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ARTICLE *in* EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY · FEBRUARY 2013

Impact Factor: 3.45 · DOI: 10.1016/j.ejmech.2013.02.005 · Source: PubMed

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European Journal of Medicinal Chemistry 63 (2013) 118–132

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Original article

Biaryl tetrazolyl ureas as inhibitors of endocannabinoid metabolism: Modulation at the *N*-portion and distal phenyl ring

Giorgio Ortar [a,*], Enrico Morera [a], Luciano De Petrocellis [b], Alessia Ligresti [c], Aniello Schiano Moriello [b], Ludovica Morera [a], Marianna Nalli [a], Rino Ragno [a,d], Adele Pirolli [a,d], Vincenzo Di Marzo [c]

[a] *Dipartimento di Chimica e Tecnologie del Farmaco, Sapienza Università di Roma, Piazzale Aldo Moro 5, 00185 Roma, Italy*
[b] *Endocannabinoid Research Group, Istituto di Cibernetica, Consiglio Nazionale delle Ricerche, via dei Campi Flegrei 34, 80078 Pozzuoli, Napoli, Italy*
[c] *Endocannabinoid Research Group, Istituto di Chimica Biomolecolare, Consiglio Nazionale delle Ricerche, via dei Campi Flegrei 34, 80078 Pozzuoli, Napoli, Italy*
[d] *Rome Center for Molecular Design (RCMD), Sapienza Università di Roma, Piazzale Aldo Moro 5, 00185 Roma, Italy*

ARTICLE INFO

Article history:
Received 6 September 2012
Received in revised form
21 December 2012
Accepted 7 February 2013
Available online 15 February 2013

Keywords:
Endocannabinoids
Fatty acid amide hydrolase (FAAH)
Monoacylglycerol lipase (MAGL)
TRPV1 channel
TRPA1 channel
Biaryl tetrazolyl ureas

ABSTRACT

In the present study, we have further extended the structure–activity relationships for the tetrazolyl ureas class of compounds as potential FAAH and/or MAGL inhibitors, by replacing the dimethylamino group of the parent compounds **1** and **2** with bulkier groups or by introducing on the distal phenyl ring of **1** and **2** a selected set of substituents. Some of the new compounds (**16**, **20**, **21**, **25**, and **28**) inhibited FAAH potently (IC_{50} = 3.0–9.7 nM) and selectively (39- to more than 141-fold) over MAGL, while tetrazole **27** turned out to be a promising dual FAAH–MAGL inhibitor of potential therapeutic use. Covalent docking studies on FAAH indicated that the binding modes of tetrazoles **1**–**32** did not display a unique pattern. The ability of tetrazoles **1**–**32** to act as TRPV1 and TRPA1 modulators was also investigated.

1. Introduction

Endogenous ligands of cannabinoid receptors (endocannabinoids) are involved as lipid messengers in a wide array of physiological and pathological conditions and the modulation of their levels appears to be of remarkable therapeutic interest in a variety of human disorders, ranging from neurological, affective and neuropsychiatric disorders and various types of chronic pain to gastrointestinal and hepatic diseases, allergic and inflammatory disorders, cancer and osteoporosis [1].

Among different metabolic pathways that can control the extent and duration of action of the two main endocannabinoids, *N*-arachidonoylethanolamine (anandamide, AEA) and 2-arachidonoylglycerol (2-AG), fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) have definitely emerged as key players [2].

In contrast, the existence of a carrier-mediated transport of AEA across the membrane preceding intracellular trafficking, accumulation, and hydrolysis of AEA has generated a pluriannual intense debate [3]. In this respect, Moore et al. described in 2005 a potent, competitive carbamoyl tetrazole inhibitor of AEA cellular uptake, LY2318912 (Fig. 1) [4], but the same group subsequently reported that LY2183240 (**1**), the parent compound of LY2318912, inhibited enzyme activity in purified FAAH preparations [5], and later Alexander and Cravatt demonstrated that **1** (actually a mixture of **1** and its 2,5-regioisomer **2**) [6] was a potent, covalent inhibitor not only of FAAH but also of several other serine hydrolases, including MAGL [7]. Mass spectral studies of the FAAH–LY2183240 adduct verified that FAAH inhibition was due to covalent modification of the catalytic Ser241 [7], analogous to the mechanism of action of carbamate-based inhibitors [8].

In order to gain further insights into the action of the carbamoyl tetrazole class of compounds, we previously investigated a first series of 1,5 and 2,5-disubstituted carbamoyl tetrazoles, including LY2318912, **1**, and **2**, and found that five of the 18 compounds, evaluated for their activity on AEA uptake, FAAH, MAGL, and DAGL, were active on AEA uptake, although all potently inhibited FAAH and some of them MAGL and DAGL as well [6]. In particular, tetrazole **2** turned out to be a more potent MAGL inhibitor than

* Corresponding author. Tel.: +39 6 49913612; fax: +39 6 49913133.
E-mail address: giorgio.ortar@uniroma1.it (G. Ortar).

0223-5234/$ – see front matter © 2013 Elsevier Masson SAS. All rights reserved.
http://dx.doi.org/10.1016/j.ejmech.2013.02.005

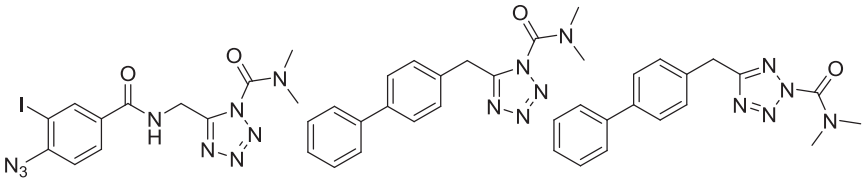


Fig. 1. Structures of compounds LY2318912, **1**, and **2**.

regioisomer **1**, a result confirmed shortly afterwards by Makriyannis et al. who demonstrated in addition that the inhibition involved a covalent interaction with Ser129 [9]. With the aim of 'designing out' cross activities of the first tetrazolyl urea series and attain a good selectivity towards the AEA uptake process, we subsequently investigated the replacement of the *N*,*N*-dimethylaminocarbonyl portion with analogous groups devoid of inherent carbamylating activity and found that this modification resulted in the identification of some tetrazole-based selective AEA uptake inhibitors [10].

In the present study, we have further extended the structure–activity relationships (SARs) for the tetrazolyl ureas class of compounds as potential FAAH and/or MAGL inhibitors, by replacing the dimethylamino group of the parent compounds **1** and **2** with bulkier groups (compounds **3**–**10**, Fig. 2) or by introducing on the distal phenyl ring of **1** and **2** a selected set of substituents (compounds **11**–**32**, Fig. 2). The distal ring was selected in order to minimize electronic effects of substituents upon the urea group and to correlate therefore potencies of compounds directly with non-covalent binding interactions.

In view of the aforementioned activity-based protein profiling (ABPP) of the reference compound **1**, revealing that it is a non-selective FAAH inhibitor acting on several other serine hydrolases [7], we have no reason to believe that the analogues described in our study behave in a different way, i.e. they represent a 'chemotype with potentially excessive protein reactivity' [7], since they were not designed to behave otherwise. Therefore, we did not perform on compounds **3**–**32** any ABPP assay and limited ourselves to investigating whether some of them inhibited FAAH selectively over MAGL (or vice versa) or behaved as dual FAAH/MAGL inhibitors.

The ability of tetrazoles **1**–**32** to act as modulators of the transient receptor potential (TRP) cation channels of vanilloid type-1 (TRPV1) [11] and ankyrin type-1 (TRPA1) [11a,b,d,12] was also investigated. A series of experiments carried out in the late 1990's has in fact established important connections between the function of endocannabinoids and that of TRP channels [11a]. Since then, the overlap between the ligand recognition properties of some TRP channels and proteins of the endocannabinoid system has been actively explored. In particular, TRPV1 was found to be activated by AEA and can therefore be activated indirectly also following FAAH inhibition and subsequent elevation of AEA levels [13], whereas TRPA1 was reported to be activated by the carbamate URB597, a well known covalent FAAH inhibitor [14].

2. Results and discussion

2.1. Chemistry

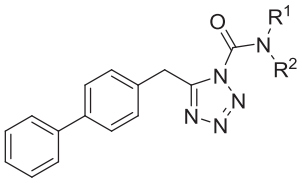
The tetrazoles in Fig. 2 were synthesized as summarized in Schemes 1 and 2. Acylation of 5-[(biphenyl-4-yl)methyl]-1*H*(2*H*)-tetrazole (**33**) [6] with the appropriate carbamoyl chlorides **34** afforded mixtures of the 1,5- (compounds **3**–**6**) and 2,5-regioisomers (compounds **7**–**10**) which could be separated by silica gel chromatography, the less polar 2,5-isomer eluting prior than 1,5-counterpart (Scheme 1) [15]. Carbamoyl chlorides were obtained from the corresponding amines by treatment with triphosgene. Non commercially available amines *N*-methyl-4-phenylbutan-1-amine and *N*-methyl-1-(naphtalen-2-yl)methanamine were prepared via reductive alkylation of 4-phenylbutan-1-amine via the ethyl carbamate [16] and by reduction of the corresponding imine, in turn obtained by reaction of 2-naphtaldehyde with methylamine [17], respectively.

The second group of carbamoyl tetrazoles (compounds **11**–**32**) was synthesized starting from the appropriate monosubstituted 4-biphenylacetonitriles **46**–**55** (Scheme 2). For the preparation of these precursors, a palladium-catalyzed Suzuki–Miyaura cross-coupling reaction between 4-iodophenylacetonitrile (**35**) and the appropriate phenylboronic acids **36**–**45** was exploited. The protection of phenolic OH group of nitriles **50**–**52** with benzyl bromide furnished nitriles **56**–**58**. Nitrile **60** was prepared from nitrile **47** via alkaline hydrolysis and conversion HOBt/EDC mediated of the resulting acid **59** into the primary amide **60**. Tetrazoles **11**–**32** were eventually obtained by refluxing monosubstituted 4-biphenylacetonitriles **46**–**49**, **53**–**58**, **60** with tri-*n*-butyltin azide [18], followed by an acidic hydrolysis step to remove the tin group from the tetrazole ring, acylation of the resulting tetrazoles **61**–**71** with *N*,*N*-dimethylcarbamoyl chloride, and, in the case of tetrazoles **15**–**17** and **26**–**28**, hydrogenolysis of the mixtures of tetrazoles **72** + **73**, **74** + **75**, and **76** + **77**. All the mixtures of the 1,5 (compounds **11**–**21**) and 2,5-regioisomers (compounds **22**–**32**) could be separated by silica gel chromatography, the less polar 2,5-isomer eluting, as in the case of tetrazoles **3**–**10**, prior than 1,5-isomer. The structure of the regioisomeric tetrazoles **3**–**32** was assigned on the basis of their ^{13}C NMR spectra. ^{13}C NMR spectra of disubstituted tetrazoles, in particular those of our two previous papers [6,10], have in fact shown that the CN_4 carbon atom of 1,5-disubstituted tetrazoles is more shielded than the corresponding atom of the 2,5-disubstituted tetrazoles.

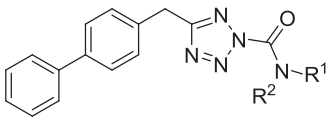
2.2. Biological evaluation

The effect of tetrazoles **3**–**32** on: (a) [^{14}C]AEA hydrolysis by rat brain membranes (which express FAAH as the only AEA hydrolyzing enzyme); (b) [^{3}H]2-AG hydrolysis by COS-7 cell cytosolic fractions (which express MAGL); (c) intracellular Ca^{2+} elevation in HEK293 cells stably transfected with either the human transient receptor potential vanilloid type 1 (TRPV1) or the rat transient receptor potential ankyrin type 1 (TRPA1) cDNAs are shown in Tables 1 and 2. Data previously recorded for tetrazoles **1** and **2** are also included in Tables 1 and 2.

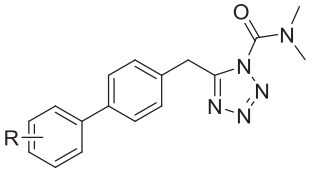
In the first set of tetrazoles (compounds **3**–**10**), one or both methyl groups of the dimethylamino moiety of the parent compounds **1** and **2** were replaced by cycloalkyl or arylalkyl groups. In particular, groups like cyclohexyl, 4-phenylbutyl, and 2-naphthylmethyl which were shown to produce significant potency improvements in the series of biphenyl-3-yl alkyl carbamates



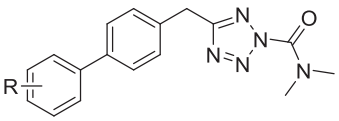
3: R^1, R^2 = *c*-C_5H_{10}
4: R^1 = Me; R^2 = *c*-C_6H_{11}
5: R^1 = Me; R^2 = 4-phenylbutyl
6: R^1 = Me; R^2 = naphtalen-2-ylmethyl



7: R^1, R^2 = *c*-C_5H_{10}
8: R^1 = Me; R^2 = *c*-C_6H_{11}
9: R^1 = Me; R^2 = 4-phenylbutyl
10: R^1 = Me; R^2 = naphtalen-2-ylmethyl



11: R = 3-COMe
12: R = 3-CO_2Me
13: R = 3-CH_2OH
14: R = 4-CH_2OH
15: R = 2-OH
16: R = 3-OH
17: R = 4-OH
18: R = 3-$CONH_2$
19: R = 2-Me
20: R = 3-Me
21: R = 4-Me

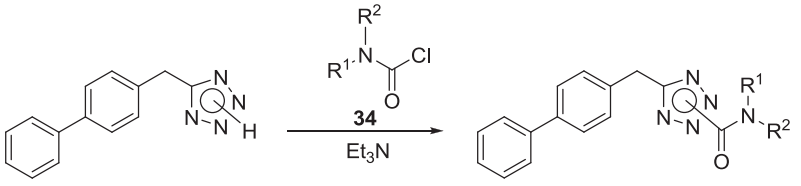


22: R = 3-COMe
23: R = 3-CO_2Me
24: R = 3-CH_2OH
25: R = 4-CH_2OH
26: R = 2-OH
27: R = 3-OH
28: R = 4-OH
29: R = 3-$CONH_2$
30: R = 2-Me
31: R = 3-Me
32: R = 4-Me

Fig. 2. Structures of carbamoyl tetrazoles synthesized and tested in this study.

[19] were selected. Unlike this series, the modifications of the dimethylamino moiety proved however to be generally detrimental to the inhibitory potency on both FAAH and MAGL, except for compounds **9** and **10** which showed IC_{50} values on FAAH of the same magnitude as their prototype **2**. The most significant decrease was observed by substituting one of the two methyl groups with a cyclohexyl group (compare compounds **1**,**2** and **4**,**8**). Furthermore, 1,5-regioisomers were negatively affected to a greater extent than 2,5-analogues. Anyway, the first group of tetrazoles, compounds **4** excluded, were endowed with some selectivity for FAAH over MAGL. Interestingly, most of tetrazoles **3**–**10** exhibited significant TRPV1 and/or TRPA1 agonist/desensitizing activities (Table 2). A special mention needs to be made for compound **8** which turned out to be a very potent TRPA1 activator, displaying a submicromolar potency (EC_{50} = 0.15 μM), ~150-fold greater than that of URB597 (EC_{50} = 24.5 μM) [14]. Although this type of activity may also cause pain in peripheral sensory afferents, TRPA1 activation in the dorsal horn of the spinal cord was shown to counteract pain by leading to inhibition of voltage-activated Na^+ channels [20]. Therefore, given the analgesic actions of FAAH inhibitors [1c,d,2b–f,i], compounds like **8** might represent useful leads for the development of intrathecal analgesics.

With the second group of tetrazoles (compounds **11**–**32**), the effect of phenyl ring substitution on FAAH and MAGL inhibition was explored, introducing on the distal phenyl ring of **1** and **2** a set of substituents. Some of the substituents we selected, in particular small polar groups like $COCH_3$, CH_2OH, OH, and $CONH_2$, have previously yielded FAAH inhibitors with potencies in the low nanomolar concentration range in the series of biphenyl-3-yl alkyl carbamates [21]. As far the FAAH inhibitory activity is concerned, in some cases 1,5-isomers proved to be more potent than 2,5-isomers (compare compounds **15**, **16**, **20**, **21** and **26**, **27**, **31**, **32**), while in other cases the opposite trend was observed (compare compounds **14**, **17**, **18** and **25**, **28**, **29**). Ortho-substituted compounds were



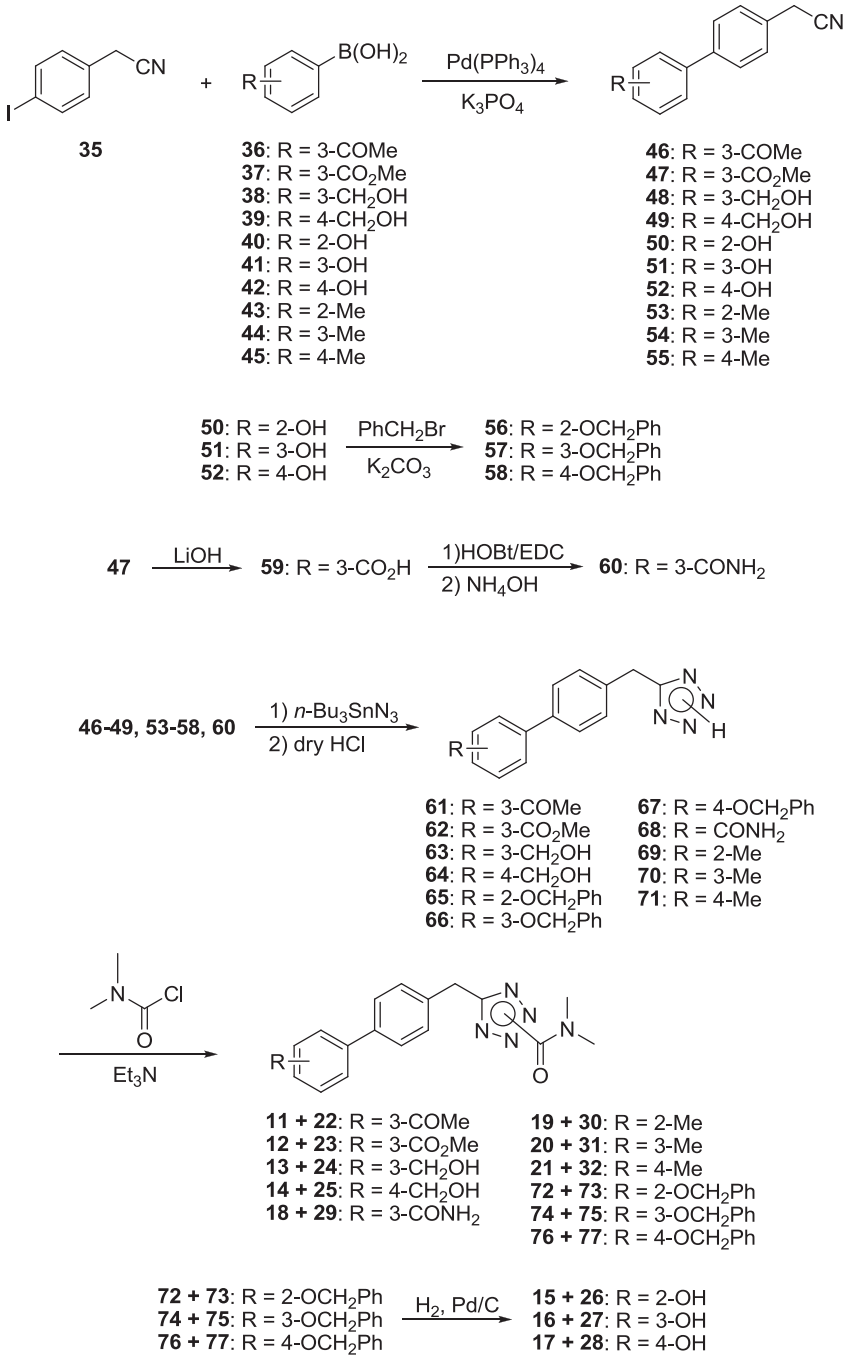
33

34

Et_3N

3 + **7**: R^1, R^2 = *c*-C_5H_{10}
4 + **8**: R^1 = Me; R^2 = *c*-C_6H_{11}
5 + **9**: R^1 = Me; R^2 = 4-phenylbutyl
6 + **10**: R^1 = Me; R^2 = naphtalen-2-ylmethyl

Scheme 1. Synthesis of carbamoyl tetrazoles **3**–**10**.



Scheme 2. Synthesis of carbamoyl tetrazoles **11**–**32**.

generally less active than the corresponding meta- and para-isomers (compare compounds **15**, **19**, **26** and **16**, **20**, **21**, **27**, **28**). Both hydrophilic and lipophilic substituents were compatible with a good inhibitory potency. Although none of compounds **11**–**32** proved to be more potent than **1**, some of them (compounds **16**, **20**, **21**, **25**, and **28**) inhibited FAAH potently ($IC_{50} = 3.0$–9.7 nM) and selectively (39- to more than 141-fold) over MAGL.

Since k_{inact}/K_i values are, unlike IC_{50} values, independent of preincubation times and substrate concentration and are considered the best measure of potency for irreversible inhibitors [22], we have also derived for one of the two most FAAH-selective inhibitors, i.e. compound **20**, the k_{inact}/K_i value from time-dependent IC_{50} values [23], using a standard software tool (XLfit®) and found that this compound displayed a k_{inact}/K_i value for FAAH of $33{,}485 \pm 9200$ M^{-1} s^{-1} which is comparable to that of the highly efficacious FAAH inhibitor PF-04457845 ($32{,}400 \pm 8600$) [22]. This observation may suggest that also the other potent FAAH inhibitors in this series may have similar k_{inact}/K_i values.

With the only partial exception of compounds **13** and **16**, none of the 1,5-regioisomers inhibited appreciably MAGL, while 2,5-isomers proved to be invariably more potent, with IC_{50} values <1 μM. Tetrazole **27**, which exhibited an excellent inhibitory activity for both FAAH and MAGL, has the potential to serve as a useful pharmacological probe for the evaluation of behavioural consequences of simultaneous elevation of the tissue levels of the two main endocannabinoids [24]. Although the issue of selectivity of

Table 1
Effect of tetrazoles **1**–**32** on [^{14}C]AEA hydrolysis by rat brain membranes and [^{3}H]2-AG hydrolysis by COS-7 cell cytosolic fractions.[a]

Compd	IC_{50} (nM)		Compd	IC_{50} (nM)	
	FAAH	MAGL		FAAH	MAGL
1	2.1[b]	>1000[b]	**2**	33.0[b]	20.0[b]
3	38.2 ± 2.4	>1000	**7**	68.1 ± 13.2	346.5 ± 2.0
4	725.0 ± 82.0	>1000	**8**	96.3 ± 10.4	601.5 ± 49.8
5	21.3 ± 2.6	>1000	**9**	11.6 ± 1.5	250.0 ± 33.5
6	86.7 ± 13.8	>1000	**10**	16.9 ± 2.5	84.5 ± 12.1
11	14.7 ± 2.2	>1000	**22**	34.2 ± 7.4	807.4 ± 35.6
12	12.1 ± 0.4	>1000	**23**	12.3 ± 2.2	498.5 ± 11.7
13	10.6 ± 1.0	520.2 ± 113.9	**24**	10.7 ± 1.7	27.0 ± 3.1
14	37.4 ± 2.3	>1000	**25**	7.5 ± 0.9	372.2 ± 24.4
15	15.6 ± 3.0	>1000	**26**	40.2 ± 13.0	98.1 ± 13.0
16	3.0 ± 0.2	170.9 ± 39.5	**27**	12.0 ± 2.1	10.0 ± 1.5
17	16.7 ± 1.6	>1000	**28**	5.4 ± 0.6	209.4 ± 33.3
18	11.0 ± 3.4	>1000	**29**	7.1 ± 1.9	137.2 ± 18.2
19	35.5 ± 4.8	>1000	**30**	51.7 ± 13.3	121.4 ± 51.5
20	9.7 ± 0.7	>1000	**31**	48.5 ± 8.0	139.2 ± 13.0
21	7.1 ± 0.4	>1000	**32**	46.2 ± 8.2	334.9 ± 25.1

[a] Data are means ± SD of $n = 4$ separate determinations.
[b] Data from Ref. [6].

inhibition for FAAH versus MAGL (or vice versa) is certainly the most important one [25], the identification of inhibitors with nanomolar potency on both targets appears to be of interest in view of their possible therapeutic exploitation in those pathological conditions in which the levels of both endocannabinoids need to be elevated. In this respect, Makriyannis et al. has very recently reported that the dual FAAH/MAGL inhibitor **2** is more protective against seizure pathology than regioisomer **1** which is more selective for FAAH than MAGL [26]. The effect of tetrazole **27** on AEA, *N*-oleoylethanolamine (OEA, an alternate FAAH substrate) [2c–g] and 2-AG levels in COS-7 cells overexpressing the human recombinant diacylglycerol lipase-α (DAGL-α) was also measured, by incubating cells with vehicle, or ionomycin with/or without pre-incubation with compound **27** (Table 3). As predicted from its activity, **27** elevated the stimulated levels of the MAGL substrate, 2-AG, and of the FAAH substrate OEA. It also slightly elevated the levels of the other FAAH substrate, AEA, but in a non-statistically significant manner. Again unsurprisingly, the effect on 2-AG was stronger, as expected from the fact that the stimulated levels of this endocannabinoid were higher due to the use of a cell line over-expressing the biosynthetic enzyme for 2-AG.

Table 3
Effect of tetrazole **27** on endocannabinoid levels in intact COS-7 cells.

	AEA	OEA	2-AG
Vehicle	0.16 ± 004	1.08 ± 0.17	2.82 ± 0.75
Ionomycin 3 μM	0.37 ± 0.06	3.99 ± 0.04	15.66 ± 2.46
27 (1 μM) + Ionomycin (3 μM)	0.41 ± 0.18	7.25 ± 0.97[a]	412.84 ± 182.78[b]

[a] Data are expressed as pmol/mg of lipid extracted and are means ± SD of $n = 4$ separate determinations.
[b] $P < 0.05$ as assessed by the ANOVA followed by the Bonferroni's test.

In contrast with the first set of new tetrazoles described here, the compounds of the second group, with the exception of **32**, displayed weak or no activity at TRPV1 and TRPA1 channels. An analogous result was essentially observed for parent compounds **1** and **2**, therefore suggesting that the presence of groups bulkier than methyl on the nitrogen of the carbamoyl moiety is required for an efficient TRPV1 and/or TRPA1 modulation.

In Figs. 3–5 the concentration–response curves for inhibition of FAAH by the FAAH-selective compounds **16**, **20**, **21**, **25**, and **28** and for inhibition of FAAH and MAGL by the dual FAAH–MAGL inhibitor **27**, respectively, are presented.

In Fig. 6 the SARs for the tetrazolyl ureas FAAH and/or MAGL inhibitors described in the present study are summarized.

2.3. Molecular modelling

Although mass spectrometry analysis has unequivocally demonstrated that tetrazole **1** covalently inhibits FAAH by carbamylation of a nucleophilic Ser241 residue with releasing the biphenylmethyl tetrazole moiety [7], no 3D-structure or molecular modelling studies have been so far reported on its possible binding mode. Molecular docking studies were therefore conducted on tetrazoles **1**–**32** with AutoDock Vina [27] docking program. AutoDock Vina usage was assessed through an extensive re-docking and cross-docking previously reported protocol [28] and using the available experimental three-dimensional structures of FAAH co-crystallized with different inhibitors (PDB IDs: 1MT5, 2VYA, 2WAP, 2WJ1, 2WJ2, 3K7F, 3K83, 3K84, 3LJ6, 3LJ7, 3OJ8, 3QJ9, 3QK5,

Table 2
Effect of tetrazoles **1**–**32** on intracellular Ca^{2+} elevation in HEK293 cells stably transfected with either the human TRPV1 or the rat TRPA1 cDNAs.[a]

Compd	TRPV1 (efficacy)[b]	TRPV1 (EC_{50}, μM)	TRPV1 (IC_{50}, μM)[c]	TRPA1 (efficacy)[d]	TRPA1 (EC_{50}, μM)	TRPA1 (IC_{50}, μM)[e]	Compd	TRPV1 (efficacy)[b]	TRPV1 (EC_{50}, μM)	TRPV1 (IC_{50}, μM)[c]	TRPA1 (efficacy)[d]	TRPA1 (EC_{50}, μM)	TRPA1 (IC_{50}, μM)[e]
1	<10	ND	>100	101.1	26.9	49.4	**2**	14.5	5.0	31.3	186.5	20.1	11.2
3	67.4	5.9	22.6	106.5	6.5	12.5	**7**	66.7	1.8	7.4	130.2	3.7	21.5
4	45.6	13.4	18.3	84.7	4.9	14.9	**8**	33.6	0.25	9.2	45.2	0.15	15.4
5	45.7	1.5	7.1	89.9	20.1	83.7	**9**	15.9	1.7	47.6	<10	ND	92.1
6	34.1	2.5	7.6	53.9	13.5	15.0	**10**	36.1	11.9	4.5	<10	ND	24.3
11	<10	ND	>100	<10	ND	>100	**22**	<10	ND	>100	<10	ND	97.2
12	<10	ND	>100	<10	ND	99.8	**23**	<10	ND	>100	<10	ND	91.2
13	<10	ND	>100	<10	ND	>100	**24**	<10	ND	>100	<10	ND	>100
14	<10	ND	>100	58.7	24.2	52.6	**25**	<10	ND	>100	67.3	24.4	70.6
15	<10	ND	>100	228.5	59.7	>100	**26**	<10	ND	>100	445.0	53.3	28.0
16	20.0	25.0	67.4	35.7	26.8	>100	**27**	<10	ND	>100	<10	ND	98.9
17	<10	ND	>100	40.5	21.7	>100	**28**	<10	ND	>100	109.4	14.6	30.2
18	21.3	32.3	>100	30.7	50.1	>100	**29**	<10	ND	91.8	<10	ND	83.1
19	<10	ND	>100	348.8	48.7	45.2	**30**	<10	ND	>100	201.6	15.9	18.9
20	<10	ND	>100	233.4	32.8	41.0	**31**	<10	ND	78.9	187.0	15.0	17.8
21	28.0	22.5	>100	388.0	19.7	15.9	**32**	33.3	4.8	20.6	275.0	10.3	10.5

[a] Data are means of $n = 4$ separate determinations. Standard errors are not shown for the sake of clarity and were never higher that 10% of the means.
[b] As percent of ionomycin (4 μM).
[c] Determined against the effect of capsaicin (0.1 μM).
[d] As percent of allyl isothiocyanate (100 μM).
[e] Determined against the effect of allyl isothiocyanate (100 μM). ND, not determined when efficacy is lower than 10%.

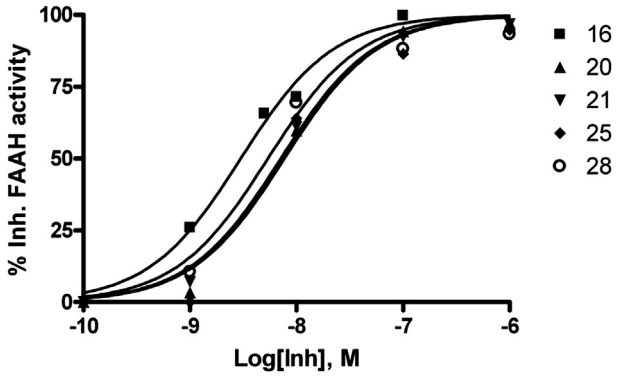


Fig. 3. Concentration-dependent inhibition of FAAH by compounds **16**, **20**, **21**, **25**, **28**.

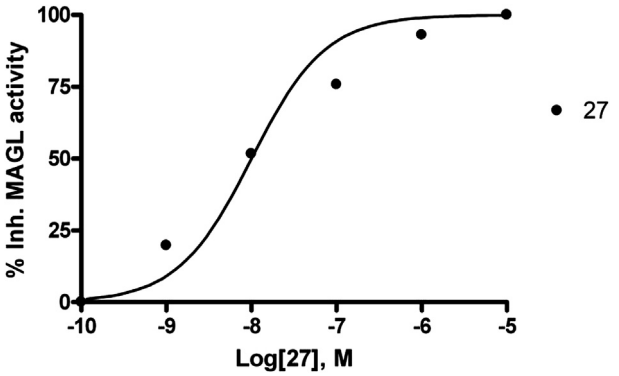


Fig. 5. Concentration-dependent inhibition of MAGL by compound **27**.

3QKV). Twelve out of 14 experimental inhibitors were covalently bound to the catalytic Ser241 [29]. Therefore, a covalent docking protocol was performed with AutoDock Vina. The inhibitors were modelled and bound to Ser241 in their transition state conformations (TS, Fig. 7).

As AutoDock Vina proved to correctly reproduce the experimental binding mode of the covalent FAAH inhibitors (see Experimental section and Supplementary material, Table SM 1), the same covalent cross-docking protocol was applied to compounds **1**–**32**. Although it would be reasonable to expect that in their TS conformation the compounds would bind by locating the biphenylmethyl tetrazole leaving groups in the cytosolic port (or cytoplasmatic access channel, CA) [2c,e,f], due to the lack of any structural insights, it was decided to investigate all the possible binding TSs. Therefore, each inhibitor/FAAH complex was built by modelling the tetrazoles **1**–**32** either in the CA cavity or in the acyl-chain binding pocket (ABP), leading to either (*S*)- or (*R*)-TS configurations. Upon cross-docking into the 12 experimental FAAH conformations on the basis of non-docking interaction energies, the (*S*)-TS configuration was found to be the preferred one (Supplementary material, Table SM 2).

Surprisingly, AutoDock Vina proposed TS binding modes of tetrazoles **1**–**32** endowed with the lowest docking energy did not display a unique pattern, and were determined by the regioisomery of compounds, the nature of nitrogen substituents of the carbamylating moiety, and the nature and position of the distal phenyl ring substituents. In seven out of 16 regioisomeric couples (Supplementary material, Table SM 3), both 1,5 and 2,5-regioisomers (**1** and **2**, **6** and **10**, **14** and **25**, **15** and **26**, **18** and **29**, **19** and **30**, and **21** and **32**) displayed the biphenylmethyl tetrazole moieties accomodated in the ABP pocket, while for the other nine couples, the leaving groups were binding in either the CA cavity (1,5-regioisomers **3**, **5**, **12**, **17**, and **20** and 2,5-regioisomers **8**, **22**, **24**, and **27**) or the ABP pocket (1,5-regioisomers **4**, **11**, **13**, and **16** and 2,5-regioisomers **7**, **9**, **15**, **28**, and **31**).

We speculate that, the orientation of the leaving groups seems to play an important role. Five out of the six single digit nanomolar active derivatives (**1**, **28**, **25**, **16**, and **21** in decreasing order of potency) were predicted to bind with their leaving moieties in the ABP pocket, with their small dimethylaminocarbonyl carbamylating portions residing in the CA channel (Supplementary material, Fig. SM 2).

Regarding the influence of the regioisomery on the binding mode of tetrazoles, the major difference among the non-bonding interactions that could account for the decreased activity of the 2,5-regioisomer **2** compared to 1,5-regioisomer **1**, was a missing π–π interaction that could be observed in **1** between the proximal phenyl ring and the Phe192 side chain (Fig. 8; phenyl centroid distance is about 3.4 Å). When examining, among the new compounds **3**–**32**, the regioisomeric couple with the highest difference in their inhibitory potencies (compounds **14** and **25**), the same π–π interaction was also observed in the compound **14**/FAAH complex. However, the presence of the 4-hydroxymethyl group on the distal phenyl group induced the adoption of a different rotamer, constraining the bonding Ser241 to a disfavoured eclipsed conformation, thus attenuating the positive π–π interaction. On the other hand, the 4-hydroxymethyl group of compound **25**, partially overlapping the membrane access channel (MAC) (Supplementary material, Fig. SM 3), points towards outside the enzyme (Fig. 9), thus possibly making some hydrophilic intermolecular stabilizing interactions that could account for its enhanced activity compared to both its analogue **2** and its regioisomer **14**.

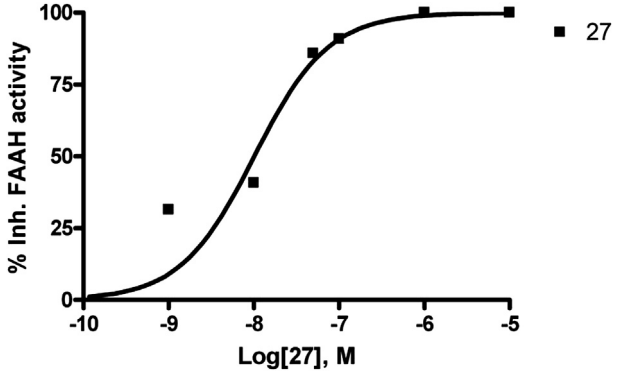


Fig. 4. Concentration-dependent inhibition of FAAH by compound **27**.

3. Conclusions

The replacement of the dimethylamino group of the parent tetrazoles **1** and **2** with bulkier groups resulted invariably in significant decreases in the inhibitory potency against both FAAH and MAGL, while the impact of the introduction of substituents at the ortho, meta or para positions of the distal phenyl ring of **1** and **2** appeared more variable. However, some of the new tetrazoles, that is **16**, **20**, **21**, **25**, and **28**, still inhibited FAAH potently and selectively versus MAGL. On the other hand, tetrazole **27**, whilst not selective versus FAAH, demonstrated also an excellent MAGL inhibitory activity in the low nanomolar range.

Molecular modelling and covalent docking studies suggested that binding modes with FAAH of tetrazoles **1**–**32** do not display a unique pattern. In twenty-three out of 32 cases, the biphenylmethyl tetrazole moiety might bind in the hydrophobic acyl chain channel

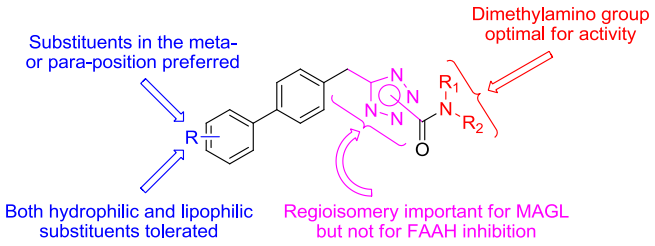


Fig. 6. Summary of SARs for tetrazolyl ureas inhibitors of FAAH and/or MAGL.

of FAAH, with the disubstituted amino group extending into the cytosolic port. The investigation of the ability of tetrazoles **1**–**32** to act as TRPV1 and TRPA1 modulators led to the identification of a potent TRPA1 activator (compound **8**) which should be tested in future studies as a spinal analgesic.

4. Experimental protocols

4.1. Chemistry

4.1.1. General

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 $CHCl_3$ solutions unless otherwise indicated. ^1H and ^{13}C NMR spectra were obtained on a Varian Gemini 200 or a Bruker Avance 400 spectrometer using $CDCl_3$ as solvent unless otherwise indicated and TMS as internal standard. Chromatographic separations were performed on Merck 60 silica gel (230–400 mesh). The preparations of *N*-methyl-4-phenylbutan-1-amine [16] and of *N*-methyl-1-(naphtalen-2-yl)methanamine [17] have been described in the literature.

4.1.2. General procedure for the preparation of carbamoyl chlorides ***34***

To a stirred solution of bis(trichloromethyl)carbonate and dry pyridine (6 equiv) in dry CH_2Cl_2 (2 mL/mmol) a solution of the appropriate amine (3 equiv) in dry CH_2Cl_2 (0.5 mL/mmol) was added. The mixture was stirred at room temperature for 2 h, acidified with 2 N HCl and extracted with CH_2Cl_2. The organic phase was washed with saturated $NaHCO_3$ and brine, dried (Na_2SO_4), and evaporated under vacuum to give the title compounds which were used in the next step without further purification.

4.1.3. General procedure for the preparation of carbamoyl tetrazoles ***3***–***10***

To a stirred solution of **33** [6] and Et_3N (2 equiv) in acetonitrile (1.5 mL/mmol) the appropriate carbamoyl chloride **34** (1.5 equiv) was added at 0 °C, and the mixture was stirred at 0 °C overnight. The mixture was diluted with brine and extracted with AcOEt. The organic phase was washed twice with brine, dried (Na_2SO_4), and evaporated under vacuum. The residue was chromatographed on silica gel using CH_2Cl_2 or CH_2Cl_2/AcOEt mixtures as eluents, the less polar 2,5-disubstituted tetrazole eluting prior to the 1,5-disubstituted regioisomer.

*4.1.3.1. (5-(Biphenyl-4-ylmethyl)-1H-tetrazol-1-yl)(piperidin-1-yl) methanone (**3**) and (5-(biphenyl-4-ylmethyl)-2H-tetrazol-2-yl)(piperidin-1-yl)methanone (**7**).* Piperidine-1-carbonyl chloride and CH_2Cl_2/AcOEt = 98/2 were used as acylating agent and as eluent for the chromatographic separation of regioisomers, respectively. Compound **7** (less polar, 33%): mp 104–105 °C; IR 2948, 2862, 1735, 1488, 1434, 1238, 988 cm^{-1}; ^1H NMR δ 1.60–1.74 (6H, m), 3.38 (2H, t, J = 5.6 Hz), 3.75 (2H, t, J = 5.5 Hz), 4.36 (2H, s), 7.32–7.57 (9H, m); ^{13}C NMR δ 23.99, 25.30, 26.00, 31.41, 46.71, 48.76, 127.04, 127.29, 127.49, 128.76, 129.35, 134.98, 140.09, 140.68, 146.57, 165.16. Anal. Calcd for $C_{20}H_{21}N_5O$: C, 69.14; H, 6.09; N, 20.16. Found: C, 69.18; H, 6.07; N, 20.19. Compound **3** (more polar, 16%): mp 102–103 °C; IR 2948, 2861, 1723, 1504, 1434, 1258, 1008 cm^{-1}; ^1H NMR δ 1.14 (2H, m), 1.49 (2H, m), 1.57 (2H, m), 3.01 (2H, t, J = 5.6 Hz), 3.58 (2H, t, J = 5.5 Hz), 7.31–7.54 (9H, m); ^{13}C NMR δ 23.73, 25.09, 25.45, 29.57, 46.01, 48.51, 126.96, 127.58, 127.63, 128.90, 129.69, 133.43, 140.37, 140.75, 146.40, 155.95. Anal. Calcd for $C_{20}H_{21}N_5O$: C, 69.14; H, 6.09; N, 20.16. Found: C, 69.19; H, 6.05; N, 20.18.

*4.1.3.2. 5-(Biphenyl-4-ylmethyl)-N-cyclohexyl-N-methyl-1H-tetrazole-1-carboxamide (**4**) and 5-(biphenyl-4-ylmethyl)-N-cyclohexyl-N-methyl-2H-tetrazole-2-carboxamide (**8**).*
Cyclohexyl(methyl)carbamoyl chloride and CH_2Cl_2/AcOEt = 98/2 were used as acylating agent and as eluent for the chromatographic separation of regioisomers, respectively. Compound **8** (less polar, 30%): oil; IR 2938, 2860, 1731, 1488, 1204, 1064 cm^{-1}; ^1H NMR δ 1.07–1.90 (10H, m), 2.87 and 3.09 (3H, 2s), 3.29 and 4.27 (1H, 2m), 4.37 (2H, s), 7.31–7.57 (9H, m); ^{13}C NMR δ 25.43, 29.41, 29.72, 29.95, 30.23, 31.51, 57.12, 59.26, 127.10, 127.32, 127.54, 128.79, 129.36, 135.16, 140.23, 140.84, 147.54, 165.09. Anal. Calcd for $C_{22}H_{25}N_5O$: C, 70.38; H, 6.71; N, 18.65. Found: C, 70.45; H, 6.66; N, 18.59. Compound **4** (more polar, 25%): oil; IR 2938, 2860, 1720, 1411, 1224, 1203, 1072 cm^{-1}; ^1H NMR δ 0.92–1.79 (10H, m), 2.47 and 2.90 (3H,

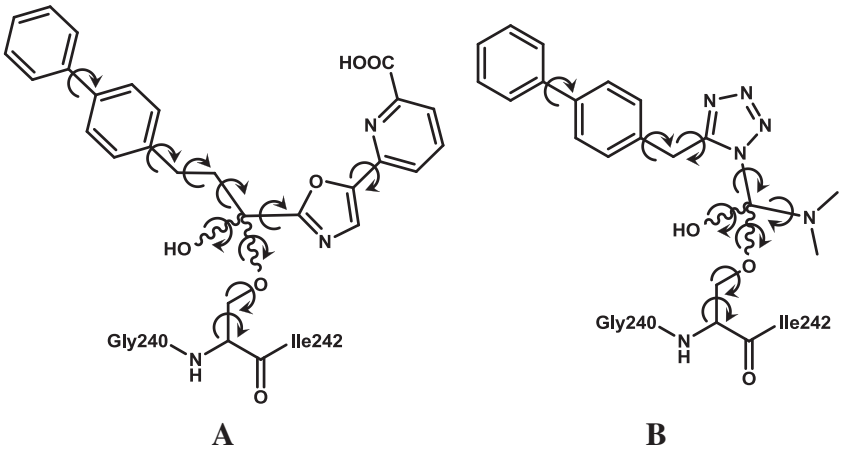


Fig. 7. Examples of the transition state of a co-crystallized inhibitor (A, PDB entry code 3K83) and of that proposed for compound **1** (B) as used in the covalent dockings. The circled arrows indicate the rotatable bonds during the covalent docking.

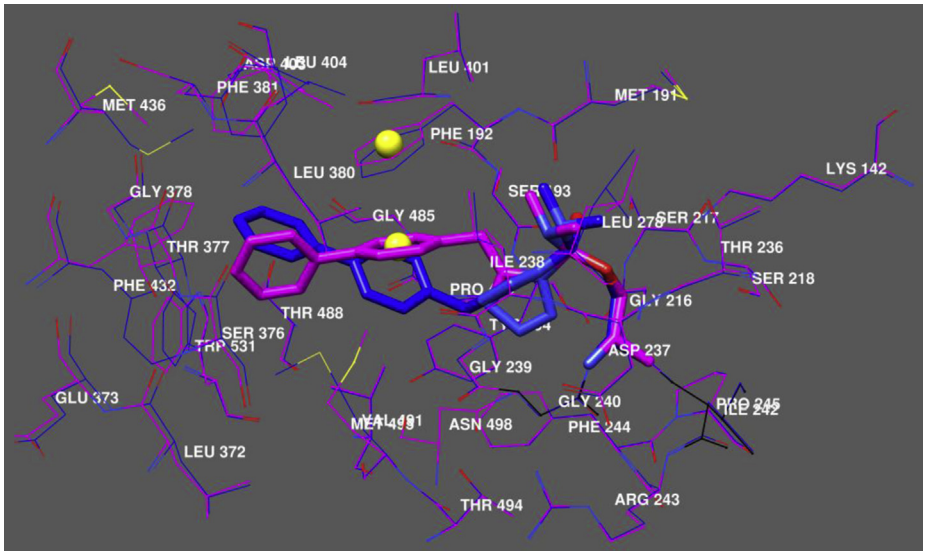


Fig. 8. Binding modes of **1** (magenta) and **2** (blue) regioisomers. In yellow are highlighted the phenyl rings centroids that account for the π–π interaction between Phe192 and the **1** proximal phenyl ring. In wire are also reported the residues in a 5 Å distance from the covalent inhibitors. For sake of clarity hydrogen atoms were undisplayed. Gly240 and Ile242 are depicted in black wire. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2s), 2.73 and 4.09 (1H, 2m), 4.48 (2H, s), 7.26–7.53 (9H, m); ^{13}C NMR δ 25.24, 29.17, 29.46, 29.69, 30.20, 31.50, 57.12, 59.26, 127.00, 127.60, 128.90, 129.48, 133.61, 140.43, 140.87, 147.38, 155.74. Anal. Calcd for $C_{22}H_{25}N_5O$: C, 70.38; H, 6.71; N, 18.65. Found: C, 70.46; H, 6.65; N, 18.58.

*4.1.3.3. 5-(Biphenyl-4-ylmethyl)-N-methyl-N-(4-phenylbutyl)-1H-tetrazole-1-carboxamide (**5**) and 5-(biphenyl-4-ylmethyl)-N-methyl-N-(4-phenylbutyl)-2H-tetrazole-2-carboxamide (**9**).*
Methyl(4-phenylbutyl)carbamoyl chloride and CH_2Cl_2 were used as acylating agent and as eluent for the chromatographic separation of regioisomers, respectively. Compound **9** (less polar, 49%): oil; IR 2940, 2862, 1736, 1488, 1406, 1230, 1067 cm^{-1}; ^{1}H NMR δ 1.48–1.74 (4H, m), 2.52 and 2.68 (2H, 2t, J = 7.5 Hz), 3.01 and 3.15 (3H, 2s), 3.26 and 3.58 (2H, 2t, J = 7.5 Hz), 4.34 (2H, s), 7.07–7.54 (14H, m); ^{13}C NMR δ 26.18, 31.46, 35.39, 37.14, 50.83, 51.42, 126.00, 127.06, 127.31, 127.52, 128.44, 128.77, 129.33, 135.06, 140.19, 140.76, 147.79, 165.13. Anal. Calcd for $C_{26}H_{27}N_5O$: C, 73.39; H, 6.40; N, 16.46. Found: C, 73.32; H, 6.46; N, 16.51. Compound **5** (more polar, 23%): oil; IR 2940, 2862, 1727, 1488, 1405, 1077 cm^{-1}; ^{1}H NMR δ 1.32–1.66 (4H, m), 2.41 and 2.62 (2H, 2t, J = 7.4 Hz), 2.65 and 3.01 (3H, 2s), 2.97 and 3.40 (2H, t, J = 7.4 Hz), 4.46 and 4.48 (2H, 2s), 7.02–7.53 (14H, m); ^{13}C NMR δ 25.96, 29.70, 35.33, 36.81, 50.17, 51.28, 126.04, 127.00, 127.51, 127.61, 128.25, 128.46, 128.90, 129.46, 129.61, 140.21, 140.70, 147.86. Calcd for $C_{26}H_{27}N_5O$: C, 73.39; H, 6.40; N, 16.46. Found: C, 73.31; H, 6.45; N, 16.50.

*4.1.3.4. 5-(Biphenyl-4-ylmethyl)-N-methyl-N-(naphthalen-2-ylmethyl)-1H-tetrazole-1-carboxamide (**6**) and 5-(biphenyl-4-ylmethyl)-N-methyl-N-(naphthalen-2-ylmethyl)-2H-tetrazole-2-carboxamide (**10**).* Methyl(naphthalen-2-ylmethyl)carbamoyl chloride and CH_2Cl_2 were used as acylating agent and as eluent for

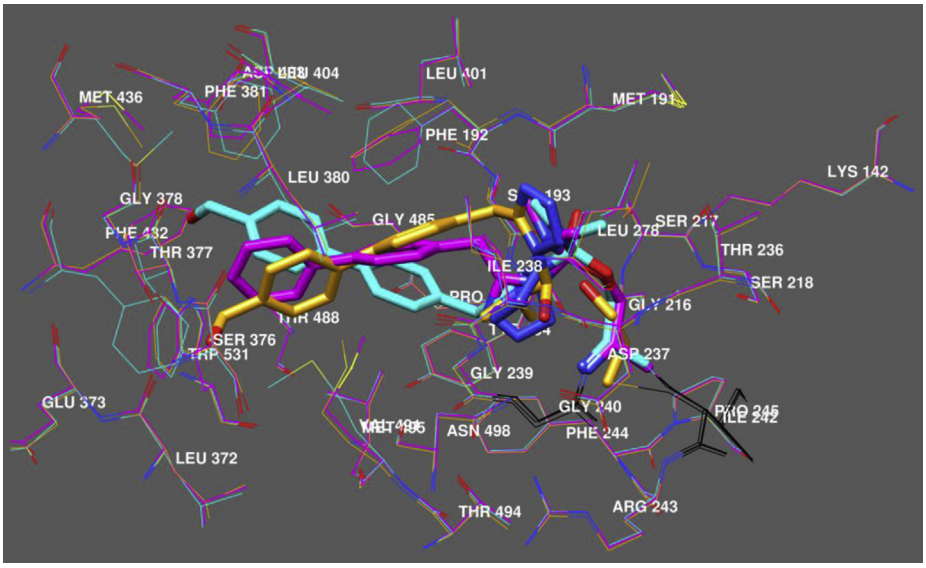


Fig. 9. Binding modes of **14** (yellow) and **25** (cyan) regioisomers. In wire are also reported the residues in a 5 Å distance from the covalent inhibitors. For sake of clarity hydrogen atoms were undisplayed. Gly240 and Ile242 are depicted in black wire. For comparison purposes the docked pose for **1** (magenta) is also reported. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the chromatographic separation of regioisomers, respectively. Compound **10** (less polar, 42%): oil; IR 3017, 2932, 1736, 1488, 1402, 1066 cm^{-1}; ^1H NMR δ 3.03 and 3.16 (3H, 2s), 4.33 and 4.37 (2H, 2s), 4.69 and 4.92 (2H, 2s), 7.30–7.85 (16H, m); ^{13}C NMR δ 31.48, 36.67, 54.40, 126.46, 126.61, 127.07, 127.31, 127.52, 127.80, 127.87, 128.76, 129.07, 129.33, 133.23, 133.37, 134.94, 140.19, 140.76, 165.31. Calcd for $C_{27}H_{23}N_5O$: C, 74.81; H, 5.35; N, 16.16. Found: C, 74.75; H, 5.39; N, 16.21. Compound **6** (more polar, 33%): mp 153–154 °C; IR 2933, 2855, 1726, 1488, 1403, 1266, 1077 cm^{-1}; ^1H NMR δ 2.67 and 3.01 (3H, 2s), 4.43 and 4.76 (2H, 2s), 4.50 and 4.55 (2H, 2s), 7.23–7.73 (16H, m); ^{13}C NMR δ 29.74, 36.33, 53.77, 124.67, 125.78, 126.50, 126.63, 126.96, 127.34, 127.51, 127.62, 127.82, 128.85, 129.11, 129.27, 129.60, 133.16, 135.18, 140.81, 155.84. Calcd for $C_{27}H_{23}N_5O$: C, 74.81; H, 5.35; N, 16.16. Found: C, 74.77; H, 5.37; N, 16.19.

*4.1.4. General procedure for the preparation of 2′- or 3′- or 4′-monosubstituted 2-(biphenyl-4-yl)acetonitriles **46**–**55***

A stirred mixture of 4-iodophenylacetonitrile (**35**), arylboronic acid (**36**–**45**) (1.1 equiv), $Pd(PPh_3)_4$ (0.03 equiv), KBr (1.1 equiv), and K_3PO_4 (2.5 equiv) in dioxane (5 mL/mmol) was purged with N_2 for 10 min at room temperature and then stirred overnight at 85 °C under N_2. The mixture was diluted with water and extracted with AcOEt. The organic phase was washed three times with water, dried (Na_2SO_4), and evaporated under vacuum. The residue was chromatographed on silica gel.

*4.1.4.1. 2-(3′-Acetylbiphenyl-4-yl)acetonitrile (**46**).* 3-Acetylphenylboronic acid (**36**) and hexane/AcOEt = 75/25 were used as arylboronic acid and as eluent for the chromatographic purification of **46** (69%), respectively: mp 71–72 °C; IR (KBr) 3057, 2916, 2247, 1676, 1584, 1412, 1359, 1300, 1240 cm^{-1}; ^1H NMR δ 2.66 (3H, s), 3.81 (2H, s), 7.42–8.17 (8H, m); ^{13}C NMR δ 23.34, 26.72, 117.66, 126.79, 127.60, 127.92, 128.55, 129.19, 129.57, 131.60, 137.80, 140.12, 140.76. Calcd for $C_{16}H_{13}NO$: C, 81.68; H, 5.57; N, 5.95. Found: C, 81.71; H, 5.55; N, 5.93.

*4.1.4.2. Methyl 4′-(cyanomethyl)biphenyl-3-carboxylate (**47**).* 3-Methoxycarbonylphenylboronic acid (**37**) and hexane/CH_2Cl_2 = 3/7 were used as arylboronic acid and as eluent for the chromatographic purification of **47** (63%), respectively: mp 155–156 °C; IR (KBr) 2921, 2247, 1715, 1443, 1309, 1298, 1250, 1113 cm^{-1}; ^1H NMR δ 3.80 (2H, s), 3.95 (3H, s), 7.41–8.26 (8H, m); ^{13}C NMR δ 23.34, 52.23, 117.69, 127.87, 128.18, 128.51, 128.71, 129.00, 129.46, 130.88, 131.41, 140.04, 140.49, 166.91. Calcd for $C_{16}H_{13}NO_2$: C, 76.48; H, 5.21; N, 5.57. Found: C, 76.50; H, 5.20; N, 5.58.

*4.1.4.3. 2-(3′(Hydroxymethyl)biphenyl-4-yl)acetonitrile (**48**).* 3-Hydroxymethylphenylboronic acid (**38**) and hexane/AcOEt = 6/4 were used as arylboronic acid and as eluent for the chromatographic purification of **48** (65%), respectively: mp 60–62 °C; IR 3605, 3424, 3009, 2932, 2255, 1484, 1415, 1020 cm^{-1}; ^1H NMR δ 3.77 (2H, s), 4.74 (2H, s), 7.34–7.60 (8H, m); ^{13}C NMR δ 23.29, 65.18, 117.82, 125.33, 126.20, 126.31, 127.84, 128.37, 128.96, 129.10, 140.51, 140.90, 141.60. Calcd for $C_{15}H_{13}NO$: C, 80.69; H, 5.87; N, 6.27. Found: C, 80.73; H, 5.84; N, 6.30.

*4.1.4.4. 2-(4′(Hydroxymethyl)biphenyl-4-yl)acetonitrile (**49**).* 4-Hydroxymethylphenylboronic acid (**39**) and hexane/AcOEt = 55/45 were used as arylboronic acid and as eluent for the chromatographic purification of **49** (38%), respectively: mp 160–162 °C; IR (KBr) 3212, 2922, 2250, 1497, 1403, 1206, 1041, 998 cm^{-1}; ^1H NMR δ 1.91 (1H, br s), 3.79 (2H, s), 4.75 (2H, s), 7.38–7.65 (8H, m); ^{13}C NMR δ 23.33, 65.00, 117.78, 127.24, 127.53, 127.77, 128.40, 128.91, 139.59, 140.36, 140.76. Calcd for $C_{15}H_{13}NO$: C, 80.69; H, 5.87; N, 6.27. Found: C, 80.72; H, 5.89; N, 6.29.

*4.1.4.5. 2-(2′-Hydroxybiphenyl-4-yl)acetonitrile (**50**).* 2-Hydroxyphenylboronic acid (**40**) and hexane/AcOEt = 75/25 were used as arylboronic acid and as eluent for the chromatographic purification of **50** (66%), respectively: mp 141–143 °C; IR 3353, 2260, 1591, 1498, 1451, 1361, 1196 cm^{-1}; ^1H NMR (CD_3OD) δ 3.90 (2H, s), 6.87–7.58 (8H, m); ^{13}C NMR (CD_3OD) δ 23.29, 117.04, 119.76, 121.03, 128.70, 129.15, 129.78, 130.47, 131.09, 131.60, 140.08, 155.49. Calcd for $C_{14}H_{11}NO$: C, 80.36; H, 5.30; N, 6.69. Found: C, 80.42; H, 5.28; N, 6.65.

*4.1.4.6. 2-(3′-Hydroxybiphenyl-4-yl)acetonitrile (**51**).* 3-Hydroxyphenylboronic acid (**41**) and hexane/AcOEt = 7/3 were used as arylboronic acid and as eluent for the chromatographic purification of **51** (75%), respectively: mp 123–125 °C; IR (KBr) 3317, 2918, 2255, 1586, 1568, 1452, 1303, 1209 cm^{-1}; ^1H NMR (CD_3OD) δ 3.88 (2H, s), 6.77–7.59 (8H, m); ^{13}C NMR (CD_3OD) δ 23.18, 114.72, 115.49, 119.22, 119.52, 128.49, 129.37, 130.87, 131.04, 142.03, 142.94, 158.80. Calcd for $C_{14}H_{11}NO$: C, 80.36; H, 5.30; N, 6.69. Found: C, 80.41; H, 5.27; N, 6.66.

*4.1.4.7. 2-(4′-Hydroxybiphenyl-4-yl)acetonitrile (**52**).* 4-Hydroxyphenylboronic acid (**42**) and hexane/AcOEt = 7/3 were used as arylboronic acid and as eluent for the chromatographic purification of **52** (70%), respectively: mp 188–190 °C; IR (KBr) 3398, 2260, 1611, 1591, 1501, 1213 cm^{-1}; ^1H NMR (CD_3OD) δ 3.88 (2H, s), 6.62 (1H, s), 6.84–7.56 (8H, m); ^{13}C NMR (CD_3OD) δ 23.15, 116.72, 116.85, 128.01, 129.03, 129.45, 130.23, 132.94, 142.09, 158.45. Calcd for $C_{14}H_{11}NO$: C, 80.36; H, 5.30; N, 6.69. Found: C, 80.42; H, 5.33; N, 6.67.

*4.1.4.8. 2-(2′-Methylbiphenyl-4-yl)acetonitrile (**53**).* 2-Methylphenylboronic acid (**43**) and hexane/AcOEt = 9/1 were used as arylboronic acid and as eluent for the chromatographic purification of **53** (87%), respectively: oil; IR 3024, 2927, 2254, 1602, 1514, 1484, 1417, 1110, 1008 cm^{-1}; ^1H NMR δ 2.26 (3H, s), 3.79 (2H, s), 7.19–7.38 (8H, m); ^{13}C NMR δ 20.40, 23.39, 117.87, 125.88, 127.57, 127.72, 128.39, 129.68, 129.94, 130.42, 135.27, 140.94, 141.90. Calcd for $C_{15}H_{13}N$: C, 86.92; H, 6.36; N, 6.76. Found: C, 86.96; H, 6.34; N, 6.77.

*4.1.4.9. 2-(3′-Methylbiphenyl-4-yl)acetonitrile (**54**).* 3-Methylphenylboronic acid (**44**) and hexane/AcOEt = 9/1 were used as arylboronic acid and as eluent for the chromatographic purification of **54** (48%), respectively: oil; IR 2924, 2860, 2255, 1604, 1486, 1460, 1378, 1115 cm^{-1}; ^1H NMR δ 2.42 (3H, s), 3.77 (2H, s), 7.11–7.60 (8H, m); ^{13}C NMR δ 21.51, 23.29, 112.29, 116.03, 121.45, 124.18, 127.83, 128.29, 128.39, 128.78, 129.36, 138.49, 140.20, 141.26. Calcd for $C_{15}H_{13}N$: C, 86.92; H, 6.36; N, 6.76. Found: C, 86.97; H, 6.33; N, 6.80.

*4.1.4.10. 2-(4′-Methylbiphenyl-4-yl)acetonitrile (**55**) [30].* 4-Methylphenylboronic acid (**45**) and hexane/AcOEt = 88/12 were used as arylboronic acid and as eluent for the chromatographic purification of **55** (35%), respectively: mp 121–122 °C; IR 3009, 2924, 2254, 1602, 1502, 1417, 1113, 1007 cm^{-1}; ^1H NMR δ 2.40 (3H, s), 3.78 (2H, s), 7.23–7.54 (8H, m); ^{13}C NMR δ 21.09, 23.28, 115.08, 117.84, 126.89, 127.60, 128.31, 129.59, 137.32, 137.48, 141.04. Calcd for $C_{15}H_{13}N$: C, 86.92; H, 6.36; N, 6.76. Found: C, 86.95; H, 6.38; N, 6.78.

*4.1.4.11. 2-(2′-Benzyloxybiphenyl-4-yl)acetonitrile (**56**).* A mixture of **50** (376 mg, 1.8 mmol), K_2CO_3 (298 mg, 2.16 mmol), benzyl bromide (246 μL, 2.07 mmol) in acetone (15 mL) was refluxed overnight, filtered, and the filtrate was evaporated under vacuum. The residue (580 mg) was chromatographed on silica gel (23 g) using hexane/AcOEt = 85/15 as eluent to give **56** (388 mg, 72%): mp 71–75 °C; IR (KBr) 3029, 2924, 2853, 2251, 1597, 1583, 1513, 1487, 1447, 1264, 1226, 1125, 1022 cm^{-1}; ^1H NMR δ 3.62 (2H, s), 5.02 (2H, s), 6.97–7.56 (13H, m); ^{13}C NMR δ 23.00, 71.15, 116.58, 117.90,

121.56, 127.11, 127.73, 127.92, 128.31, 128.62, 128.98, 129.32, 130.00, 131.91, 136.72, 137.88. Calcd for $C_{21}H_{17}NO$: C, 84.25; H, 5.72; N, 4.68. Found: C, 84.32; H, 5.67; N, 4.72.

*4.1.4.12. 2-(3′-Benzyloxybiphenyl-4-yl)acetonitrile (**57**).* Prepared from **51** following the same procedure that was used for the preparation of **56** using hexane/AcOEt = 85/15 as eluent for the chromatographic purification of **57** (86%): mp 96–97 °C; IR (KBr) 2932, 2868, 2255, 1605, 1581, 1563, 1480, 1294, 1198, 1008 cm^{-1}; ^1H NMR δ 3.76 (2H, s), 5.11 (2H, s), 6.97–7.59 (13H, m); ^{13}C NMR δ 23.28, 70.15, 113.85, 113.96, 117.76, 119.81, 127.51, 127.83, 128.02, 128.33, 128.62, 129.02, 129.92, 136.92, 140.89, 141.73, 159.26. Calcd for $C_{21}H_{17}NO$: C, 84.25; H, 5.72; N, 4.68. Found: C, 84.29; H, 5.69; N, 4.65.

*4.1.4.13. 2-(4′-Benzyloxybiphenyl-4-yl)acetonitrile (**58**).* Prepared from **52** following the same procedure that was used for the preparation of **56** using hexane/CH_2Cl_2 = 65/35 as eluent for the chromatographic purification of **58** (70%): mp 165–167 °C; IR (KBr) 2923, 2863, 2251, 1607, 1499, 1249 cm^{-1}; ^1H NMR δ 3.76 (2H, s), 5.11 (2H, s), 7.03–7.56 (13H, m); ^{13}C NMR δ 23.29, 70.14, 115.29, 127.37, 127.46, 128.03, 128.12, 128.19, 128.33, 128.63, 132.98, 136.90, 140.69, 158.65. Calcd for $C_{21}H_{17}NO$: C, 84.25; H, 5.72; N, 4.68. Found: C, 84.31; H, 5.68; N, 4.71.

*4.1.4.14. 4′-(Cyanomethyl)biphenyl-3-carboxylic acid (**59**).* A solution of **47** (338 mg, 1.35 mmol) and 1 N LiOH (2.06 mL) in THF/H_2O = 5/1 (23 mL) was stirred at room temperature overnight. After acidification to pH 4 with 2 N HCl, the mixture was concentrated under vacuum and extracted with AcOEt. The organic phase was washed with brine until neutral, dried (Na_2SO_4), and evaporated under vacuum to give the acid **59** (310 mg, 97%), which was used in the next step without further purification.

*4.1.4.15. 4′-(Cyanomethyl)biphenyl-3-carboxamide (**60**).* To a stirred solution of **59** (300 mg, 1.26 mmol) in DMF (5 mL) 1-hydroxybenzotriazole (HOBt, 204 mg, 1.33 mmol) and *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC, 255 mg, 1.33 mmol) were added at 0 °C. The mixture was stirred for 15 min at 0 °C and for 1 h at room temperature. Then, a 25% NH_3 aqueous solution (153 μL, 1.89 mmol) wad 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, brine, saturated $NaHCO_3$, and brine, dried (Na_2SO_4), and evaporated under vacuum to give **60** (298 mg, 100%): mp 193–195 °C; IR (KBr) 3328, 3147, 2254, 1668, 1628, 1580, 1447, 1389, 1121 cm^{-1}; ^1H NMR (DMSO-d_6) δ 4.11 (2H, s), 7.47–8.19 (8H, m); ^{13}C NMR (DMSO-d_6) δ 22.01, 119.08, 125.56, 126.67, 127.27, 128.65, 128.89, 129.27, 130.72, 134.92, 138.84, 139.38, 167.71. Calcd for $C_{15}H_{12}N_2O$: C, 76.25; H, 5.12; N, 11.86. Found: C, 76.31; H, 5.09; N, 11.82.

*4.1.5. General procedure for the preparation of tetrazoles **61–71***

A solution of the appropriate 2′-, 3′- or 4′-monosubstituted 4-biphenylacetonitrile (**46–49**, **53–58**, **60**) and *n*-Bu_3SnN_3 [18] (1.5 equiv) in 1,4-dioxane (2 mL/mmol) was refluxed overnight, cooled at room temperature, and gaseous HCl was added over 15 min. The mixture was evaporated under vacuum and the residue was triturated with hot hexane. The resulting solid was filtered off, washed with hexane, and dried under vacuum.

*4.1.5.1. 1-(4′-((1H(2H)-Tetrazol-5-yl)methyl)biphenyl-3-yl)ethanone (**61**).* Yield 76%; mp 159–161 °C; IR (KBr) 2855, 2783, 2617, 1684, 1437, 1359, 1236, 1051, 790 cm^{-1}; ^1H NMR (CD_3OD) δ 2.64 (3H, s), 4.37 (2H, s), 7.39–8.19 (8H, m); ^{13}C NMR (CD_3OD) δ 26.85, 29.85, 127.39, 128.34, 128.47, 130.25, 130.36, 132.48, 136.38, 138.84, 140.23, 142.00, 156.85. Calcd for $C_{16}H_{14}N_4O$: C, 69.05; H, 5.07; N, 20.13. Found: C, 69.11; H, 5.03; N, 20.09.

*4.1.5.2. Methyl 4′-((1H(2H)-tetrazol-5-yl)methyl)biphenyl-3-carboxylate (**62**).* Yield 89%; mp 169–171 °C; IR (KBr) 2956, 2852, 2691, 2613, 1719, 1446, 1409, 1297, 1251, 1043 cm^{-1}; ^1H NMR (CD_3OD) δ 3.92 (3H, s), 4.38 (2H, s), 7.40–8.23 (8H, s); ^{13}C NMR (CD_3OD) δ 29.67, 52.60, 128.23, 128.41, 129.12, 130.12, 130.30, 131.72, 132.27, 136.43, 139.72, 141.68, 167.69. Calcd for $C_{16}H_{14}N_4O_2$: C, 65.30; H, 4.79; N, 19.04. Found: C, 65.36; H, 4.75; N, 18.99.

*4.1.5.3. (4′-((1H(2H)-Tetrazol-5-yl)methyl)biphenyl-3-yl)methanol (**63**).* Yield 86%; mp 116–118 °C; IR (KBr) 3158, 2852, 1563, 1493, 1251, 1040 cm^{-1}; ^1H NMR (CD_3OD) δ 4.34 (2H, s), 4.66 (2H, s), 7.30–7.61 (8H, m); ^{13}C NMR (CD_3OD) δ 30.02, 65.18, 126.48, 126.84, 127.09, 128.57, 129.92, 130.23, 135.65, 141.63, 141.85, 143.37, 157.19. Calcd for $C_{15}H_{14}N_4O$: C, 67.65; H, 5.30; N, 21.04. Found: C, 67.71; H, 5.27; N, 21.00.

*4.1.5.4. (4′-((1H(2H)-Tetrazol-5-yl)methyl)biphenyl-4-yl)methanol (**64**).* Yield 66%; mp 154–156 °C; IR (KBr) 3160, 2856, 1567, 1498, 1249, 1031 cm^{-1}; ^1H NMR (DMSO-d_6) δ 4.34 (2H, s), 4.54 (2H, s), 7.34–7.65 (8H, m); ^{13}C NMR (DMSO-d_6) δ 28.45, 62.51, 115.34, 126.17, 126.74, 126.93, 129.13, 134.81, 137.98, 138.76, 141.74. Calcd for $C_{15}H_{14}N_4O$: C, 67.65; H, 5.30; N, 21.04. Found: C, 67.70; H, 5.28; N, 21.08.

*4.1.5.5. 5-((2′-(Benzyloxy)biphenyl-4-yl)methyl)-1H(2H)-tetrazole (**65**).* Yield 88%; mp 118–119 °C; IR (KBr) 3029, 2865, 2615, 1581, 1485, 1448, 1221, 1106, 1020, 752 cm^{-1}; ^1H NMR (CD_3OD) δ 4.35 (2H, s), 5.05 (2H, s), 7.00–7.54 (13H, m); ^{13}C NMR (CD_3OD) δ 30.12, 71.64, 114.72, 122.44, 128.29, 128.71, 129.28, 129.40, 129.92, 131.32, 131.74, 132.06, 135.12, 138.68, 139.38, 157.02. Calcd for $C_{21}H_{18}N_4O$: C, 73.67; H, 5.30; N, 16.36. Found: C, 73.70; H, 5.28; N, 16.33.

*4.1.5.6. 5-((3′-(Benzyloxy)biphenyl-4-yl)methyl)-1H(2H)-tetrazole (**66**).* Yield 90%; mp 144–145 °C; IR (KBr) 2867, 2608, 1581, 1565, 1479, 1407, 1294, 1197, 1049 cm^{-1}; ^1H NMR (CD_3OD) δ 4.32 (2H, s), 5.09 (2H, s), 6.93–7.56 (13H, m); ^{13}C NMR (CD_3OD) δ 30.03, 71.09, 114.72, 114.93, 120.64, 128.61, 128.88, 129.51, 130.20, 130.94, 135.75, 138.71, 141.49, 143.26, 160.66. Calcd for $C_{21}H_{18}N_4O$: C, 73.67; H, 5.30; N, 16.36. Found: C, 73.73; H, 5.27; N, 16.31.

*4.1.5.7. 5-((4′-(Benzyloxy)biphenyl-4-yl)methyl)-1H(2H)-tetrazole (**67**).* Yield 83%; mp 216–218 °C; IR (KBr) 3034, 2864, 1607, 1500, 1252 cm^{-1}; ^1H NMR (DMSO-d_6) δ 4.32 (2H, s), 5.15 (2H, s), 7.08–7.64 (13H, m); ^{13}C NMR (DMSO-d_6) δ 28.40, 69.15, 115.16, 126.38, 126.57, 127.54, 127.57, 127.72, 128.34, 128.50, 129.07, 132.19, 136.96, 138.45, 157.89. Calcd for $C_{21}H_{18}N_4O$: C, 73.67; H, 5.30; N, 16.36. Found: C, 73.75; H, 5.26; N, 16.30.

*4.1.5.8. 4′-((1H(2H)-Tetrazol-5-yl)methyl)biphenyl-3-carboxamide (**68**).* Yield 75%; mp 222–224 °C; IR (KBr) 3304, 3132, 2253, 1670, 1627, 1580, 1447, 1415, 1390, 1122 cm^{-1}; ^1H NMR (DMSO-d_6) δ 4.36 (2H, s), 7.38–8.15 (8H, m); ^{13}C NMR (DMSO-d_6) δ 28.46, 125.48, 126.49, 127.03, 128.81, 129.16, 129.21, 134.89, 135.35, 138.24, 139.57, 167.66. Calcd for $C_{15}H_{13}N_5O$: C, 64.51; H, 4.69; N, 25.07. Found: C, 64.59; H, 4.65; N, 25.01.

*4.1.5.9. 5-((2′-Methylbiphenyl-4-yl)methyl)-1H(2H)-tetrazole (**69**).* Yield 82%; mp 188–189 °C; IR (KBr) 2978, 2719, 2615, 1575, 1483, 1430, 1256, 1106, 1049, 759 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.22 (3H, s), 4.36 (2H, s), 7.17–7.35 (8H, m); ^{13}C NMR (DMSO-d_6) δ 20.07, 28.49,

125.85, 127.23, 128.42, 129.19, 129.38, 130.25, 134.46, 134.59, 139.90, 140.73, 155.16. Calcd for $C_{15}H_{14}N_4$: C, 71.98; H, 5.64; N, 22.38. Found: C, 72.03; H, 5.60; N, 22.34.

*4.1.5.10. 5-((3′-Methylbiphenyl-4-yl)methyl)-1H(2H)-tetrazole (**70**).* Yield 71%; mp 149–152 °C; IR (KBr) 2856, 2717, 1572, 1485, 1409, 1258, 1050, 766 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.37 (3H, s), 4.34 (2H, s), 7.16–7.64 (8H, m); ^{13}C NMR (DMSO-d_6) δ 21.01, 28.43, 123.62, 126.87, 127.13, 127.20, 127.98, 128.71, 129.10, 134.91, 134.93, 137.95, 138.95, 139.59. Calcd for $C_{15}H_{14}N_4$: C, 71.98; H, 5.64; N, 22.38. Found: C, 72.06; H, 5.59; N, 22.31.

*4.1.5.11. 5-((4′-Methylbiphenyl-4-yl)methyl)-1H(2H)-tetrazole (**71**).* Yield (63%); mp 214–215 °C; IR (KBr) 2856, 2659, 2613, 1575, 1499, 1408, 1259, 1194, 1049 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.34 (3H, s), 4.33 (2H, s), 7.25–7.62 (8H, m); ^{13}C NMR (DMSO-d_6) δ 20.55, 28.45, 126.30, 126.62, 126.71, 129.11, 129.41, 134.69, 136.64, 136.73, 138.76. Calcd for $C_{15}H_{14}N_4$: C, 71.98; H, 5.64; N, 22.38. Found: C, 72.04; H, 5.61; N, 22.33.

*4.1.6. General procedure for the preparation of carbamoyl tetrazoles **11**–**14**, **18**–**25**, **29**–**32**, **72**–**77***

To a stirred solution of the appropriate tetrazole (**61**–**71**) and Et_3N (2 equiv) in acetonitrile (1.5 mL/mmol) Me_2NCOCl (1.5 equiv) was added at 0 °C, and the mixture was stirred at 0 °C overnight. The mixture was diluted with brine and extracted with AcOEt. The organic phase was washed twice with brine, dried (Na_2SO_4), and evaporated under vacuum. The residue was chromatographed on silica gel using CH_2Cl_2/acetone or CH_2Cl_2/MeOH mixtures as eluents, the less polar 2,5-disubstituted tetrazole eluting prior to the 1,5-disubstituted regioisomer.

*4.1.6.1. 5-((3′-Acetylbiphenyl-4-yl)methyl)-N,N-dimethyl-1H-tetrazole-1-carboxamide (**11**) and 5-((3′-acetylbiphenyl-4-yl)methyl)-N,N-dimethyl-2H-tetrazole-2-carboxamide (**22**).*
CH_2Cl_2/acetone = 98/2 was used as eluent for the chromatographic separation of regioisomers. Compound **22** (less polar, 38%): oil; IR 3007, 2930, 1743, 1684, 1496, 1393, 1238, 1068 cm^{-1}; ^1H NMR δ 2.64 (3H, s), 3.10 (3H, s), 3.24 (3H, s), 4.37 (2H, s), 7.43–8.15 (8H, m); ^{13}C NMR δ 26.70, 31.44, 38.13, 39.18, 126.85, 127.25, 127.59, 129.08, 129.54, 131.60, 135.63, 137.72, 139.13, 141.23, 147.81, 165.05, 197.89. Calcd for $C_{19}H_{19}N_5O_2$: C, 65.32; H, 5.48; N, 20.04. Found: C, 65.25; H, 5.52; N, 20.10. Compound **11** (more polar, 16%): mp 153–154 °C; IR 3005, 2940, 1731, 1684, 1497, 1397, 1238, 1088 cm^{-1}; ^1H NMR δ 2.65 (3H, s), 2.78 (3H, s), 3.10 (3H, s), 4.50 (2H, s), 7.36–8.15 (8H, m); ^{13}C NMR δ 26.72, 29.72, 37.55, 38.95, 126.69, 127.55, 127.59, 129.18, 129.65, 131.54, 134.00, 137.80, 139.65, 140.90, 147.93, 155.73, 197.88. Calcd for $C_{19}H_{19}N_5O_2$: C, 65.32; H, 5.48; N, 20.04. Found: C, 65.28; H, 5.51; N, 20.07.

*4.1.6.2. Methyl 4′-((1-(dimethylcarbamoyl)-1H-tetrazol-5-yl)methyl) biphenyl-3-carboxylate (**12**) and methyl 4′-((2-(dimethylcarbamoyl)-2H-tetrazol-5-yl)methyl)biphenyl-3-carboxylate (**23**).*
CH_2Cl_2/acetone = 98/2 was used as eluent for the chromatographic separation of regioisomers. Compound **23** (less polar, 28%): oil; IR 3007, 2953, 1742, 1495, 1443, 1394, 1311, 1252 cm^{-1}; ^1H NMR δ 3.09 (3H, s), 3.23 (3H, s), 3.92 (3H, s), 4.37 (2H, s), 7.42–8.25 (8H, m); ^{13}C NMR δ 31.38, 38.10, 39.14, 52.14, 127.47, 128.07, 128.36, 128.88, 129.46, 130.73, 131.35, 135.54, 138.93, 140.88, 147.77, 164.99, 166.92. Calcd for $C_{19}H_{19}N_5O_3$: C, 62.46; H, 5.24; N, 19.17. Found: C, 62.38; H, 5.28; N, 19.22. Compound **12** (more polar, 16%): mp 153–154 °C; IR 3007, 2953, 1727, 1444, 1397, 1310, 1251 cm^{-1}; ^1H NMR δ 2.76 (3H, s), 3.10 (3H, s), 3.95 (3H, s), 4.50 (2H, s), 7.35–8.24 (8H, m); ^{13}C NMR δ 29.68, 37.52, 38.90, 52.23, 127.52, 128.09, 128.62, 128.99, 129.61, 130.87, 131.33, 133.88, 139.55, 140.57, 147.91, 155.69, 166.93. Calcd for $C_{19}H_{19}N_5O_3$: C, 62.46; H, 5.24; N, 19.17. Found: C, 62.41; H, 5.27; N, 19.20.

*4.1.6.3. 5-((3′-(Hydroxymethyl)biphenyl-4-yl)methyl)-N,N-dimethyl-1H-tetrazole-1-carboxamide (**13**) and 5-((3′-(hydroxymethyl) biphenyl-4-yl)methyl)-N,N-dimethyl-2H-tetrazole-2-carboxamide (**24**).* CH_2Cl_2/acetone = 96/4 was used as eluent for the chromatographic separation of regioisomers. Compound **24** (less polar, 31%): oil; IR 3603, 3435, 3009, 2932, 1742, 1484, 1393, 1068 cm^{-1}; ^1H NMR δ 3.09 (3H, s), 3.23 (3H, s), 4.35 (2H, s), 4.73 (2H, s), 7.26–7.60 (8H, m); ^{13}C NMR δ 31.40, 38.14, 39.19, 65.26, 125.63, 125.90, 126.28, 127.52, 128.99, 129.34, 135.05, 139.90, 140.97, 141.53, 147.81, 165.12. Calcd for $C_{18}H_{19}N_5O_2$: C, 64.08; H, 5.68; N, 20.76. Found: C, 64.00; H, 5.72; N, 20.81. Compound **13** (more polar, 8%): oil; IR 3603, 3435, 3007, 2938, 1732, 1483, 1396, 1068 cm^{-1}; ^1H NMR δ 2.69 (3H, s), 3.06 (3H, s), 4.45 (2H, s), 4.75 (2H, s), 7.27–7.56 (8H, m); ^{13}C NMR δ 29.59, 37.45, 38.80, 65.11, 125.51, 126.16, 127.48, 129.07, 129.46, 129.63, 133.34, 140.44, 140.52, 141.69, 147.88, 155.72. Calcd for $C_{18}H_{19}N_5O_2$: C, 64.08; H, 5.68; N, 20.76. Found: C, 63.99; H, 5.73; N, 20.82.

*4.1.6.4. 5-((4′-(Hydroxymethyl)biphenyl-4-yl)methyl)-N,N-dimethyl-1H-tetrazole-1-carboxamide (**14**) and 5-((4′-(hydroxymethyl) biphenyl-4-yl)methyl)-N,N-dimethyl-2H-tetrazole-2-carboxamide (**25**).* CH_2Cl_2/acetone = 95/5 was used as eluent for the chromatographic separation of regioisomers. Compound **25** (less polar, 16%): mp 91–93 °C; IR (KBr) 3299, 2922, 1734, 1499, 1394, 1166, 1002 cm^{-1}; ^1H NMR δ 2.08 (1H, br s), 3.09 (3H, s), 3.23 (3H, s), 4.35 (2H, s), 4.71 (2H, s), 7.39–7.55 (8H, m); ^{13}C NMR δ 31.39, 38.15, 39.20, 64.96, 127.15, 127.44, 129.35, 134.98, 139.74, 139.98, 140.09, 165.12. Calcd for $C_{18}H_{19}N_5O_2$: C, 64.08; H, 5.68; N, 20.76. Found: C, 64.02; H, 5.71; N, 20.79. Compound **14** (more polar, 9%): mp 134–135 °C; IR (KBr) 3279, 2926, 1731, 1495, 1392, 1252, 1087 cm^{-1}; ^1H NMR δ 1.94 (1H, br s), 2.71 (3H, s), 3.07 (3H, s), 4.48 (2H, s), 4.74 (2H, s), 7.27–7.56 (8H, m); ^{13}C NMR δ 29.64, 37.46, 38.83, 64.94, 127.12, 127.40, 127.52, 129.51, 133.33, 139.61, 140.28, 140.38, 155.75. Calcd for $C_{18}H_{19}N_5O_2$: C, 64.08; H, 5.68; N, 20.76. Found: C, 64.04; H, 5.69; N, 20.81.

*4.1.6.5. 5-((3′-Carbamoylbiphenyl-4-yl)methyl)-N,N-dimethyl-1H-tetrazole-1-carboxamide (**18**) and 5-((3′-carbamoylbiphenyl-4-yl) methyl)-N,N-dimethyl-2H-tetrazole-2-carboxamide (**29**).*
CH_2Cl_2/MeOH = 97/3 was used as eluent for the chromatographic separation of regioisomers. Compound **29** (less polar, 28%): mp 161–163 °C; IR (KBr) 3310, 3133, 1739, 1673, 1629, 1604, 1417, 1389, 1126, 1069 cm^{-1}; ^1H NMR ($CDCl_3/CD_3OD$ = 9/1) δ 3.12 (3H, s), 3.26 (3H, s), 4.37 (2H, s), 7.35–8.06 (8H, m); ^{13}C NMR ($CDCl_3/CD_3OD$ = 9/1) δ 31.45, 38.23, 39.28, 126.32, 126.37, 127.65, 129.15, 129.57, 130.59, 134.02, 135.57, 139.25, 141.22, 148.02, 165.23, 170.44. 170.49. Calcd for $C_{18}H_{18}N_6O_2$: C, 61.70; H, 5.18; N, 23.99. Found: C, 61.64; H, 5.21; N, 24.04. Compound **18** (more polar, 13%): oil; IR 3530, 3413, 2929, 2855, 1731, 1677, 1586, 1441, 1396, 1369, 1230, 1088 cm^{-1}; ^1H NMR δ 2.77 (3H, s), 3.10 (3H, s), 4.48 (2H, s), 6.12 (1H, br s), 6.40 (1H, br s), 7.27–8.05 (8H, m); ^{13}C NMR δ 29.69, 37.55, 38.93, 126.16, 126.29, 127.56, 129.14, 129.60, 130.43, 133.92, 134.10, 139.57, 140.91, 147.89, 155.71, 169.27. Calcd for $C_{18}H_{18}N_6O_2$: C, 61.70; H, 5.18; N, 23.99. Found: C, 61.62; H, 5.23; N, 24.05.

*4.1.6.6. N,N-Dimethyl-5-((2′-methylbiphenyl-4-yl)methyl)-1H-tetrazole-1-carboxamide (**19**) and N,N-dimethyl-5-((2′-methylbiphenyl-4-yl)methyl)-2H-tetrazole-2-carboxamide (**30**).* $CHCl_3$/acetone = 99/1 was used as eluent for the chromatographic separation of regioisomers. Compound **30** (less polar, 38%): oil; IR 3034, 2943, 1743, 1601, 1484, 1393, 1166, 1068 cm^{-1}; ^1H NMR δ 2.24 (3H, s), 3.09 (3H, s), 3.22 (3H, s), 4.36 (2H, s), 7.19–7.39 (8H, m); ^{13}C NMR δ 20.42,

31.41, 38.08, 39.13, 125.76, 127.27, 128.58, 129.54, 129.71, 130.31, 134.41, 135.26, 140.77, 141.36, 147.80, 165.90. Calcd for $C_{18}H_{19}N_5O$: C, 67.27; H, 5.96; N, 21.79. Found: C, 67.21; H, 6.01; N, 21.74. Compound **19** (more polar, 12%): oil; IR 3034, 2937, 1729, 1602, 1483, 1396, 1258, 1088 cm^{-1}; ^1H NMR δ 2.24 (3H, s), 2.70 (3H, s), 3.08 (3H, s), 4.49 (2H, s), 7.15–7.32 (8H, m); ^{13}C NMR δ 20.43, 29.69, 37.43, 38.73, 125.91, 127.55, 128.82, 129.66, 130.44, 132.83, 135.12, 141.06, 141.51, 147.98, 155.78. Calcd for $C_{18}H_{19}N_5O$: C, 67.27; H, 5.96; N, 21.79. Found: C, 67.20; H, 6.00; N, 21.75.

*4.1.6.7. N,N-Dimethyl-5-((3′-methylbiphenyl-4-yl)methyl)-1H-tetrazole-1-carboxamide (**20**) and N,N-dimethyl-5-((3′-methylbiphenyl-4-yl)methyl)-2H-tetrazole-2-carboxamide (**31**).* CH_2Cl_2/acetone = 98/2 was used as eluent for the chromatographic separation of regioisomers. Compound **31** (less polar, 11%): oil; IR 3023, 2939, 1742, 1604, 1490, 1394, 1216 cm^{-1}; ^1H NMR δ 2.40 (3H, s), 3.10 (3H, s), 3.24 (3H, s), 4.36 (2H, s), 7.14–7.55 (8H, m); ^{13}C NMR δ 21.51, 31.40, 38.13, 39.19, 124.14, 127.50, 127.82, 128.05, 128.66, 129.25, 134.81, 138.32, 140.23, 140.65, 165.15. Calcd for $C_{18}H_{19}N_5O$: C, 67.27; H, 5.96; N, 21.79. Found: C, 67.19; H, 6.01; N, 21.73. Compound **20** (more polar, 10%): oil; IR 3011, 2930, 1732, 1604, 1485, 1397, 1256, 1089 cm^{-1}; ^1H NMR δ 2.41 (3H, s), 2.67 (3H, s), 3.06 (3H, s), 4.48 (2H, s), 7.17–7.56 (8H, m); ^{13}C NMR δ 21.52, 29.61, 37.39, 38.75, 124.07, 127.47, 127.73, 128.34, 128.79, 129.41, 133.14, 138.50, 140.22, 140.76, 147.91, 155.73. Calcd for $C_{18}H_{19}N_5O$: C, 67.27; H, 5.96; N, 21.79. Found: C, 67.18; H, 6.02; N, 21.72.

*4.1.6.8. N,N-Dimethyl-5-((4′-methylbiphenyl-4-yl)methyl)-1H-tetrazole-1-carboxamide (**21**) and N,N-dimethyl-5-((4′-methylbiphenyl-4-yl)methyl)-2H-tetrazole-2-carboxamide (**32**).* CH_2Cl_2/acetone = 98/2 was used as eluent for the chromatographic separation of regioisomers. Compound **32** (less polar, 31%): oil; IR 3013, 2941, 1741, 1602, 1500, 1394, 1223, 1068 cm^{-1}; ^1H NMR δ 2.38 (3H, s), 3.09 (3H, s), 3.23 (3H, s), 4.35 (2H, s), 7.22–7.54 (8H, m); ^{13}C NMR δ 21.07, 31.40, 38.12, 39.17, 126.86, 127.30, 129.28, 134.62, 137.07, 137.79, 140.05, 147.80, 165.18. Calcd for $C_{18}H_{19}N_5O$: C, 67.27; H, 5.96; N, 21.79. Found: C, 67.19; H, 5.92; N, 21.82. Compound **21** (more polar, 18%): oil; IR 3014, 2928, 1731, 1602, 1500, 1398, 1257, 1088 cm^{-1}; ^1H NMR δ 2.39 (3H, s), 2.67 (3H, s), 3.05 (3H, s), 4.47 (2H, s), 7.23–7.55 (8H, m); ^{13}C NMR δ 21.08, 29.61, 37.39, 38.74, 126.78, 127.24, 129.43, 129.60, 132.93, 137.35, 137.45, 140.56, 147.91, 155.74. Calcd for $C_{18}H_{19}N_5O$: C, 67.27; H, 5.96; N, 21.79. Found: C, 67.20; H, 5.91; N, 21.85.

*4.1.6.9. 5-((2′-(Benzyloxy)biphenyl-4-yl)methyl)-N,N-dimethyl-1H-tetrazole-1-carboxamide (**72**) and 5-((2′-(benzyloxy)biphenyl-4-yl)methyl)-N,N-dimethyl-2H-tetrazole-2-carboxamide (**73**).* Prepared according to the general procedure and used in the next step as a mixture without further purification.

*4.1.6.10. 5-((3′-(Benzyloxy)biphenyl-4-yl)methyl)-N,N-dimethyl-1H-tetrazole-1-carboxamide (**74**) and 5-((3′-(benzyloxy)biphenyl-4-yl)methyl)-N,N-dimethyl-2H-tetrazole-2-carboxamide (**75**).* Prepared according to the general procedure and used in the next step as a mixture without further purification.

*4.1.6.11. 5-((4′-(Benzyloxy)biphenyl-4-yl)methyl)-N,N-dimethyl-1H-tetrazole-1-carboxamide (**76**) and 5-((4′-(benzyloxy)biphenyl-4-yl)methyl)-N,N-dimethyl-2H-tetrazole-2-carboxamide (**77**).* Prepared according to the general procedure and used in the next step as a mixture without further purification.

*4.1.6.12. 5-((2′-(Hydroxy)biphenyl-4-yl)methyl)-N,N-dimethyl-1H-tetrazole-1-carboxamide (**15**) and 5-((2′-(hydroxy)biphenyl-4-yl)methyl)-N,N-dimethyl-2H-tetrazole-2-carboxamide (**26**).* A stirred solution of the crude mixture of **72** and **73** (524 mg, ~1.27 mmol) in AcOEt (8 mL) and MeOH (2 mL) was hydrogenated in the presence of 10% Pd/C (175 mg) at room temperature and atmospheric pressure for 15 h. The suspension was filtered through a short pad of silica gel. The filtrate was evaporated under vacuum and the residue (347 mg) was chromatographed on silica gel (35 g) using CH_2Cl_2/acetone = 95/5 as eluent for the chromatographic separation of regioisomers. Compound **26** (less polar, 150 mg, 37%): oil; IR 3556, 3011, 1744, 1483, 1394, 1180 cm^{-1}; ^1H NMR δ 3.08 (3H, s), 3.21 (3H, s), 4.33 (2H, s), 6.52 (1H, br s), 6.93–7.45 (8H, m); ^{13}C NMR δ 31.40, 38.14, 39.18, 116.02, 120.74, 127.78, 129.09, 129.48, 129.55, 130.31, 135.21, 136.38, 147.79, 152.73, 164.98. Calcd for $C_{17}H_{17}N_5O_2$: C, 63.15; H, 5.30; N, 21.66. Found: C, 63.08; H, 5.25; N, 21.72. Compound **15** (91 mg, more polar, 22%): mp 142–144 °C; IR 3560, 3009, 1730, 1483, 1397, 1257, 1088 cm^{-1}; ^1H NMR δ 2.69 (3H, s), 3.05 (3H, s), 4.44 (2H, s), 6.93–7.48 (8H, s); ^{13}C NMR δ 29.66, 37.43, 38.79, 116.17, 120.81, 127.59, 129.14, 129.36, 129.69, 130.33, 133.09, 137.40, 147.81, 152.85, 155.75. Calcd for $C_{17}H_{17}N_5O_2$: C, 63.15; H, 5.30; N, 21.66. Found: C, 63.09; H, 5.27; N, 21.70.

*4.1.6.13. 5-((3′-(Hydroxy)biphenyl-4-yl)methyl)-N,N-dimethyl-1H-tetrazole-1-carboxamide (**16**) and 5-((3′-(hydroxy)biphenyl-4-yl)methyl)-N,N-dimethyl-2H-tetrazole-2-carboxamide (**27**).* The title compounds were obtained from the mixture of **74** and **75** following the same procedure that was employed for the preparation of **15** and **26** using CH_2Cl_2/acetone = 96/4 as eluent for the chromatographic separation of regioisomers. Compound **27** (less polar, 81 mg, 20%): oil; IR 3595, 3316, 3006, 2940, 1740, 1598, 1494, 1393, 1307, 1174 cm^{-1}; ^1H NMR δ 3.09 (3H, s), 3.23 (3H, s), 4.32 (2H, s), 6.52 (1H, br s), 6.80–7.48 (8H, m); ^{13}C NMR δ 31.35, 38.21, 39.23, 114.10, 114.46, 119.24, 127.45, 129.25, 129.91, 134.92, 139.78, 142.23, 147.86, 156.36, 165.19. Calcd for $C_{17}H_{17}N_5O_2$: C, 63.15; H, 5.30; N, 21.66. Found: C, 63.07; H, 5.35; N, 21.72. Compound **16** (62 mg, more polar, 15%): mp 158–160 °C; IR 3595, 3312, 3015, 2939, 1732, 1599, 1480, 1397, 1307, 1088 cm^{-1}; ^1H NMR δ 2.66 (3H, s), 3.06 (3H, s), 4.46 (2H, s), 6.82–7.55 (8H, s); ^{13}C NMR δ 29.63, 37.45, 38.79, 114.06, 114.75, 118.54, 127.56, 129.48, 130.04, 133.13, 140.83, 141.81, 147.98, 155.96, 157.38. Calcd for $C_{17}H_{17}N_5O_2$: C, 63.15; H, 5.30; N, 21.66. Found: C, 63.09; H, 5.26; N, 21.71.

*4.1.6.14. 5-((4′-(Hydroxy)biphenyl-4-yl)methyl)-N,N-dimethyl-1H-tetrazole-1-carboxamide (**17**) and 5-((4′-(hydroxy)biphenyl-4-yl)methyl)-N,N-dimethyl-2H-tetrazole-2-carboxamide (**28**).* The title compounds were obtained from the mixture of **76** and **77** following the same procedure that was employed for the preparation of **15** and **26** using CH_2Cl_2/acetone = 95/5 as eluent for the chromatographic separation of regioisomers. Compound **28** (less polar, 27%): mp 222 °C; IR (KBr) 3260, 2926, 1741, 1611, 1499, 1386, 1271, 1172 cm^{-1}; ^1H NMR (CD_3OD) δ 3.03 (3H, s), 3.21 (3H, s), 4.32 (2H, s), 6.82–7.50 (8H, m); ^{13}C NMR (CD_3OD) δ 31.93, 38.20, 39.39, 116.66, 127.75, 128.97, 130.25, 133.32, 135.73, 141.27, 158.25, 166.72. Calcd for $C_{17}H_{17}N_5O_2$: C, 63.15; H, 5.30; N, 21.66. Found: C, 63.11; H, 5.27; N, 21.70. Compound **17** (more polar, 9%): mp 205 °C; IR (KBr) 3143, 1736, 1608, 1497, 1383, 1251, 1084 cm^{-1}; ^1H NMR (CD_3OD) δ 2.67 (3H, s), 3.08 (3H, s), 4.46 (2H, s), 6.87–7.61 (8H, m); ^{13}C NMR (CD_3OD) δ 29.84, 37.54, 38.91, 116.25, 127.41, 128.50, 129.90, 132.24, 132.64, 141.35, 156.54, 157.56. Calcd for $C_{17}H_{17}N_5O_2$: C, 63.15; H, 5.30; N, 21.66. Found: C, 63.10; H, 5.28; N, 21.69.

4.2. Biological evaluation

4.2.1. Assay of fatty amide hydrolase (FAAH)

The effect of compounds on the enzymatic hydrolysis of anandamide was obtained using membranes prepared from rat brain, incubated with the test compounds and [^{14}C]AEA (2.4 μM) in

50 mM Tris–HCl, pH 9, for 30 min at 37 °C. [^{14}C]Ethanolamine produced from [^{14}C]AEA 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). Data are expressed as the concentration exerting 50% inhibition of AEA hydrolysis (IC_{50}), calculated by GraphPad.

4.2.2. Assay of 2-AG hydrolysis

The 10,000 g cytosolic fraction from COS-7 cells, which contains high levels of MAGL, was incubated in Tris–HCl 50 mM, at pH 7.0 at 37 °C for 20 min, with synthetic [^{3}H]2-AG (1.0 mCi/mmol, 25 μM). After the incubation, lipids were extracted with 2 volumes of $CHCl_3/CH_3OH = 2/1$ (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.

4.2.3. Z′-Factor evaluation

The *Z*′-factor was used to evaluate the quality of the assays on FAAH and MAGL and was calculated from the following equation: $Z' = 1 - (3\sigma_{c+} + 3\sigma_{c-})/|\mu_{c-} - \mu_{c+}|$ where σ_{c+} is the standard deviation of the positive control (URB597 0.1 μM for FAAH test and OMDM169 [31] 1.0 μM for MAGL test) and μ_{c+} is the means of sample signals, while σ_{c-} and μ_{c-} are values of the negative control, i.e. the test without inhibitor. *Z*′-Factor was found to be 0.77 ± 0.05 for FAAH and 0.78 ± 0.10 for MAGL denoting an excellent assay [32].

4.2.4. Assay of endocannabinoid levels in intact COS-7 cells

For each data point, about 10^6 COS-7 cells overexpressing the human recombinant DAGL-α [33] were incubated for 20 min at 37 °C with vehicle, or ionomycin 3 μM with or without preincubation (10 min) with compound **27**. Cells were then homogenized in 5 volumes of $CHCl_3/CH_3OH$/Tris–HCl 50 mM pH 7.4 (2/1/1) containing 5 pmol of d^8-AEA, d^5-2-AG and d^4-OEA as internal standards. Homogenates were centrifuged at 13,000× *g* for 15 min (4 °C), the aqueous phase plus debris was collected and extracted again twice with 1 volume of $CHCl_3$. Lipid-containing organic phases from the three extractions were pooled and the organic solvents evaporated under vacuum. Lyophilized extracts were resuspended in chloroform/methanol = 99/1 (by volume). The solutions were then purified by open bed chromatography on silica gel as described in Bisogno et al. [34] and analyzed by isotope dilution liquid chromatography (LC)-atmospheric pressure chemical ionization (APCI)-mass spectrometry (MS) (LC-APCI-MS) using a Shimadzu HPLC apparatus (LC-10ADVP) coupled to a Shimadzu (LCMS-2010) quadrupole MS via a Shimadzu APCI interface [35]. MS analyzes were carried out in the selected ion-monitoring mode. AEA, 2-AG, and OEA levels were quantified on the basis of their area ratio with deuterated internal standards signal area. For 2-AG, the areas of the peaks corresponding to 1(3)-and 2-isomers were added together. Amounts in pmols were normalized per mg of lipid extract. All determinations are at least in triplicate, data were statistically evaluated by one-way ANOVA followed by the Bonferroni's test.

4.2.5. TRPV1 and TRPA1 channel assays

HEK293 (human embryonic kidney) cells were grown as monolayers in EMEM supplemented with nonessential amino acids, 10% fetal bovine serum, and 2 mM glutamine, maintained under 5% CO_2 at 37 °C plated on 100-mm diameter Petri dishes. Cells were transfected at approximately 80% confluence with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) by using a plasmid pcDNA3 (Invitrogen) containing the human TRPV1-cDNA, or the rat TRPA1-cDNA, according to the manufacturer's protocol. Stably transfected clones were selected by G-418 (Invitrogen; 600 μg/ml). The effect of the substances on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was determined by using Fluo-4-AM, a selective intracellular fluorescent probe for Ca^{2+}. On the day of the experiment, cells were loaded for 1 h at room temperature with the methyl ester Fluo-4-AM (4 μM; Invitrogen) in EMEM, then were washed twice in Tyrode's buffer (145 mM NaCl, 2.5 mM KCl, 1.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM D-glucose, and 10 mM HEPES, pH 7.4), resuspended in Tyrode's buffer, and transferred to the quartz cuvette of the spectrofluorimeter (Perkin–Elmer LS50B; PerkinElmer Life and Analytical Sciences, Waltham, MA) under continuous stirring. $[Ca^{2+}]_i$ was determined before and after the addition of various concentrations of test compounds by measuring cell fluorescence (λ_{EX} = 488 nm, λ_{EM} = 516 nm). Curve fitting (sigmoidal dose–response variable slope) and parameter estimation were performed with GraphPad Prism® (GraphPad Software Inc., San Diego, CA). Potency was expressed as the concentration of test substances exerting a half-maximal agonist effect (i.e. half-maximal increases in $[Ca^{2+}]_i$) (EC_{50}). The efficacy of TRPV1 agonists was first determined by normalizing their effect to the maximum Ca^{2+} influx effect on $[Ca^{2+}]_i$ observed with application of 4 μM ionomycin (Sigma). The effects of TRPA1 agonists are expressed as a percentage of the effect obtained with 100 μM allyl isothiocyanate (AITC). For both TRPA1 and TRPV1 agonism, the values of the effect on $[Ca^{2+}]_i$ in wild type HEK293 (i.e. not transfected with any TRP construct) were taken as baselines and subtracted from the values obtained from transfected cells. Antagonist/desensitizing behaviour was evaluated against capsaicin (0.1 μM) for TRPV1 and against AITC (100 μM) for TRPA1, by adding the test compounds in the quartz cuvette 5 min before stimulation of cells with agonists. Data are expressed as the concentration exerting a half-maximal inhibition of agonist-induced $[Ca^{2+}]_i$ elevation (IC_{50}), which was calculated again using GraphPad Prism® software. The effect on $[Ca^{2+}]_i$ exerted by agonist alone was taken as 100%. Dose response curves were fitted by a sigmoidal regression with variable slope. All determinations were performed at least in triplicate. Statistical analysis of the data was performed by analysis of variance at each point using ANOVA followed by the Bonferroni's test.

4.3. Molecular modelling

4.3.1. FAAH experimental structure preparation

Fourteen FAAH experimental structures (Supplementary material, Table SM 1) co-crystallized with 12 covalent and 2 reversible inhibitors were retrieved from Brookheaven Data Bank (PDB) [36]. In 50% of the covalent complexes (PDB IDs: 1MT5, 2BYA, 2WAP, 3LJ6, 3LJ7, and 3QKV) the found experimental PDB structures displayed only a portion of them bound to the catalytic Ser214 oxygen and all in the acyl-chain binding pocket (ABP), while the other 50% (PDB IDs: 2WJ1, 2WJ2, 3K7F, 3K83, 3K84, 3OJ8) clearly all displayed the more hydrophilic molecule ends to be in the cytosolic port region (CA) and their hydrophobic ones all overlapped in the ABP region. Therefore for the covalent docking studies the TS for 1MT5, 2BYA, 2WAP, 3LJ6, 3LJ7, and 3QKV were modelled by building the leaving groups in the CA channel. A third pocked is also visible for the non-covalent inhibitors 3QJ9, 3QK5 (membrane access channel, MAC) partially overlapped with ABP.

The structures of the 14 complexes were arbitrary superimposed using as template the better resolved complex (PDB ID = 2WJ1, R = 1.84 Å). The superimpositions of the FAAH complexes were carried out by means of the program UCSF Chimera [37] using the implementation of MatchMaker [38]. All crystal waters were discarded, and hydrogen atoms were added using the Leap module of AMBER suite [39]. All ligands were individually inspected, and the correct protonation states at pH 7.4 were

considered, i.e., lysines, arginines, aspartates, and glutamates were assumed to be in the ionized form and the parameter were calculated by means of the Antechamber module of AMBER suite. The complexes were solvated (SOLVATEOCT command) in a box extending 10 Å with water molecules (TIP3 model) and neutralized with Na^+ and Cl^- ions. The solvated complexes were then refined by a single point minimization using the Sander module of AMBER suite. The minimized complexes were realigned with MatchMaker using the same reference complex.

4.3.2. Covalent docking protocol

For the docking, the AutoDock Vina program [27] was used in a similar fashion as described by Ahn [40]. A full docking protocol similarly as described [28] was performed for docking program assessment. In Table SM 1 (Supplementary material) are reported the root mean square deviation errors (RMSDs) supporting that AutoDock Vina could reproduce the experimental binding modes. In the herein applied protocol, covalent docking was applied by simply docking a water molecule while setting as flexible residue the covalent inhibitors bound to the nucleophile Ser241 residue. AutoDock 4 [41] was also tested as possible alternative docking method. RMSDs were however much higher than those obtained with AutoDock Vina. To select the binding, all the AutoDock Vina poses were collected in a single bin and clustered using the clustering feature of the AutoDock program. To inspect the binding modes, the program UCSF Chimera 1.5.3 was used.

Acknowledgements

We are grateful to Dr. Ciro Nappi (ICIB) for helping us in the mathematical treatment of the experimental data to calculate k_{inact}/K_i values.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.02.005.

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