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Original article

Carbamoyl tetrazoles as inhibitors of endocannabinoid inactivation: A critical revisitation

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Abstract

We have synthesized a series of 18 1,5- and 2,5-disubstituted carbamoyl tetrazoles, including LY2183240 (**1**) and LY2318912 (**7**), two compounds previously described as potent inhibitors of the cellular uptake of the endocannabinoid anandamide, and their regioisomers **2** and **8**. We confirm that compound **1** is a potent inhibitor of both the cellular uptake and, like the other new compounds synthesized here, the enzymatic hydrolysis of anandamide. With the exception of **9**, **12**, **15**, and the 2,5-regioisomer of LY2183240 **2**, the other compounds were all found to be weakly active or inactive on anandamide uptake. Several compounds also inhibited the enzymatic hydrolysis of the other main endocannabinoid, 2-arachidonoylglycerol, as well as its enzymatic release from *sn*-1-oleoyl-2-arachidonoyl-glycerol, at submicromolar concentrations. Four of the novel compounds, i.e. **3**, **4**, **17**, and **18**, inhibited anandamide hydrolysis potently ($IC_{50} = 2.1-5.4$ nM) and selectively over all the other targets tested ($IC_{50} \geq 10$ μM), thus representing new potentially useful tools for the inhibition of fatty acid amide hydrolase.

Keywords: Endocannabinoids; Anandamide cellular uptake inhibitors; Fatty acid amide hydrolase inhibitors; Carbamoyl tetrazoles

1. Introduction

Endocannabinoids, i.e. endogenous agonists of CB_1 and CB_2 cannabinoid receptors [1,2], are involved in an extraordinarily large number of physiological and pathological conditions in mammals [3,4]. Increasing evidence suggests that the modulation of endocannabinoid levels might be of valuable therapeutic relevance in the treatment of a variety of pathological conditions including anorexia, anxiety, inflammation, nausea and emesis, neuropsychiatric disorders, pain and spasticity [5]. The levels of the two main endocannabinoids, anandamide (AEA) [6] and 2-arachidonoylglycerol (2-AG) [7,8], are regulated by specific biosynthetic and degradative pathways, most of which have been now fully characterized and the relevant enzymes cloned [9]. The termination of the action of AEA is regulated by its cellular uptake, followed by intracellular hydrolysis by fatty acid amide hydrolase (FAAH). The mechanism of AEA transport, first characterized in 1994 by Di Marzo et al. [10], has been proposed to be an ATP-independent process of facilitated diffusion. However, a protein able to specifically bind AEA and facilitate its migration across the cell membrane has not yet been identified, its existence is still controversial, and alternative hypotheses have been proposed, including passive diffusion, endocytosis, and intracellular sequestration [11–17]. Much of the discussion in recent years has been centred on the issue of whether or not AEA uptake is driven uniquely by the FAAH-catalyzed hydrolysis of the compound.

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While the nature of the mechanism responsible for AEA uptake is still a matter of debate, a large number of arachidonic or oleic acid-based compounds that effectively inhibit this process have been described over the last decade. Although the ability of many of them to inhibit also FAAH has hampered the accurate characterization of their selectivity for either target, the recent individuation of novel selective AEA transport inhibitors that do not appear to inhibit FAAH such as UCM707 [18], AM1172 [13] and OMDM1,2 [19] has stimulated further studies to distinguish between the relative contributions of the putative AEA transporter and FAAH in AEA removal process.

From a medicinal chemistry perspective, the identification of potent and selective inhibitors of AEA transport lacking a long acyl side chain would be advantageous in terms of more precise target identification and of their potential of being made into therapeutic drugs. In this respect, Hopkins and Wang reported in 2004 in abstract form a non-arachidonoyl compound, SEP-0200228 that inhibited AEA uptake in human monocytes with an IC_{50} of 90 nM [20]. However, a full publication of this study has not yet appeared. More recently, Moore et al. described a potent, competitive small molecule inhibitor of AEA uptake, LY2318912, which has allowed the identification of a high-affinity AEA binding site distinct from FAAH in RBL-2H3 cell plasma membranes [21]. They also tested the effect of the parent compound of LY2318912, LY2183240, in a pharmacological test of persistent pain.

As underlined by Mechoulam and Deutsch in the commentary accompanying Moore et al.'s paper [22], 'the identification of a high-affinity binding site involved in the transport of AEA by Eli Lilly group represents a major step in understanding endocannabinoid metabolism and will present drug researchers with a very valuable tool'. However, these authors also raised a number of questions regarding the possibility that the novel compounds developed at Eli Lilly might act via additional mechanisms other than inhibition of AEA cellular uptake. Indeed, the Eli Lilly group subsequently reported that LY2183240 does inhibit enzyme activity in purified FAAH preparations [23], whereas very recently Alexander and Cravatt have shown that LY2183240 is a potent, covalent inhibitor not only of FAAH but also of several other serine proteases, including the monoacylglycerol lipase (MAGL) that catalyses the hydrolysis of 2-arachidonoylglycerol [24].

The compound described by Alexander and Cravatt is, however, in all evidence a mixture of regioisomeric 1,5- and 2,5-disubstituted tetrazoles (see Section 2), while neither details of the syntheses of LY2183240 and LY2318912 nor any information on the activity of their 2,5-regioisomers has been presented in the Eli Lilly papers. This despite the fact that the main procedure for the preparation of disubstituted tetrazoles, i.e. the alkylation (arylation, acylation) of 5-substituted tetrazoles, affords generally mixtures of regioisomers and difficulties are often encountered in their separation and/or when assigning their regiochemistry [25].

In light of these uncertainties of both synthetic and biological nature and in an effort to gain further insight into the actions of tetrazolic ureas, in particular the individual regioisomers, we have synthesized a series of nine regioisomeric couples of 1,5- and 2,5-disubstituted carbamoyl tetrazoles, including LY2183240, LY2318912 and their regioisomers (Fig. 1), by varying the substituent in the 5-position, and have investigated the effects of the regiochemistry and of the 5-substitution on the activity of these compounds on both AEA uptake and FAAH. The capability of these compounds to also inhibit the MAGL as well as the diacylglycerol lipase catalysing the biosynthesis of 2-AG [3] has also been assessed.

2. Chemistry

The tetrazole compounds in Fig. 1 were prepared as detailed below and as summarized in Schemes 1–3, following some of the newer methods for 5-substituted tetrazole

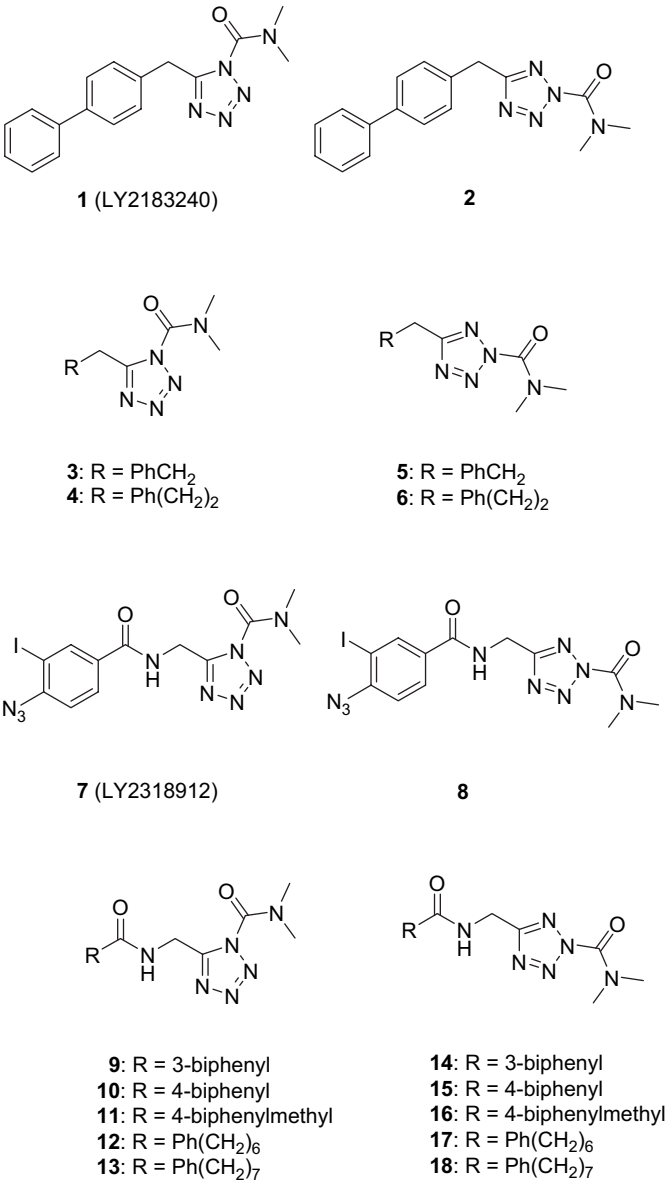
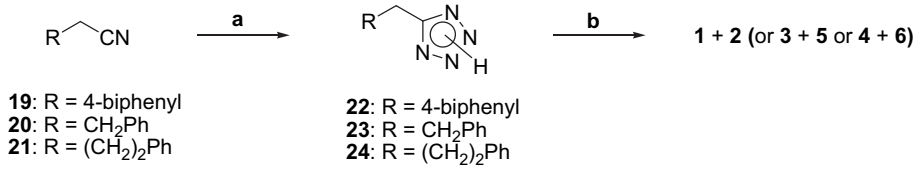


Fig. 1. Structures of carbamoyl tetrazoles synthesized and tested in this study.



Scheme 1. Synthesis of tetrazoles **1**–**6**. Reagents and conditions: (a) *n*-Bu_3SnN_3, dioxane, reflux, 20 h, then dry HCl, rt, 15 min; (b) Me_2NCOCl, Et_3N, MeCN, rt, 15 h.

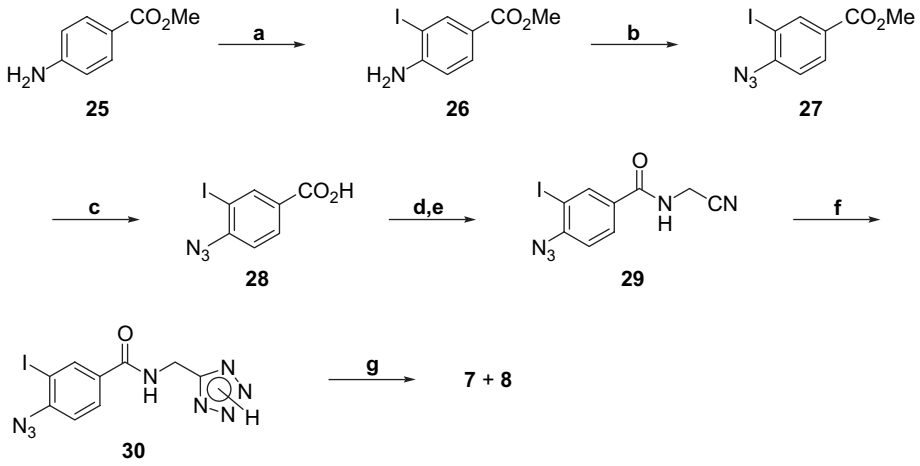
formation from organic nitriles [26]. Refluxing the commercially available 4-biphenylacetonitrile **19** with tri-*n*-butyltin azide [27], followed by an acidic hydrolysis step to remove the tin group from the tetrazole ring and acylation of the resulting tetrazole **22** [28] with *N*,*N*-dimethylcarbamoyl chloride gave a 0.58:1 mixture of 1,5- and 2,5-regioisomers **1** (LY2183240) and **2** which could be separated by silica gel chromatography, the less polar 2,5-disubstituted tetrazole **2**, eluting prior to the 1,5-disubstituted tetrazole **1** (Scheme 1). A mixture of regioisomers was also obtained using the same conditions employed by Alexander and Cravatt [24] for the carbamoylation of **22** and, indeed, the ^{1}H NMR spectrum reported by them for the compound assumed to be pure **1** was the sum of those of **1** and **2**.

The structures of the regioisomeric tetrazoles were first tentatively assigned on the basis of ^{13}C NMR spectra. ^{13}C NMR studies of disubstituted tetrazoles have shown that the CN_4 carbon atom of 1,5-disubstituted tetrazoles is more shielded than the corresponding carbon atom of the 2,5-disubstituted tetrazoles [29–31], but exceptions to this general rule can be found [32]. Based on these reported observations, the tetrazole which was first eluted was designated the 2,5-disubstituted tetrazole **2**, since it showed a CN_4 ^{13}C resonance at δ 164.99 ppm, while the more polar tetrazole **1** had a CN_4 ^{13}C resonance at δ 155.53 ppm. However, more spectroscopic evidence for the regiochemical assignment of the two regioisomers could not be obtained by a heteronuclear multiple bond correlation (HMBC) experiments, since both the compounds showed similar HMBC correlations. The 2,5-disubstituted structure of **2** was thus unambiguously determined by X-ray crystal structural analysis. Perspective view of **2** with atom numbering is shown in Fig. 2, while crystal data collection procedures, structure determination methods and refinement results are summarized in Table 1.

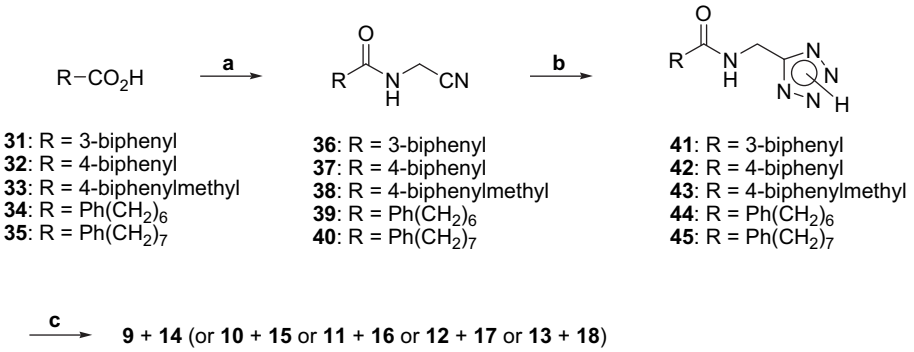
Repetition of the above procedure on nitriles **20** and **21** furnished similar and separable mixtures of 1,5- and 2,5-regioisomers **3** + **5** and **4** + **6**, respectively.

As concerns the preparation of regioisomers **7** (LY2318912) and **8**, methyl 4-aminobenzoate (**25**) was converted into methyl 4-amino-3-iodobenzoate (**26**) according to Spivey et al. [33]. Reaction of **26** with sodium nitrite and sodium azide in acidic medium followed by alkaline hydrolysis of the ester **27** as described previously [34] afforded 4-azido-3-iodobenzoic acid (**28**). Reaction of the acyl chloride derived from **28** by the action of $SOCl_2$ with aminoacetonitrile bisulfate furnished the amide **29**. Refluxing of **29** with tri-*n*-butyltin azide followed by acidic hydrolysis and acylation of tetrazole **30** with *N*,*N*-dimethylcarbamoyl chloride afforded a 0.52:1 mixture of 1,5- and 2,5-regioisomers **7** and **8** which could be separated by silica gel chromatography, again the less polar 2,5-disubstituted tetrazole **8** eluting prior to the 1,5-disubstituted tetrazole **7** (Scheme 2).

Repetition of the three-step procedure on acids **31**–**35** furnished mixtures of 1,5- and 2,5-regioisomers which could be separated in the case of tetrazoles **9** + **14**, **12** + **17** and **13** + **18**, while from the mixture of regioisomers **10** + **15**



Scheme 2. Synthesis of tetrazoles **7** and **8**. Reagents and conditions: (a) ICl, AcOH, rt, 1 h; (b) 4 N HCl, $NaNO_2$, AcOH/H_2O, 0–5 °C, 15 min, then NaN_3, H_2O, rt, 15 min; (c) 1 M LiOH, THF/H_2O, rt, 15 h; (d) $SOCl_2$, 100 °C, 1 h; (e) $H_2NCH_2CN{\cdot}H_2SO_4$, Et_3N, CH_2Cl_2, rt, 15 h; (f) *n*-Bu_3SnN_3, dioxane, reflux, 20 h, then dry HCl, rt, 15 min; (h) Me_2NCOCl, Et_3N, MeCN, rt, 15 h.



Scheme 3. Synthesis of tetrazoles **9**–**18**. Reagents and conditions: (a) HOBt/EDC, rt, 1 h, then $H_2NCH_2CN \cdot H_2SO_4$, Et_3N, CH_2Cl_2, rt, 15 h; (b) *n*-Bu_3SnN_3, dioxane, reflux, 20 h, then dry HCl, rt, 15 min; (c) Me_2NCOCl, Et_3N, MeCN, rt, 15 h.

only 2,5-regioisomer **15** could be isolated in a pure form, owing to the propensity of 1,5-regioisomer **10** to isomerise during chromatographic separation, and attempts to separate regioisomers **11** + **16** were unsuccessful (Scheme 3).

3. Biological evaluation

Apart from testing for the first time 13 new compounds (two of which as a mixture of regioisomers) and the 2,5-isomers **2**, **8** of the Eli Lilly compounds, whose biological activity was not reported in Moore et al.'s paper [21], we have also re-examined in this study the activity of the previously described 1,5-isomers **1**, **7**, using different conditions for the assay of [^{14}C]anandamide cellular uptake. These conditions, by using higher concentrations of the [^{14}C]anandamide substrate, have proved in the past to be more restrictive than other assays regarding potency and/or efficacy of competitive uptake inhibitors [35] and, by using incubation times with intact rat basophilic leukemia RBL-2H3 cell that are shorter (90 s) than those used by Moore et al., minimize the interference of FAAH inhibition by the compounds on their net effect on [^{14}C]anandamide uptake [11]. We have also used a 10 min pre-incubation of cells with the compound to maximize their potential competition with the specific site(s) involved in anandamide cellular uptake. In view of the results published by Alexander and Cravatt, showing that carbamoyl tetrazoles act as inhibitors for several serine hydrolases, including FAAH and MAGL [24], we have used membranes from rat brain to assess the FAAH inhibitory activity. This method is now to be considered even more suitable than in the past since recent data indicate that in some mammals, but not in rats or mice, a second FAAH isoform (FAAH-2) is also abundantly expressed [36]. In order to determine whether the compounds would also act as inhibitors of 2-AG hydrolysis via the MAGL, and of 2-AG biosynthesis via the *sn*-1-selective diacylglycerol lipases (DAGLs), we have used the cytosolic and membrane fractions, respectively, of COS cells overexpressing DAGLα, since COS cells exhibit high levels of a cytosolic

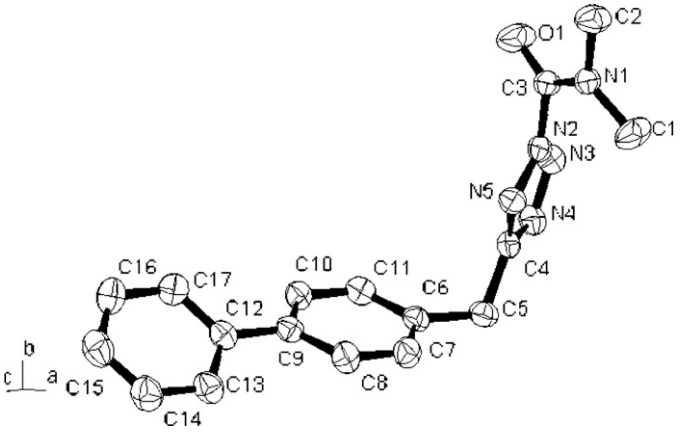


Fig. 2. ORTEP projection of the tetrazole **2**.

Table 1
Crystal data of compound **2**

Empirical formula	$C_{17}H_{17}N_5O$
Formula weight	307.36
Crystal size (mm)	0.4 × 0.3 × 0.3
Z	4
Density (calculated) (g/cm^3)	1.294
Crystal system	Monoclinic
Space group	$P2_1/c$
a (Å)	6.112 (4)
b (Å)	8.907 (4)
c (Å)	29.07 (2)
β (°)	94.27 (9)
Volume (Å^3)	1578.2 (17)
Wavelength (Å)	1.54178
Temperature (K)	293
Reflections number (total)	2483
Reflections number (observed)	1946
Reflections threshold expression	$F > 3.0\sigma\|F\|$
Diffraction measurement method	ϑ–2ϑ
θ_{min}, θ_{max}	1.50–31.04
μ (mm^{-1})	0.698
Refine number parameters	208
R factor	0.065
w*R* factor	0.083
Goodness of fit	0.88
Shift/error-max	0.000
Shift/su_mean	0.000
Final e.s.d. of ρ	0.64
Computing structure solution	*SIR200*[a]
Computing structure refinement	*SIR2004*[a]

[a] Ref. [39].

constitutive MAGL activity whereas their overexpressed DAGL activity is concentrated in the membranes [37]. Only 15 of the 18 compounds could be tested in a regioisomerically pure form. Compound **10** was unstable and easily got converted to **15**, and therefore could not be tested. The two regioisomers **11** and **16** were tested in mixture, as they could not be separated chromatographically.

4. Results and discussion

The results of the pharmacological assays shown in Table 2 can be summarized as follows: (1) we could confirm that compound **1** is a potent inhibitor of both the cellular uptake and the enzymatic hydrolysis of anandamide [21,24]; (2) with the exception of **9**, **12**, **15**, and the 2,5-regioisomer of LY2183240 (**2**), which exhibited lower potency than **1**, but were still capable of efficaciously inhibiting anandamide cellular uptake with IC_{50} values between 0.1 and 1 μM, the other compounds were all weakly active or inactive in this assay; (3) all compounds inhibited anandamide hydrolysis by rat brain membranes with IC_{50} values ranging from 2.1 to 33 nM; (4) several compounds also inhibited the enzymatic hydrolysis of the other endocannabinoid, 2-AG, as well as its enzymatic release from *sn*-1-oleoyl-2-arachidonoyl-glycerol, at submicromolar concentrations; (5) four of the novel compounds, i.e. **3**, **4**, **17**, and **18**, inhibited anandamide hydrolysis potently (IC_{50} = 2.1–5.4 nM) and with high (500–2000-fold) selectivity over all the other targets tested.

Table 2
Effect of the compounds synthesized in this study on [^{14}C]anandamide hydrolysis by rat brain membranes (which express FAAH as the only anandamide hydrolysing enzyme), [^{14}C]anandamide uptake by intact RBL-2H3 cells (where a putative anandamide transporter has been characterized pharmacologically), [^{14}C]2-AG hydrolysis by COS cell cytosolic fractions (which express MAGL), and *sn*-1-[^{14}C]oleoyl-2-arachidonoyl-glycerol (DAG) conversion into 2-AG and [^{14}C]oleic acid by COS cells membranes overexpressing human recombinant DAGLα[a]

Compound	AEA hydrolysis	AEA uptake	2-AG hydrolysis	DAG hydrolysis to 2-AG
1	0.0021	0.015	8.10	>10
2	0.0330	0.998	0.02	0.51
3	0.0054	>10 (15.8%)	10	10
4	0.0027	>10 (18.0%)	10	10
5	0.0035	>10 (10.9%)	0.48	0.60
6	0.0032	>10 (16.0%)	0.11	0.50
7	0.0230	>1 (32.4%)	0.35	0.13
8	0.0250	>1 (9.0%)	>10	4.40
9	0.0320	0.090	0.85	0.34
11 + **16**	0.0290	>10 (35.2%)	0.10	1.00
12	0.0037	0.851	0.02	0.20
13	0.0100	>10 (37.5%)	0.04	0.57
14	0.0230	>10 (23.0%)	10	>10
15	0.0060	0.985	>10	>10
17	0.0021	>10 (30.0%)	>10	10
18	0.0032	>10 (18.8%)	10	10

[a] Data are expressed as IC_{50} (μM) and in the case of weak inhibitors as the maximal percent inhibition observed (between parentheses), and are means of $n = 3$–4 separate determinations. Standard errors are not shown for the sake of clarity and were never higher than 10% of the means.

Although we have confirmed here that the most potent anandamide uptake inhibitor identified by Moore et al., i.e. compound **1**, is also the most potent compound in this assay under our conditions, we could not confirm here the same potency ($IC_{50} = 0.27$ nM, i.e. ~55-fold higher) published previously for this compound. The discrepancy is even more evident with compound **7**, which was previously reported to inhibit AEA uptake in the same cells with an IC_{50} of 7.2 nM and was found here to exhibit ~140-fold lower potency. The exact reasons for these differences are not known, but are very likely due to the fact that we used experimental conditions different from those used in the previous study (namely much shorter incubation times and higher concentrations of substrate [^{14}C]anandamide).

Furthermore, as predicted by Mechoulam and Deutsch [22], and later shown by both the Eli Lilly group [23] and Alexander and Cravatt [24], we found that all compounds also potently inhibit rat brain FAAH. However, additional data also indicate that the effect of compound **1** on AEA uptake is only in part due to its capability of inhibiting AEA hydrolysis. First, we observed that under the incubation conditions used here, the extent of temperature-sensitive [^{14}C]anandamide uptake (16.2 ± 3.2% of total incubated anandamide) was significantly higher than that of [^{14}C]anandamide hydrolysis (1.9 ± 0.1% of total incubated anandamide, means ± SD of $N = 10$). Furthermore, when this was tested in the same experiment in intact RBL-2H3 cells, compound **1** inhibited the uptake of [^{14}C]anandamide more efficaciously than it inhibited the formation of [^{14}C]ethanolamine from [^{14}C]anandamide (respectively, 66.5 ± 11.4 and 74.2 ± 8.1% inhibition of uptake and 41.1 ± 6.1 and 36.7 ± 5.0% inhibition of hydrolysis at 0.1 and 1 μM, respectively, $p < 0.01$ in both cases, $N = 4$). These findings suggest that, in intact cells, compound **1** inhibits anandamide hydrolysis, which is an intracellular process, because it inhibits anandamide uptake and not *vice versa*. Thus, although part of the inhibitory effect on anandamide uptake by compound **1** is clearly due to its inhibition of FAAH, which contributes to driving cellular uptake, this compound seems capable of influencing the former process also in a way independent from anandamide hydrolysis. This conclusion is also supported by the data recently published by Dickason-Chesterfield et al., who also found a clear dissociation between the FAAH- and anandamide uptake-inhibitory activities of several compounds, including compound **1** [23].

As could be predicted again by the study by Alexander and Cravatt [24], several of the carbamoyl tetrazoles synthesized in this study also potently inhibited the hydrolysis of [^{14}C]2-AG by a MAGL-like activity present in the cytosol of COS cells. Furthermore, we showed here for the first time that these compounds can also be good inhibitors of 2-AG biosynthesis from *sn*-1-[^{14}C]oleoyl-2-arachidonoyl-glycerol. However, we agree only in part with the conclusions drawn by these authors that 'the promiscuity of LY2183240 designates the heterocyclic urea as a chemotype with potentially excessive protein reactivity for drug design'. In fact, we did find that out of the 17 tetrazoles screened, four compounds exhibited potent activity for FAAH selectively over all other targets tested here.

These compounds, i.e. **3**, **4**, **17**, and **18**, might represent new useful FAAH inhibitors exploitable for therapeutic use in pathologies as diverse as those listed in Section 1.

Our results in 12 carbamoyl tetrazoles, which represent six 1,5- and 2,5-regioisomeric couples, together with those obtained with **1** and **7** and their 2,5-regioisomers **2** and **8** allow us to delineate for the first time some structure–activity relationships for the interactions of this class of compounds with the putative anandamide membrane transporter, FAAH, MAGL and DAGLα, and hence provide new information for the future design of new pharmacological tools interacting with these targets. In particular, the following conclusions can be drawn from the data in Table 2: (1) regarding the inhibition of anandamide cellular uptake, most 1,5-isomers are significantly more potent than the corresponding 2,5-isomers. Furthermore, conformationally less flexible substituents in the 5-position seem to yield better inhibitors than more flexible ones. In any case, the features of 5-substitution necessary for potent uptake inhibition appear to be rather stringent, as seemingly minor structural differences produce remarkable variations of the activity (e.g. compounds **14**, **15** and **12**, **13**); (2) by contrast, 2,5-isomers can also be more potent FAAH inhibitors than their 1,5-counterparts; the requirements of the 5-substitution are less critical and conformationally more flexible substituents appear to afford more potent inhibitors. These observations represent yet another indirect piece of evidence in favour of the existence of a FAAH-independent mechanism for anandamide uptake; (3) regarding the inhibition of MAGL and DAGL, 1,5-isomers were significantly more potent than the corresponding 2,5-isomers in the case of compounds with an amide function in the 5-side chain, while the opposite seems to apply for compounds with non-functionalized side chains; (4) with a few exceptions, carbamoyl tetrazoles seem to be significantly more potent at inhibiting MAGL than DAGL. Nevertheless, this property is not likely to be exploited for the development of selective MAGL inhibitors since the most potent inhibitors of 2-AG hydrolysis synthesized in the present study were as potent as FAAH inhibitors.

5. Conclusions

We have provided here new methods for the syntheses of carbamoyl tetrazoles with activity as inhibitors of endocannabinoid inactivation, as well as new data on the structure–activity relationships for the interactions of these compounds with the putative anandamide membrane transporter, FAAH, MAGL and DAGLα. Our data provide further indirect evidence that a specific and FAAH-independent process underlies part of the cellular uptake of anandamide by RBL-2H3 cells, but argue against the possibility of developing selective inhibitors of such process from carbamoyl tetrazoles. On the other hand, we have shown here that it might be possible to develop selective as well as potent FAAH inhibitors from the chemical modification of the compounds previously reported by Eli Lilly. Four such new inhibitors i.e. **3**, **4**, **17**, and **18**, have been described here.

6. Experimental protocols

6.1. Chemistry

All chemical reagents were commercially available unless otherwise indicated. Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. IR spectra were recorded on a Perkin-Elmer 1000 FT-IR spectrophotometer as KBr disks unless otherwise indicated. ^{1}H and ^{13}C NMR spectra were obtained on a Varian Mercury 300 spectrometer using $CDCl_3$ as solvent unless otherwise indicated and TMS as internal standard. Analyses indicated by the symbols of the elements or functions were within ±0.4% of the theoretical values (Table 3).

Compounds **26** [33], **27** [34], **28** [34], and **31** [38] have been described in the literature.

6.1.1. 5-((Biphenyl-4-yl)methyl)-1H-tetrazole (22)

A solution of 4-biphenylacetonitrile (**19**) (1.449 g, 7.5 mmol) and *n*-Bu_3SnN_3 [27] (3.736 g, 11.25 mmol) in 1,4-dioxane (15 mL) was refluxed for 20 h, cooled at room temperature, and gaseous HCl was added over 15 min. The mixture was diluted with water, neutralized with 2 N NaOH, and extracted with AcOEt. The organic phase was washed with water, dried (Na_2SO_4), and evaporated under vacuum to leave a residue (5.20 g) which was triturated with hot hexane. The resulting solid was filtered off, washed with hexane, and dried under vacuum to give 1.460 g (82%) of **22**. Mp 215–217 °C; IR 3117, 3029, 2853, 2727, 2623, 1575, 1487, 1424, 1256, 1106, 1053 cm^{-1}; ^{1}H NMR (300 MHz) δ 4.37 (2H, s), 7.36–7.67 (9H, m); ^{13}C NMR (75 MHz) δ 28.53, 126.53, 126.94, 127.37, 128.85, 129.21, 135.05, 138.93, 139.69, 155.17. Anal. ($C_{14}H_{12}N_4$) C, H, N.

6.1.2. 5-((Biphenyl-4-yl)methyl)-N,N-dimethyl-1H-tetrazole-1-carboxamide (1) and 5-((biphenyl-4-yl)methyl)-N,N-dimethyl-2H-tetrazole-2-carboxamide (2)

To a stirred solution of **22** (1.460 g, 6.18 mmol) and Et_3N (1.73 mL, 12.36 mmol) in acetonitrile (10 mL) Me_2NCOCl (0.85 mL, 9.27 mmol) was added at 0 °C, and the mixture was stirred at 0 °C for 15 h. The mixture was diluted with water and extracted with AcOEt. The organic phase was washed twice with brine, dried (Na_2SO_4), and evaporated under vacuum. The residue (1.91 g) was chromatographed on silica gel (200 g) with CH_2Cl_2/AcOEt = 99/1 as eluent to give **2** (1.041 g, 55%) followed by **1** (609 mg, 32%). Compound **2**: mp 80–81 °C; IR ($CHCl_3$) 3010, 1732, 1488, 1396, 1257, 1231, 1088 cm^{-1}; ^{1}H NMR (300 MHz) δ 3.11 (3H, s), 3.25 (3H, s), 4.37 (2H, s), 7.33–7.58 (9H, m); ^{13}C NMR (75 MHz) δ 31.26, 37.98, 39.00, 126.89, 127.14, 127.35, 128.60, 129.16, 134.78, 140.00, 140.55, 147.66, 164.99. Anal. ($C_{17}H_{17}N_5O$) C, H, N. Compound **1**: mp 87–88 °C; IR ($CHCl_3$) 3011, 1742, 1489, 1393, 1235, 1166, 1130, 1068 cm^{-1}; ^{1}H NMR (300 MHz) δ 2.69 (3H, s), 3.07 (3H, s), 4.48 (2H, s), 7.32–7.57 (9H, m); ^{13}C NMR (75 MHz) δ 29.43, 37.22, 38.57, 126.75, 127.26, 127.37, 128.66, 129.26, 133.07, 140.07, 140.46, 147.71, 155.53. Anal. ($C_{17}H_{17}N_5O$) C, H, N.

Table 3
Elemental analysis data

Compound	Molecular formula	Calculated C, H, N	Found C, H, N
1	$C_{17}H_{17}N_5O$	C, 66.43; H, 5.58; N, 22.79	C, 66.49; H, 5.49; N, 22.65
2	$C_{17}H_{17}N_5O$	C, 66.43; H, 5.58; N, 22.79	C, 66.37; H, 5.62; N, 22.87
3	$C_{12}H_{15}N_5O$	C, 58.76; H, 6.16; N, 28.55	C, 58.59; H, 6.31; N, 28.67
4	$C_{13}H_{17}N_5O$	C, 60.21; H, 6.61; N, 27.01	C, 60.37; H, 6.44; N, 26.83
5	$C_{12}H_{15}N_5O$	C, 58.76; H, 6.16; N, 28.55	C, 59.02; H, 6.03; N, 28.18
6	$C_{13}H_{17}N_5O$	C, 60.21; H, 6.61; N, 27.01	C, 60.40; H, 6.47; N, 27.33
7	$C_{12}H_{12}IN_9O_2$	C, 32.67; H, 2.74; N, 28.57	C, 32.72; H, 2.72; N, 28.57
8	$C_{12}H_{12}IN_9O_2$	C, 32.67; H, 2.74; N, 28.57	C, 32.59; H, 2.70; N, 28.49
9	$C_{18}H_{18}N_6O_2$	C, 61.70; H, 5.18; N, 23.99	C, 61.48; H, 5.09; N, 24.08
12	$C_{18}H_{26}N_6O_2$	C, 60.32; H, 7.31; N, 23.45	C, 60.14; H, 7.28; N, 23.47
13	$C_{19}H_{28}N_6O_2$	C, 61.27; H, 7.58; N, 22.56	C, 61.03; H, 7.49; N, 22.48
14	$C_{18}H_{18}N_6O_2$	C, 61.70; H, 5.18; N, 23.99	C, 61.54; H, 5.07; N, 24.16
15	$C_{18}H_{18}N_6O_2$	C, 61.70; H, 5.18; N, 23.99	C, 61.67; H, 5.19; N, 23.90
17	$C_{18}H_{26}N_6O_2$	C, 60.32; H, 7.31; N, 23.45	C, 59.97; H, 7.44; N, 23.22
18	$C_{19}H_{28}N_6O_2$	C, 61.27; H, 7.58; N, 22.56	C, 61.30; H, 7.67; N, 22.67
22	$C_{14}H_{12}N_4$	C, 71.17; H, 5.12; N, 23.71	C, 71.39; H, 5.18; N, 23.64
23	$C_9H_{10}N_4$	C, 62.05; H, 5.79; N, 32.16	C, 62.16; H, 5.65; N, 32.47
24	$C_{10}H_{12}N_4$	C, 63.81; H, 6.43; N, 29.77	C, 63.58; H, 6.19; N, 29.75
29	$C_9H_6IN_5O$	C, 33.05; H, 1.85; N, 21.41	C, 32.89; H, 1.97; N, 21.37
30	$C_9H_7IN_8O$	C, 29.21; H, 1.91; N, 30.28	C, 29.36; H, 1.87; N, 30.35
36	$C_{15}H_{12}N_2O$	C, 76.25; H, 5.12; N, 11.86	C, 76.47; H, 5.07; N, 11.68
37	$C_{15}H_{12}N_2O$	C, 76.25; H, 5.12; N, 11.86	C, 76.32; H, 5.02; N, 12.01
38	$C_{16}H_{14}N_2O$	C, 76.78; H, 5.64; N, 11.19	C, 76.68; H, 5.60; N, 11.13
39	$C_{15}H_{20}N_2O$	C, 73.74; H, 8.25; N, 11.47	C, 73.56; H, 8.32; N, 11.53
40	$C_{16}H_{22}N_2O$	C, 70.04; H, 8.08; N, 10.21	C, 69.78; H, 7.99; N, 10.34
41	$C_{15}H_{13}N_5O$	C, 64.51; H, 4.69; N, 25.07	C, 64.47; H, 4.39; N, 25.40
42	$C_{15}H_{13}N_5O$	C, 64.51; H, 4.69; N, 25.07	C, 64.39; H, 4.51; N, 24.93
43	$C_{16}H_{15}N_5O$	C, 65.52; H, 5.15; N, 23.88	C, 65.72; H, 5.07; N, 23.80
44	$C_{15}H_{21}N_5O$	C, 62.70; H, 7.37; N, 24.37	C, 62.76; H, 7.45; N, 24.12
45	$C_{16}H_{23}N_5O$	C, 63.76; H, 7.69; N, 23.24	C, 63.46; H, 7.58; N, 23.24

*6.1.3. 5-Phenethyl-1H-tetrazole (**23**)*

The title compound was prepared from 3-phenylpropanenitrile (**20**) following the same procedure that was used for the synthesis of **22**. Yield 70%; mp 80–85 °C; IR 3134, 3029, 2859, 2730, 2626, 1560, 1497, 1454, 1258, 1108, 1050 cm^{-1}; ^1H NMR (300 MHz) δ 3.15 (2H, t, $J = 7.6$ Hz), 3.38 (2H, t, $J = 7.6$ Hz), 7.13–7.25 (5H, m); ^{13}C NMR (75 MHz) δ 25.29, 33.67, 126.72, 128.30, 128.67, 139.10, 156.11. Anal. ($C_9H_{10}N_4$) C, H, N.

*6.1.4. N,N-Dimethyl-5-phenethyl-1H-tetrazole-1-carboxamide (**3**) and N,N-dimethyl-5-phenethyl-2H-tetrazole-2-carboxamide (**5**)*

The title compounds were prepared from **23** following the same procedure that was used for the synthesis of **1**, **2** using hexane/AcOEt = 1/1 as eluent for the chromatographic separation of regioisomers. Compound **5** (less polar, 23%): oil; IR ($CHCl_3$) 3009, 2941, 1740, 1498, 1393, 1311, 1252, 1168, 1130, 1070 cm^{-1}; ^1H NMR (300 MHz) δ 3.01 (3H, s), 3.22 (3H, s), 3.11–3.34 (4H, m), 7.14–7.32 (5H, m); ^{13}C NMR (75 MHz) δ 27.12, 33.79, 38.05, 39.00, 126.39, 128.37, 128.52, 140.09, 147.92, 165.53. Anal. ($C_{12}H_{15}N_5O$) C, H, N. Compound **3** (more polar, 17%): oil; IR ($CHCl_3$) 3014, 2941, 1728, 1497, 1396, 1257, 1236, 1088, 1076 cm^{-1}; ^1H NMR (300 MHz) δ 2.76 (3H, s), 3.09 (3H, s), 3.13 (2H, t, $J = 7.6$ Hz), 3.48 (2H, t, $J = 7.6$ Hz), 7.13–7.31 (5H, m); ^{13}C NMR (75 MHz) δ 25.65, 33.61, 37.58, 39.12, 126.68, 128.58, 139.24, 147.84, 156.37. Anal. ($C_{12}H_{15}N_5O$) C, H, N.

*6.1.5. 5-(3-Phenylpropyl)-1H-tetrazole (**24**)*

The title compound was prepared from 4-phenylbutyronitrile (**21**) following the same procedure that was used for the synthesis of **22**. Yield 55%; mp 80–85 °C; IR 3130, 3024, 2864, 2730, 2622, 1568, 1495, 1450, 1421, 1256, 1107, 1047 cm^{-1}; ^1H NMR (300 MHz) δ 2.19 (2H, m), 2.71 (2H, t, $J = 7.5$ Hz), 3.08 (2H, t, $J = 7.7$ Hz), 7.11–7.27 (5H, m); ^{13}C NMR (75 MHz) δ 22.91, 29.03, 34.99, 126.25, 128.42, 128.52, 140.54, 156.77. Anal. ($C_{10}H_{12}N_4$) C, H, N.

*6.1.6. N,N-Dimethyl-5-(3-phenylpropyl)-1H-tetrazole-1-carboxamide (**4**) and N,N-dimethyl-5-(3-phenylpropyl)-2H-tetrazole-2-carboxamide (**6**)*

The title compounds were prepared from **24** following the same procedure that was used for the synthesis of **1**, **2** using preparative layer chromatography (silica gel, 0.5 cm thick) and hexane/acetone = 7/3 as eluent for the separation of regioisomers. Compound **6** (less polar, 38%): oil; IR ($CHCl_3$) 3024, 2942, 2864, 1740, 1603, 1497, 1453, 1393, 1310, 1167, 1071 cm^{-1}; ^1H NMR (300 MHz) δ 2.16 (2H, m), 2.70 (2H, t, $J = 7.5$ Hz), 2.99 (2H, t, $J = 7.6$ Hz) 3.09 (3H, s), 3.24 (3H, s), 7.14–7.33 (5H, m); ^{13}C NMR (75 MHz) δ 24.76, 29.26, 35.11, 38.06, 39.10, 126.03, 128.42, 128.49,

141.19, 147.96, 166.14. Anal. ($C_{13}H_{17}N_5O$) C, H, N. Compound **4** (more polar, 23%): oil; IR ($CHCl_3$) 3024, 2942, 2863, 1728, 1603, 1497, 1453, 1396, 1258, 1088 cm^{-1}; ^1H NMR (300 MHz) δ 2.17 (2H, m), 2.74 (2H, t, $J = 7.5$ Hz), 3.06 (5H, m), 3.20 (3H, s), 7.15–7.32 (5H, m); ^{13}C NMR (75 MHz) δ 23.59, 28.48, 35.02, 37.76, 39.33, 126.17, 128.48, 140.80, 148.00, 156.89. Anal. ($C_{13}H_{17}N_5O$) C, H, N.

*6.1.7. 4-Azido-N-(cyanomethyl)-3-iodobenzamide (**29**)*

A solution of **28** [34] (7.250 g, 25.08 mmol) in $SOCl_2$ (30 mL) was stirred at 100 °C for 2 h. Excess $SOCl_2$ was evaporated under vacuum and the residue of the acid chloride (7.711 g, 100%) was dissolved in dry CH_2Cl_2 (50 mL), and $H_2NCH_2CN \cdot H_2SO_4$ (4.253 g, 27.59 mmol) and Et_3N (17.6 mL, 125.71 mmol) were added under stirring at 0 °C. The mixture was stirred at room temperature for 15 h, diluted with water, and extracted with AcOEt. The organic phase was washed with 2 N HCl, water until neutral, 2 N NaOH, and water until neutral, dried (Na_2SO_4), and evaporated under vacuum to leave a residue (5.239 g, 64%) of **29** as a white solid. Mp 65–68 °C; IR 3337, 3230, 3059, 3000, 2116, 1643, 1588, 1538, 1474, 1298 cm^{-1}; ^1H NMR (300 MHz) δ 4.38 (2H, d, $J = 5.8$ Hz), 6.81 (1H, m), 7.18 (1H, d, $J = 8.4$ Hz), 7.83 (1H, dd, $J = 8.4$, 2.0 Hz), 8.21 (1H, d, $J = 2.0$ Hz); ^{13}C NMR (75 MHz) δ 28.15, 87.68, 115.87, 118.28, 128.66, 130.18, 139.11, 146.07, 165.06. Anal. ($C_9H_6IN_5O$) C, H, N.

*6.1.8. N-((1H-Tetrazol-5-yl)methyl)-4-azido-3-iodobenzamide (**30**)*

The title compound was prepared from **29** following the same procedure that was used for the synthesis of **22**. Yield (39%); mp 188–190 °C; IR 3287, 2855, 2125, 1636, 1447, 1301, 1035 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ 3.38 (1H, br s), 4.76 (2H, s), 7.47 (1H, d, $J = 8.1$ Hz), 7.98 (1H, dd, $J = 8.1$, 1.8 Hz), 8.36 (1H, d, $J = 1.8$ Hz), 9.35 (1H, m); ^{13}C NMR (75 MHz, DMSO-d_6) δ 33.14, 33.24, 87.71, 118.99, 129.00, 131.11, 138.42, 144.30, 154.48, 164.37, 164.45. Anal. ($C_9H_7IN_8O$) C, H, N.

*6.1.9. 5-((4-Azido-3-iodobenzamido)methyl)-N,N-dimethyl-1H-tetrazole-1-carboxamide (**7**) and 5-((4-azido-3-iodobenzamido)methyl)-N,N-dimethyl-2H-tetrazole-2-carboxamide (**8**)*

The title compounds were prepared from **30** following the same procedure that was used for the synthesis of **1**, **2** using hexane/AcOEt = 45/55 as eluent for the chromatographic separation of regioisomers. Compound **8** (less polar, 29%): mp 125 °C; IR 3274, 2928, 2121, 1739, 1651, 1534, 1477, 1291, 1085 cm^{-1}; ^1H NMR (300 MHz) δ 3.17 (3H, s), 3.26 (3H, s), 4.99 (2H, d, $J = 5.8$ Hz), 7.10 (1H, d, $J = 8.4$ Hz), 7.81 (1H, dd, $J = 8.4$, 2.2 Hz), 8.00 (1H, m), 8.20 (1H, d, $J = 2.2$ Hz); ^{13}C NMR (75 MHz) δ 34.22, 37.85, 39.38, 87.33, 117.87, 128.32, 130.53, 138.99, 145.26, 147.73, 154.49, 165.16. Anal. ($C_{12}H_{12}IN_9O_2$) C, H, N. Compound **7** (more polar, 15%): mp 119 °C; IR 3279, 2933, 2123, 1735, 1638, 1536, 1390, 1314, 1131 cm^{-1}; ^1H NMR (300 MHz) δ 3.13 (3H, s), 3.27 (3H, s), 4.97 (2H, d, $J = 6.0$ Hz), 7.13 (1H, d, $J = 8.4$ Hz), 7.48 (1H, m), 7.86 (1H, dd, $J = 8.4$, 2.1 Hz), 8.25 (1H, d, $J = 2.1$ Hz); ^{13}C NMR (75 MHz) δ 35.25, 38.11, 39.13, 87.33, 117.87, 128.44, 131.02, 138.93, 145.06, 147.39, 163.12, 165.06. Anal. ($C_{12}H_{12}IN_9O_2$) C, H, N.

*6.1.10. N-(Cyanomethyl)biphenyl-3-carboxamide (**36**)*

To a stirred solution of biphenyl-3-carboxylic acid (**31**) [35] (223 mg, 1.12 mmol) in DMF (4.5 mL) were added at 0 °C HOBt (181 mg, 1.18 mmol) and EDC (226 mg, 1.18 mmol). The mixture was stirred for 15 min at 0 °C and for 1 h at room temperature, and $H_2NCH_2CN \cdot H_2SO_4$ (207 mg, 1.35 mmol) and Et_3N (0.78 mL, 5.61 mmol) were added and the mixture was stirred overnight at room temperature. The mixture was diluted with brine and extracted with AcOEt. The organic phase was washed with 2 N HCl solution, brine, saturated $NaHCO_3$ and brine, dried (Na_2SO_4), and evaporated under vacuum. The residue (349 mg) was chromatographed on silica gel (11 g) using CH_2Cl_2/AcOEt = 95/5 as eluent to give 224 mg (85%) of **36** as an oil; IR ($CHCl_3$) 3453, 3339, 3065, 2943, 1676, 1509, 1476, 1304, 1257, 1202, 1145, 1091 cm^{-1}; ^1H NMR (300 MHz) δ 4.31 (2H, d, $J = 5.6$ Hz), 7.30–7.99 (10H, m); ^{13}C NMR (75 MHz) δ 28.10, 116.36, 125.93, 126.06, 127.09, 127.91, 128.94, 129.23, 131.01, 133.06, 139.79, 141.83, 167.82. Anal. ($C_{15}H_{12}N_2O$) C, H, N.

*6.1.11. N-((1H-Tetrazol-5-yl)methyl)biphenyl-3-carboxamide (**41**)*

The title compound was prepared from **36** following the same procedure that was used for the synthesis of **22**. Yield 86%; oil; IR ($CHCl_3$) 3410, 3338, 3031, 2960, 2925, 1656, 1518, 1476, 1306, 1225, 1088 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3/CD_3OD$ = 9/1) δ 4.86 (2H, s), 7.32–8.05 (9H, m); ^{13}C NMR (75 MHz, $CDCl_3/CD_3OD$ = 9/1) δ 33.62, 126.01, 126.12, 127.13, 127.17, 127.85, 128.95, 129.10, 130.66, 133.84, 140.07, 141.70, 168.77. Anal. ($C_{15}H_{13}N_5O$) C, H, N.

*6.1.12. 5-(Biphenyl-3-ylcarboxamidomethyl)-N,N-dimethyl-1H-tetrazole-1-carboxamide (**9**) and 5-(biphenyl-3-ylcarboxamidomethyl)-N,N-dimethyl-2H-tetrazole-2-carboxamide (**14**)*

The title compounds were prepared from **41** following the same procedure that was used for the synthesis of **1**, **2** using hexane/acetone = 65/35 as eluent for the chromatographic separation of regioisomers. Compound **14** (less polar, 14%): oil; IR ($CHCl_3$) 3451, 3033, 2944, 1731, 1667, 1510, 1396, 1252, 1091 cm^{-1}; ^1H NMR (300 MHz) δ 3.10 (3H, s), 3.20 (3H, s), 5.03 (2H, d, $J = 5.8$ Hz), 7.27–8.02 (10H, m); ^{13}C NMR (75 MHz) δ 34.34, 38.61, 39.47, 125.82, 126.02, 127.16, 127.83, 128.91, 129.11, 130.71, 133.65, 140.02, 141.82, 148.06, 154.55, 167.70. Anal. ($C_{18}H_{18}N_6O_2$) C, H, N. Compound **9** (more polar, 10%): oil; IR ($CHCl_3$) 3452, 3414, 3022, 2941, 1746, 1675, 1509, 1392, 1251, 1072, 1000 cm^{-1}; ^1H NMR (300 MHz) δ 3.09 (3H, s), 3.23 (3H, s), 5.03 (2H, d, $J = 5.6$ Hz), 7.26–8.02 (10H, m); ^{13}C NMR (75 MHz) δ 35.42, 38.11, 39.20, 125.97, 126.28, 127.18,

127.77, 128.88, 129.05, 130.54, 134.28, 140.14, 141.78, 163.42, 167.66. Anal. ($C_{18}H_{18}N_6O_2$) C, H, N.

*6.1.13. N-(Cyanomethyl)biphenyl-4-carboxamide (**37**)*

The title compound was prepared from acid **32** following the same procedure that was used for the synthesis of **36**. Yield 76%; mp 197–198 °C; IR 3307, 2923, 1645, 1608, 1531, 1484, 1413, 1309 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$/CD_3OD = 1/1) δ 4.32 (2H, d, J = 8.2 Hz), 7.38–7.95 (9H, m); ^{13}C NMR (75 MHz, $CDCl_3$/CD_3OD = 1/1) δ 28.02, 116.69, 127.38, 127.45, 128.20, 128.39, 129.17, 131.58, 140.03, 145.34, 168.50. Anal. ($C_{15}H_{12}N_2O$) C, H, N.

*6.1.14. N-((1H-Tetrazol-5-yl)methyl)biphenyl-4-carboxamide (**42**)*

The title compound was prepared from **37** following the same procedure that was used for the synthesis of **22**. Yield 67%; mp 275–276 °C; IR 3288, 2856, 1638, 1532, 1484, 1433, 1310, 1246, 1222, 1054 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ 3.28 (1H, br s), 4.49 (2H, d, J = 5.6 Hz), 7.07–7.73 (9H, m), 9.02 (1H, t, J = 5.6 Hz); ^{13}C NMR (75 MHz, DMSO-d_6) δ 33.23, 126.44, 126.78, 128.02, 128.93, 131.45, 132.31, 139.00, 143.02, 154.72, 166.26. Anal. ($C_{15}H_{13}N_5O$) C, H, N.

*6.1.15. 5-(Biphenyl-4-ylcarboxamidomethyl)-N,N-dimethyl-2H-tetrazole-2-carboxamide (**15**)*

The title compound was prepared from **42** following the same procedure that was used for the synthesis of **1**, **2** using hexane/AcOEt = 45/55 as eluent for the chromatographic separation of regioisomers. Attempts to isolate 1,5-regioisomer **10** in a pure form failed owing to its propensity to isomerise. Compound **15** (49%): mp 186–188 °C; IR 3289, 2857, 1725, 1627, 1552, 1487, 1397, 1307, 1249, 1084 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ 2.91 (3H, s), 3.08 (3H, s), 4.90 (2H, d, J = 5.4 Hz), 6.88 (1H, br s), 7.41–8.00 (9H, m); ^{13}C NMR (75 MHz, DMSO-d_6) δ 30.24, 33.36, 36.91, 124.69, 126.44, 126.69, 127.81, 128.82, 131.77, 138.86, 143.09, 147.31, 151.28, 154.17. Anal. ($C_{18}H_{18}N_6O_2$) C, H, N.

*6.1.16. 2-(Biphenyl-4-yl)-N-(cyanomethyl)acetamide (**38**)*

The title compound was prepared from 2-(biphenyl-4-yl)-acetic acid (**33**) following the same procedure that was used for the synthesis of **36**. Yield 69%; mp 146–147 °C; IR (KBr) 3249, 3046, 1683, 1644, 1541, 1486, 1407, 1256, 1160, 1029 cm^{-1}; ^1H NMR (300 MHz, CD_3OD) δ 3.61 (2H, s), 4.43 (2H, s), 7.34–7.56 (9H, m); ^{13}C NMR (75 MHz, CD_3OD) δ 27.62, 42.43, 116.53, 127.24, 127.65, 127.71, 129.09, 129.88, 133.65, 140.56, 140.95, 172.80. Anal. ($C_{16}H_{14}N_2O$) C, H, N.

*6.1.17. N-((1H-Tetrazol-5-yl)methyl)-2-(biphenyl-4-yl)acetamide (**43**)*

The title compound was prepared from **38** following the same procedure that was used for the synthesis of **22**. Yield 67%; mp 187–189 °C; IR 3334, 3034, 1646, 1548, 1517, 1487, 1423, 1261, 1231, 1160, 1076 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ 3.60 (2H, s), 4.09 (2H, m), 7.30–7.59 (9H, m); ^{13}C NMR (75 MHz, DMSO-d_6) δ 27.53, 42.42, 116.36, 127.13, 127.56, 127.66, 128.97, 129.81, 133.36, 140.51, 140.73, 172.26. Anal. ($C_{16}H_{15}N_5O$) C, H, N.

*6.1.18. 5-((2-(Biphenyl-4-yl)acetamido)methyl)-N,N-dimethyl-1H-tetrazole-1-carboxamide (**11**) and 5-((2-(biphenyl-4-yl)acetamido)methyl)-N,N-dimethyl-2H-tetrazole-2-carboxamide (**16**)*

The title compounds were obtained as an inseparable 0.82:1 mixture from **43** following the same procedure that was used for the synthesis of **1**, **2**. Yield 31%; IR ($CHCl_3$) 3433, 3009, 2942, 1740, 1676, 1504, 1489, 1395, 1257, 1165, 1064 cm^{-1}; ^1H NMR (300 MHz) δ 3.01 (3H, s), 3.14 and 3.19 (3H, 2s), 3.60 and 3.66 (2H, 2s), 4.76 and 4.77 (2H, 2d, J = 5.8 Hz), 6.72 and 7.03 (1H, 2m), 7.26–7.58 (9H, m); ^{13}C NMR (75 MHz) δ 33.96, 35.00, 37.83, 38.11, 39.10, 39.32, 42.73, 42.97, 126.98, 127.38, 127.41, 127.57, 128.81, 129.79, 129.88, 133.37, 133.57, 140.23, 140.26, 140.47, 140.55, 147.56, 147.81, 154.46, 163.25, 171.27, 171.60.

*6.1.19. N-(Cyanomethyl)-7-phenylheptanamide (**39**)*

The title compound was prepared from 7-phenylheptanoic acid (**34**) following the same procedure that was used for the synthesis of **36** using hexane/AcOEt = 7/3 as eluent for the chromatographic purification. Yield 61%; oil; IR ($CHCl_3$) 3452, 3318, 3034, 2934, 2858, 1686, 1602, 1501, 1414, 1234, 1204 cm^{-1}; ^1H NMR (300 MHz) δ 1.31–1.36 (4H, m), 1.61 (4H, m) 2.21 (2H, t, J = 7.4 Hz), 2.59 (2H, t, J = 7.6 Hz), 4.10 (2H, d, J = 5.8 Hz), 6.60 (1H, m), 7.14–7.30 (5H, m); ^{13}C NMR (75 MHz) δ 25.22, 27.41, 28.86, 28.98, 31.20, 35.81, 35.88, 116.33, 125.64, 128.25, 128.38, 142.59, 173.57. Anal. ($C_{15}H_{20}N_2O$) C, H, N.

*6.1.20. N-((1H-Tetrazol-5-yl)methyl)-7-phenylheptanamide (**44**)*

The title compound was prepared from **39** following the same procedure that was used for the synthesis of **22**. Yield 56%; mp 124–132 °C; IR 3297, 3025, 2934, 2854, 1659, 1521, 1434, 1333, 1250, 1235, 1080, 1053 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$/CD_3OD = 9/1) δ 1.27–1.38 (4H, m), 1.52–1.66 (4H, m) 2.21 (2H, t, J = 7.6 Hz), 2.58 (2H, t, J = 7.5 Hz), 4.66 (2H, s), 7.11–7.31 (5H, m); ^{13}C NMR (75 MHz, $CDCl_3$/CD_3OD = 9/1) δ 25.46, 28.95, 29.11, 31.27, 32.71, 35.89, 36.06, 125.69, 128.31, 128.43, 142.69, 154.76, 175.12. Anal. ($C_{15}H_{21}N_5O$) C, H, N.

*6.1.21. N,N-Dimethyl-5-((7-phenylheptanamido)methyl)-1H-tetrazole-1-carboxamide (**12**) and N,N-dimethyl-5-((7-phenylheptanamido)methyl)-2H-tetrazole-2-carboxamide (**17**)*

The title compounds were prepared from **44** following the same procedure that was used for the synthesis of **1**, **2** using hexane/acetone = 65/35 as eluent for the chromatographic separation of regioisomers. Compound **17** (less polar, 38%): mp 74–76 °C; IR 3310, 3062, 3027, 2926, 2853, 1727, 1653, 1542, 1420, 1397, 1251, 1087 cm^{-1}; ^1H NMR

(300 MHz) δ 1.27–1.35 (4H, m), 1.52–1.62 (4H, m) 2.21 (2H, t, $J = 7.5$ Hz), 2.58 (2H, t, $J = 7.6$ Hz), 3.10 (3H, s), 3.22 (3H, s), 4.80 (2H, d, $J = 5.8$ Hz), 6.94 (1H, m), 7.13–7.30 (5H, m); ^{13}C NMR (75 MHz) δ 25.38, 28.88, 29.03, 31.18, 33.69, 35.82, 36.09, 37.91, 39.42, 125.62, 128.24, 128.38, 142.63, 147.91, 154.65, 173.72. Anal. ($C_{18}H_{26}N_6O_2$) C, H, N. Compound **12** (more polar, 26%): mp 55–57 °C; IR 3312, 3066, 3029, 2937, 2846, 1732, 1651, 1548, 1410, 1383, 1277, 1215, 1134, 1073 cm^{-1}; ^{1}H NMR (300 MHz) δ 1.26–1.39 (4H, m), 1.54–1.69 (4H, m) 2.26 (2H, t, $J = 7.5$ Hz), 2.60 (2H, t, $J = 7.7$ Hz), 3.09 (3H, s), 3.24 (3H, s), 4.80 (2H, d, $J = 5.6$ Hz), 6.40 (1H, m), 7.13–7.31 (5H, m); ^{13}C NMR (75 MHz) δ 25.47, 28.95, 29.09, 31.25, 34.87, 35.87, 36.38, 38.17, 39.17, 125.63, 128.26, 128.41, 142.70, 147.61, 163.46, 173.31. Anal. ($C_{18}H_{26}N_6O_2$) C, H, N.

6.1.22. N-(Cyanomethyl)-8-phenyloctanamide (*40*)

The title compound was prepared from 8-phenyloctanoic acid (**35**) following the same procedure that was used for the synthesis of **36** using hexane/AcOEt = 7/3 as eluent for the chromatographic purification. Yield 47%; mp 39–40 °C; IR ($CHCl_3$) 3452, 3323, 3034, 2932, 2857, 2337, 1686, 1602, 1498, 1453, 1348, 1263, 1263, 1206, 1031 cm^{-1}; ^{1}H NMR (300 MHz) δ 1.30 (6H, m), 1.59 (4H, m), 2.20 (2H, t, $J = 7.5$ Hz), 2.57 (2H, t, $J = 7.6$ Hz), 4.10 (2H, d, $J = 5.8$ Hz), 6.52 (1H, m), 7.11–7.30 (5H, m); ^{13}C NMR (75 MHz) δ 25.30, 27.42, 29.08, 29.13, 31.34, 35.90, 35.95, 116.30, 125.62, 128.26, 128.39, 142.76, 173.54. Anal. ($C_{16}H_{22}N_2O$) C, H, N.

6.1.23. N-((1H-Tetrazol-5-yl)methyl)-8-phenyloctanamide (*45*)

The title compound was prepared from **40** following the same procedure that was used for the synthesis of **22**. Yield 79%; mp 118–120 °C; IR 3291, 3024, 2930, 2852, 1659, 1526, 1433, 1333, 1251, 1231, 1083, 1053 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3/CD_3OD = 9/1$) δ 1.29 (6H, m), 1.57–1.61 (4H, m) 2.26 (2H, t, $J = 7.6$ Hz), 2.58 (2H, t, $J = 7.7$ Hz), 4.70 (2H, m), 7.13–7.31 (5H, m), 7.67 (1H, m); ^{13}C NMR (75 MHz, $CDCl_3/CD_3OD = 9/1$) δ 25.48, 29.11, 31.35, 32.46, 32.57, 35.92, 36.12, 36.16, 125.63, 128.27, 128.40, 142.76, 153.20, 175.43. Anal. ($C_{16}H_{23}N_5O$) C, H, N.

6.1.24. N,N-Dimethyl-5-((7-phenyloctanamido)methyl)-1H-tetrazole-1-carboxamide (*13*) and N,N-dimethyl-5-((7-phenyloctanamido)methyl)-2H-tetrazole-2-carboxamide (*18*)

The title compounds were prepared from **45** following the same procedure that was used for the synthesis of **1**, **2** using preparative layer chromatography (silica gel, 0.5 cm thick) and hexane/acetone = 60/40 as eluent for the chromatographic separation of regioisomers. Compound **18** (less polar, 38%): mp 74–76 °C; IR 3302, 3074, 2927, 2852, 1728, 1655, 1548, 1413, 1397, 1334, 1251, 1123, 1087 cm^{-1}; ^{1}H NMR (300 MHz) δ 1.29 (6H, m), 1.55–1.61 (4H, m) 2.21 (2H, t, $J = 7.6$ Hz), 2.58 (2H, t, $J = 7.7$ Hz), 3.09 (3H, s), 3.22 (3H, s), 4.81 (2H, d, $J = 6.0$ Hz), 6.95 (1H, m), 7.14–7.30 (5H, m); ^{13}C NMR (75 MHz) δ 25.45, 29.07, 29.13, 31.36, 33.69 35.90, 36.12, 37.90, 39.42, 125.58, 128.22, 128.37, 142.75, 147.91, 154.64, 173.75. Anal. ($C_{19}H_{28}N_6O_2$) C, H, N. Compound **13** (more polar, 26%): mp 55–57 °C; IR 3294, 3068, 2927, 2849, 1732, 1655, 1549, 1490, 1384, 1300, 1248, 1133, 1075 cm^{-1}; ^{1}H NMR (300 MHz) δ 1.32 (6H, m), 1.61–1.68 (4H, m) 2.26 (2H, t, $J = 7.6$ Hz), 2.59 (2H, t, $J = 7.6$ Hz), 3.09 (3H, s), 3.25 (3H, s), 4.81 (2H, d, $J = 5.8$ Hz), 6.37 (1H, m), 7.12–7.30 (5H, m); ^{13}C NMR (75 MHz) δ 25.51, 29.11, 29.18, 31.38, 34.86, 35.93, 36.41, 38.17, 39.18, 125.58, 128.23, 128.39, 142.79, 147.59, 163.42, 173.30. Anal. ($C_{19}H_{28}N_6O_2$) C, H, N.

6.2. X-ray structure determination of tetrazole *2*

X-ray diffraction data for compound **2** (slow crystallisation from CH_2Cl_2/hexane) were collected on a Rigaku AFC5R diffractometer at 298 K, using graphite-monochromated Cu Kα radiation ($\lambda = 1.54178$ Å) and a rotating anode generator. The structure was solved by direct methods and refined by full-matrix least-squares with anisotropic thermal parameters, using the *SIR2004* structure determination package [39]. The hydrogen atoms were idealised (C–H = 0.96 Å). Each H atom was assigned the equivalent isotropic temperature factor of the parent atom and allowed to ride it. The final difference Fourier map with a root-mean-square deviation of electron density of 0.64 eÅ^{-3} showed no significant features.

6.3. Biological evaluation

6.3.1. Assay of AEA cellular reuptake

The effect of compounds on the uptake of [^{14}C]anandamide by rat basophilic leukemia (RBL-2H3) cells was studied by using 2.4 μM (10,000 cpm) of [^{14}C]anandamide [40]. The method consisted of incubating the cells with [^{14}C]anandamide for 90 s at 37 °C, in the presence or absence of varying concentrations of the inhibitors, which were administered to cells 10 min prior to incubation with [^{14}C]anandamide. In all cases, residual [^{14}C]anandamide in the incubation medium after extraction with $CHCl_3/CH_3OH = 2/1$ (by volume), determined by scintillation counting of the lyophilized organic phase, was used as a measure of the amount of anandamide that was taken up by cells. In some cases, the formation of [^{14}C]ethanolamine from [^{14}C]anandamide was measured by counting the water phase of the incubation medium extract. Data are expressed as the concentration exerting 50% inhibition of anandamide uptake (IC_{50}) calculated by GraphPad®. Non-specific binding of [^{14}C]anandamide to cells and plastic dishes was determined in the presence of 100 μM anandamide and was never higher than 40% of total uptake.

6.3.2. Assay of fatty acid amide hydrolase (FAAH)

The effect of compounds on the enzymatic hydrolysis of anandamide was studied as described previously [40] using membranes prepared from either rat brain, incubated with the test compounds and [^{14}C]anandamide (2.4 μM) in 50 mM tris–HCl, pH 9, for 30 min at 37 °C. [^{14}C]ethanolamine

produced from [^{14}C]anandamide hydrolysis was measured by scintillation counting of the aqueous phase after extraction of the incubation mixture with 2 volumes of $CHCl_3/CH_3OH = 2/1$ (by volume). With active compounds, data are expressed as the concentration exerting 50% inhibition of AEA hydrolysis (IC_{50}), calculated by GraphPad.

6.3.3. Assay of DAGL activity

Confluent COS cells, overexpressing DAGLα, were harvested in tris–HCl buffer, pH 7 and homogenized in a homogenizer (Dounce). The homogenates were centrifuged at 4 °C at 800 g (5 min) and then at 10 000 g (25 min). The 10 000 g membrane fraction was incubated in incubation buffer (tris–HCl 50 mM and $CaCl_2$ 1 mM) at pH 7.0 at 37 °C for 20 min, with synthetic *sn*-1-[^{14}C]oleoyl-2-arachidonoyl-glycerol (1.0 mCi/mmol, 25 μM). After the incubation, lipids were extracted with 2 volumes of $CHCl_3/MeOH = 2/1$ (by volume), and the extracts were lyophilized under vacuum. Extracts were fractionated by TLC on silica on plastic plates using $CHCl_3/MeOH/NH_4OH$ (85/15/1 by volume) as the eluting system. Bands corresponding to [^{14}C]oleic acid were cut, and their radioactivity was counted with a β-counter.

6.3.4. Assay of MAGL activity

The 10 000 g cytosolic fraction from COS cells was incubated in tris–HCl 50 mM, at pH 7.0 at 37 °C for 20 min, with synthetic 2-[^{3}H] arachidonoyl-glycerol (1.0 mCi/mmol, 25 μM). After the incubation, lipids were extracted with 2 volumes of chloroform/methanol = 2/1 (by volume), and the extracts were lyophilized under vacuum. Extracts were fractionated by TLC on silica on plastic plates using $CHCl_3/MeOH/NH_4OH$ (85/15/1 by volume) as the eluting system. Bands corresponding to [^{3}H]arachidonic acid were cut, and their radioactivity was counted with a β-counter.

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