, 500.1 MHz) δ 8.70 (s, 1H), 8.16 (d, $J = 6.8$ Hz, 1H), 7.46 (pt, $J = 8.1$, 6.8 Hz, 1H), 7.37 (pt, $J = 8.1$, 6.8 Hz, 1H), 7.28 (d, $J = 8.1$ Hz, 1H), 5.12 (s, 1H), 3.25–3.21 (m, 2H), 1.54–1.48 (m, 2H), 1.38–1.31 (m, 2H), 0.93 (t, $J = 7.1$ Hz, 3H); ^{13}C NMR ($CDCl_3$, 125.1 MHz) δ 158.2, 153.9, 148.2, 133.1, 130.6, 130.3, 126.2, 124.2, 123.6, 41.1, 31.8, 19.8, 13.7; Anal. ($C_{13}H_{15}N_3O_2S$) C, H, N.

6.1.8.36. n-*Butylcarbamic acid 3-(1,2,3-thiadiazol-4-yl)phenyl ester (**49**).* Pale yellow solid (244 mg, 81%): mp 101.6–102.9 °C; ^{1}H NMR ($CDCl_3$, 500.1 MHz) δ 8.65 (s, 1H), 7.89 (d, $J = 7.9$ Hz, 1H), 7.83 (s, 1H), 7.49 (pt, $J = 8.1$, 7.9 Hz, 1H), 7.22 (d, $J = 8.1$ Hz, 1H), 5.07 (s, 1H), 3.32–3.28 (m, 2H), 1.61–1.55 (m, 2H), 1.45–1.38 (m, 2H), 0.97 (t, $J = 7.4$ Hz, 3H); ^{13}C NMR ($CDCl_3$, 125.1 MHz) δ 162.0, 154.3, 151.6, 131.9, 130.6, 130.0, 124.1, 122.6, 120.6, 41.0, 31.8, 19.8, 13.7; Anal. ($C_{13}H_{15}N_3O_2S$) C, H, N.

*6.1.8.37. Dodecylcarbamic acid 2-(1,2,3-thiadiazol-4-yl)phenyl ester (**50**).* White solid (187 mg, 96%): mp 107.1–108.4 °C; ^{1}H NMR ($CDCl_3$, 500.1 MHz) δ 8.70 (s, 1H), 8.17 (d, $J = 7.4$ Hz, 1H), 7.47 (pt, $J = 8.1$, 7.4 Hz, 1H), 7.38 (pt, $J = 8.1$, 7.4 Hz, 1H), 7.29 (d, $J = 8.1$ Hz, 1H), 5.08 (s, 1H), 3.25–3.21 (m, 2H), 1.55–1.52 (m, 2H), 1.30–1.26 (m, 18H), 0.88 (t, $J = 6.6$ Hz, 3H); ^{13}C NMR ($CDCl_3$, 125.1 MHz) δ 158.3, 153.9, 148.2, 133.0, 130.6, 130.3, 126.2, 124.2, 123.6, 41.4, 31.9, 29.8, 29.61, 29.57, 29.5, 29.3, 29.2, 26.7, 22.7, 14.1; Anal. ($C_{21}H_{31}N_3O_2S$) C, H, N.

*6.1.8.38. Dodecylcarbamic acid 3-(1,2,3-thiadiazol-4-yl)phenyl ester (**51**).* White solid (99 mg, 90%): mp 106.4–107.4 °C; ^{1}H NMR ($CDCl_3$, 500.1 MHz) δ 8.65 (s, 1H), 7.89 (d, $J = 7.8$ Hz, 1H), 7.83 (s, 1H), 7.50 (pt, $J = 8.1$, 7.8 Hz, 1H), 7.23 (d, $J = 8.1$ Hz, 1H), 5.06 (s, 1H), 3.29 (m, 2H), 1.62–1.59 (m, 2H), 1.37–1.27 (m, 18H), 0.88 (t, $J = 6.5$ Hz, 3H); ^{13}C NMR ($CDCl_3$, 125.1 MHz) δ 162.1, 154.3, 151.7, 132.0, 130.4, 130.1, 124.2, 122.7, 120.7, 41.4, 31.9, 29.8, 29.62, 29.58, 29.5, 29.3, 29.3, 26.8, 14.1; Anal. ($C_{21}H_{31}N_3O_2S$) C, H, N.

*6.1.9. General procedure for the preparation of the alkyl carbamic acid 4-nitrophenyl esters **34** and **36**;* n-*butylcarbamic acid 4-nitrophenyl ester (**34**) [52,55]*

4-Nitrophenyl chloroformate (670 mg, 3.3 mmol, 110 mol%) in CH_2Cl_2 (10 mL) was added to a mixture of butylamine (0.3 mL, 3.0 mmol, 100 mol%) and pyridine (0.27 mL, 3.3 mmol, 110 mol%). The mixture was gently refluxed for 1 h, cooled and evaporated to dryness. The residue was purified by FC (eluent EtOAc/PE, 1:4) to

give **34** (232 mg, 32%) as a white solid: mp 96.7–97.4 °C; ^{1}H NMR ($CDCl_3$, 500.1 MHz) δ 8.24 (d, J = 9.1 Hz, 2H), 7.32 (d, J = 9.1 Hz, 2H), 5.09 (s, 1H), 3.32–3.28 (m, 2H), 1.61–1.55 (m, 2H), 1.45–1.37 (m, 2H), 0.97 (t, J = 7.4 Hz, 3H); ^{13}C NMR ($CDCl_3$, 125.1 MHz) δ 156.0, 153.1, 144.6, 125.0, 121.9, 41.0, 31.7, 19.8, 13.6; Anal. ($C_{11}H_{14}N_2O_4$) C, H, N.

*6.1.9.1. Cyclohexylcarbamic acid 4-nitrophenyl ester (**36**) [61].* Pale yellow solid (261 mg, 99%): mp 146.3–147.1 °C; ^{1}H NMR ($CDCl_3$, 500.1 MHz) δ 8.24 (d, J = 9.1 Hz, 2H), 7.32 (d, J = 9.1 Hz, 2H), 4.99 (d, J = 6.1 Hz, 1H), 3.61–3.54 (m, 1H), 2.04–2.01 (m, 2H), 1.79–1.73 (m, 2H), 1.67–1.17 (m, 6H); ^{13}C NMR ($CDCl_3$, 125.1 MHz) δ 156.1, 152.1, 144.6, 125.1, 121.9, 50.4, 33.1, 25.3, 24.7; Anal. ($C_{13}H_{16}N_2O_4$) C, H, N.

*6.1.10. General procedure for the preparation of the carbamic acid esters **45**, and **47** via esterification; 3-methyl-4-(hexylcarbamoyloxy)benzoic acid methyl ester (**45**)*

4-Hydroxy-3-methyl benzoic acid (152 mg, 100 mol%) in methanol (10 mL) and a catalytic amount of conc. H_2SO_4 was refluxed overnight and the remaining methanol was evaporated giving 177 mg (100%) of the corresponding methyl ester. This was used directly without further purification to the preparation of the title compound.

To a mixture of 4-hydroxy-3-methyl benzoic acid methyl ester (83 mg, 0.5 mmol, 100 mol%) and a catalytic amount of Et_3N in dry toluene (2 mL) was added hexyl isocyanate (80 μL, 110 mol%). After gently refluxing overnight the reaction mixture was cooled and the solvent evaporated. Purification by FC (eluent EtOAc/PE, 1:1) and crystallization (EtOAc/hexane, 1:2) gave **45** (81 mg, 55%) as white crystals: mp 52.4–53.5 °C; ^{1}H NMR ($CDCl_3$, 500.1 MHz) δ 7.91 (s, 1H), 7.87 (d, J = 8.4 Hz, 1H), 7.15 (d, J = 8.4 Hz, 1H), 5.08 (s, 1H), 3.89 (s, 3H), 3.29–3.24 (m, 2H), 2.25 (s, 3H), 1.61 (m, 2H), 1.38 (m, 6H), 0.90 (t, J = 6.8 Hz, 2H); ^{13}C NMR ($CDCl_3$, 125.1 MHz) δ 166.6, 153.7, 153.2, 132.5, 130.7, 128.5, 127.2, 122.2, 52.1, 41.3, 31.4, 29.8, 26.4, 22.5, 16.1, 14.0; Anal. ($C_{16}H_{23}NO_4$) C, H, N.

*6.1.10.1. 3,5-Di-tert-butyl-4-(hexylcarbamoyloxy)benzoic acid methyl ester (**47**).* White crystals (50 mg, 26%): mp 104.1–104.9 °C; ^{1}H NMR ($CDCl_3$, 500.1 MHz) δ 8.00 (s, 2H), 5.09 (s, 1H), 3.90 (s, 3H), 3.29–3.25 (m, 2H), 1.67–1.31 (m, 29H); ^{13}C NMR ($CDCl_3$, 125.1 MHz) δ 167.2, 154.7, 152.3, 143.8, 127.9, 126.7, 52.0, 41.3, 35.7, 31.4, 31.2, 29.8, 26.4, 22.6, 14.0; Anal. ($C_{23}H_{37}NO_4$) C, H, N.

*6.1.11. 4-Nitrophenylcarbamic acid cyclohexyl ester (**52**)*

To a mixture of cyclohexanol (47 μL, 0.5 mmol, 100 mol%) and a catalytic amount of Et_3N in dry toluene (2 ml) was added 4-nitrophenyl isocyanate (90 mg, 0.55 mmol, 110 mol%). The reaction mixture was gently refluxed and monitored by TLC until cyclohexanol could not be detected on TLC. The reaction mixture was cooled and solvent evaporated. Purification by FC (eluent EtOAc/PE, 1:1) gave compound **52** (145 mg, 100%) as white solid: mp 115.2–116.4 °C; ^{1}H NMR ($CDCl_3$, 500.1 MHz) δ 8.18 (d, J = 8.9, 2H), 7.55 (d, J = 8.9, 2H), 7.03 (s, 1H), 4.81–4.76 (m, 1H), 1.94–1.91 (m, 2H), 1.76–1.73 (m, 2H), 1.57–1.23 (m, 6H); ^{13}C NMR ($CDCl_3$, 125.1 MHz) δ 152.4, 144.2, 142.8, 125.2, 117.6, 74.6, 31.7, 25.2, 23.7; Anal. ($C_{13}H_{16}N_2O_4$) C, H, N.

*6.1.12. 1-Hexyl-3-(4-(1,2,3-thiadiazol-4-yl)phenyl) urea (**53**)*

To a mixture of 4-(1,2,3-thiadiazol-4-yl)phenylamine hydrochloride (**58d**, 89 mg, 0.41 mmol, 100 mol%) and Et_3N (63 μL, 110 mol%) in dry toluene (2 mL) was added hexyl isocyanate (65 μL, 0.451 mmol, 110 mol%). The reaction mixture was gently refluxed for 5 h and then stirred overnight at rt. Solvent was evaporated and the resulting crude product was purified by FC (eluent EtOAc/PE, 1:1) and crystallization (EtOAc) to give compound **53** (85 mg, 68%) as yellow solid: mp 159.5–160.7 °C; ^{1}H NMR (DMSO-d_6, 500.1 MHz) δ 9.47 (s, 1H), 8.68 (s, 1H), 8.03 (d, J = 8.6 Hz, 2H), 7.59 (d, J = 8.6 Hz, 2H), 6.25 (t, J = 5.6 Hz, 1H), 3.15–3.11 (m, 2H), 1.49–1.45 (m, 2H), 1.37–1.27 (m, 6H), 0.93–0.88 (m, 3H); ^{13}C NMR (DMSO-d_6, 125.1 MHz) δ 163.0, 156.0, 142.6, 132.0, 128.6, 124.6, 118.7, 32.0, 31.0, 30.6, 27.0, 23.0, 14.9; Anal. ($C_{15}H_{20}N_4OS \cdot 1/14$ *n*-hexylamine) C, H, N.

6.2. Assay of MGL activity

6.2.1. Animals and preparation of rat cerebellar membranes for MGL assay

Four-week-old male Wistar rats were used in these studies. All animal experiments were approved by the local ethics committee. The animals lived in a 12-h light/12-h dark cycle (lights on at 0700 h), with water and food available ad libitum. The rats were decapitated eight hours after lights on (1500 h), the whole brains were removed, dipped in isopentane on dry ice and stored at −80 °C.

The membranes were prepared as previously described [71–73]. Briefly, cerebella were dissected and homogenized in nine volumes of ice-cold 0.32 M sucrose with a glass Teflon homogenizer. The crude homogenate was centrifuged at low speed (1000 × *g* for 10 min at 4 °C) and the pellet was discharged. The supernatant was centrifuged at high speed (100,000 × *g* for 10 min at 4 °C). The pellet was resuspended in ice-cold deionized water and washed twice, repeating the high-speed centrifugation. Finally, the membranes were resuspended in 50 mM Tris–HCl, pH 7.4 with 1 mM EDTA and aliquoted for storage at −80 °C until use. The protein concentration of the final preparation was measured by the Bradford method [74].

6.2.2. In vitro assay for MGL activity in rat cerebellar membranes

The assay for MGL has been described previously [34]. Briefly, experiments were carried out with preincubations (80 μL, 30 min at 25 °C) containing 10 μg membrane protein, 44 mM Tris–HCl (pH 7.4), 0.9 mM EDTA, 0.5% (wt/vol) BSA and 1.25% (vol/vol) DMSO as a solvent for inhibitors. The preincubated membranes were kept at 0 °C just prior to the experiments. The incubations (90 min at 25 °C) were initiated by adding 40 μL of the preincubated membrane cocktail, giving a final volume of 400 μL. The final volume contained 5 μg membrane protein, 54 mM Tris–HCl (pH 7.4), 1.1 mM EDTA, 100 mM NaCl, 5 mM $MgCl_2$, 0.5% (wt/vol) BSA and 50 μM of 2-AG. At time-points of 0 and 90 min, 100 μl aliquots were removed from the incubation, acetonitrile (200 μL) was added to stop the enzymatic reaction, and the pH of the samples was simultaneously decreased with phosphoric acid (added to acetonitrile) to 3.0, in order to stabilize 2-AG against chemical acyl migration reaction to 1(3)-AG. The samples were centrifuged at 23,700*g* for 4 min at rt prior to HPLC analysis of the supernatant.

6.2.3. HPLC method for in vitro assay for MGL

The analytical HPLC was performed as previously described [34]. Briefly, the analytical HPLC system consisted of a Merck Hitachi (Hitachi Ltd., Tokyo, Japan) L-7100 pump, D-7000 interface module, L-7455 diode-array UV detector (190–800 nm, set at 211 nm) and L-7250 programmable autosampler. The separations were accomplished on a Zorbax SB-C18 endcapped reversed-phase precolumn (4.6 × 12.5 mm, 5 μm) and column (4.6 × 150 mm, 5 μm) (Agilent, U.S.A). The injection volume was 50 μl. A mobile phase mixture of 28% phosphate buffer (30 mM, pH 3.0) in acetonitrile was used at a flow rate of 2.0 ml min^{-1}. Retention times were 5.8 min for 2-AG, 6.3 min for 1(3)-AG and 10.2 min for arachidonic acid. The relative concentrations of 2-AG, 1(3)-AG and arachidonic acid were determined by the corresponding peak areas. This was justified by the equivalence of response factors for the studied compounds, and

was supported by the observation that the sum of the peak areas was constant throughout the experiments.

6.3. Human recombinant MGL (hrMGL) assay

The endpoint enzymatic assay was developed to quantify human recombinant MGL (Cayman Chemical, cat# 10008354) activity with tritium-labelled 2-oleoylglycerol [glycerol-1,2,3-^{3}H] (American Radiolabeled Chemicals Inc., St Louis, MO, USA). The assay buffer was 50 mM Tris–HCl, pH 7.4; 1 mM EDTA and test compounds were dissolved in DMSO (the final DMSO concentration was not more than 5% v/v). The incubations were performed in the presence of 0.5% (w/v) BSA (essentially fatty acid-free). hrMGL (0.015 μL) was preincubated with test compounds for 10 min at 37 °C (60 μL). At the 10 min time point, 2-oleoylglycerol was added to achieve the final concentration of 50 μM (containing 112×10^{-3} μCi of 40 Ci/mmol [^{3}H]2-OG) with the final incubation volume of 100 μL. The incubations proceeded for 10 min at 37 °C. Ethyl acetate (400 μL) was added at the 20 min time point to stop the enzymatic reaction. Additionally, 100 μL of buffer (50 mM Tris–HCl, pH 7.4; 1 mM EDTA) was added. The samples were centrifuged at 16,000*g* for 4 min at rt, and aliquots (100 μL) were taken from the aqueous phase, which contained glycerol-1,2,3-^{3}H, and measured for radioactivity by liquid scintillation counting (Wallac 1450 MicroBeta; Wallac Oy, Finland).

6.4. Assay of FAAH activity

6.4.1. Animals and preparation of rat brain homogenate for FAAH assay

Eight-week-old male Wistar rats were used in these studies. All animal experiments were approved by the local ethics committee. The animals lived in a 12-h light/12-h dark cycle (lights on at 0700 h) with water and food available ad libitum.

The rats were decapitated, forebrains were dissected and homogenized in one volume (v/w) of ice-cold 0.1 M potassium phosphate buffer (pH 7.4) with a Potter–Elvehjem homogenizer (Heidolph). The homogenate was centrifuged at 10,000*g* for 20 min (at 4 °C). The protein concentration of the supernatant was determined by the method of Bradford with BSA as a standard [74]. Aliquots of the supernatant were stored at −80 °C until use.

6.4.2. In vitro assay for FAAH activity

The assay for FAAH has been described previously [50]. The endpoint enzymatic assay was developed to quantify FAAH activity with tritium-labelled arachidonoylethanolamide [ethanolamine 1-^{3}H] (American Radiolabeled Chemicals Inc., St Louis, MO, USA). The assay buffer was 0.1 M potassium phosphate (pH 7.4) and test compounds were dissolved in DMSO (the final DMSO concentration was not more than 5% v/v). The incubations were performed in the presence of 0.5% (w/v) BSA (essentially fatty acid-free). Test compounds were preincubated with rat brain homogenate protein (18 μg) for 10 min at 37 °C (60 μL). At the 10 min time point, *N*-arachidonoylethanolamine was added to achieve the final concentration of 2 μM (containing 50×10^{-3} μCi of 60 Ci/mmol [^{3}H]AEA) with the final incubation volume of 100 μL. The incubations proceeded for 10 min at 37 °C. Ethyl acetate (400 μL) was added at the 20 min time point to stop the enzymatic reaction. Additionally, 100 μL of unlabeled ethanolamine (1 mM) was added. Samples were centrifuged at 16,000*g* for 4 min at rt, and aliquots (100 μL) were taken from the aqueous phase, which contained ethanolamine 1-^{3}H, and measured for radioactivity by liquid scintillation counting (Wallac 1450 MicroBeta; Wallac Oy, Finland).

6.5. Reversibility studies

The reversibility of FAAH inhibition by compounds **26**, CAY10435 and **4** was determined by measuring the recovery of enzymatic activity after a rapid and large dilution of enzyme–inhibitor complex. In the preincubation mixture (70 μL) the amount of rat brain homogenate was 20-fold the amount required for the activity assay, and the inhibitor concentration was approximately 20-fold the IC_{50} value. After the preincubation, 1.8 μL of enzyme–inhibitor mixture was taken and immediately diluted 500-fold into the assay buffer containing AEA. The final incubation concentration of AEA was 2 μM (including ∼20 nM [^{3}H]AEA). The samples of 100 μL were taken at 0, 5, 10, 15, 20, 30, 45 and 60 min time-points and added to tubes containing 400 μL of cold ethyl acetate. Additionally, 100 μl of assay buffer was added. Samples were centrifuged at 16,000*g* for 4 min at rt, and aliquots (100 μL) were taken from the aqueous phase, which contained ethanolamine 1-^{3}H, and measured for radioactivity by liquid scintillation counting (Wallac 1450 MicroBeta; Wallac Oy, Finland).

The reversibility of hrMGL inhibition by compound **26** and MAFP was determined analogously to the method described for the reversibility of FAAH. Briefly, in the preincubation mixture (30 μL) the hrMGL concentration was 50-fold the concentration required for the activity assay, and the inhibitor concentration was approximately 30-fold the IC_{50} value. After the preincubation, 1.8 μL of enzyme–inhibitor mixture was taken and immediately diluted 500-fold into the assay buffer containing 2-OG. The final incubation concentration of 2-OG was 50 μM (containing 1.2 μCi of 40 Ci/mmol [^{3}H]2-OG). The samples of 100 μL were taken at 0, 5, 10, 15, 20, 30, 45 and 60 min time-points and added to tubes containing 400 μL of cold ethyl acetate. Additionally, 100 μl of assay buffer was added. Samples were centrifuged at 16,000*g* for 4 min at rt, and aliquots (100 μL) were taken from the aqueous phase, which contained glycerol-1,2,3-^{3}H, and measured for radioactivity by liquid scintillation counting (Wallac 1450 MicroBeta; Wallac Oy, Finland).

6.6. Uptake of AEA by intact RBL2H3 cells

The uptake assay using RBL2H3 basophilic leukaemia cells was undertaken as described previously [75] using tritium-labelled arachidonoylethanolamide [arachidonoyl 5,6,8,9,11,12,14,15-^{3}H] (American Radiolabeled Chemicals Inc., St Louis, MO, USA). The cells were preincubated with compound **26** and/or URB597 for 10 min at 37 °C prior to addition of [^{3}H]AEA (assay concentration 100 nM) and incubation for a further 10 min at 37 °C. In these cells, the uptake of AEA is driven by its subsequent FAAH-catalysed hydrolysis to arachidonic acid [76], and the difference between the observed activity in the absence and presence of an FAAH inhibitor like URB597 represents the component of uptake due to FAAH.

6.7. Hydrolysis of AEA and 2-AG by intact RBL2H3 cells

The assay used was that of Paylor et al. [76] using tritium-labelled arachidonoylethanolamide [ethanolamine 1-^{3}H] and 2-arachidonoylglycerol [glycerol-1,2,3-^{3}H] (American Radiolabeled Chemicals Inc., St Louis, MO, USA). The cells were preincubated with compound 25 and/or URB597 for 10 min at 37 °C prior to addition of [^{3}H]AEA or [^{3}H]2-AG, as appropriate, (assay concentration 100 nM) and incubation for a further 20 min at 37 °C.

6.8. Data analyses

The results from the enzyme inhibition experiments are presented as mean ± 95% confidence intervals of at least three

independent experiments performed in duplicate. Data analyses for the concentration–response curves were calculated as non-linear regressions using the built-in equation "sigmoidal dose–response curve, variable Hill slope" of GraphPad Prism 4.0 for Windows.

Acknowledgements

We thank Minna Glad, Erja Kivioja, Tiina Koivunen and Helly Rissanen for technical help. The Graduate School for Organic Chemistry and Chemical Biology (GSOCCB), The National Technology Agency of Finland, The Academy of Finland (grant 110277), and The Research and Science Foundation of Farmos have provided financial support for this study. In addition, CJF thanks Mrs. Eva Hallin and Ingrid Persson for technical expertise with the experiments utilizing RBL2H3 cells, and the Swedish Research Council (Grant no. 12158, medicine) for financial support.

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