Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry Letters 18 (2008) 2820–2824
Bioorganic & Medicinal Chemistry Letters

New tetrazole-based selective anandamide uptake inhibitors

Giorgio Ortar,[a,*] Aniello Schiano Moriello,[b] Maria Grazia Cascio,[b] Luciano De Petrocellis,[c] Alessia Ligresti,[b] Enrico Morera,[a] Marianna Nalli[a] and Vincenzo Di Marzo[b]

[a]*Dipartimento di Studi Farmaceutici, 'Sapienza' Università di Roma, piazzale Aldo Moro 5, 00185 Roma, Italy*
[b]*Istituto di Chimica Biomolecolare, Consiglio Nazionale delle Ricerche, via dei Campi Flegrei 34, 80078 Pozzuoli (Napoli), Italy*
[c]*Istituto di Cibernetica, Consiglio Nazionale delle Ricerche, via dei Campi Flegrei 34, 80078 Pozzuoli (Napoli), Italy*

Received 13 February 2008; revised 31 March 2008; accepted 1 April 2008
Available online 4 April 2008

Abstract—A new series of 1,5- and 2,5-disubstituted tetrazoles have been synthesized and evaluated as inhibitors of anandamide cellular uptake. Some of them inhibit the uptake process with a relatively high potency (IC_{50} = 2.3–5.1 μM) and selectively over other proteins involved in endocannabinoid action and metabolism.

The endocannabinoid signalling system is composed of two G-protein coupled receptors, CB_1 and CB_2, their endogenous agonists (endocannabinoids), and a series of proteins responsible for the synthesis and the inactivation of endocannabinoids.[1] This system has been found to be involved in an increasing number of physiological and pathological conditions, and an enormous interest has therefore arisen about the potential therapeutic applications of compounds acting on its components.[2] Endocannabinoids are synthesized and released 'on demand', act near their site of synthesis, and are then rapidly inactivated by cellular uptake followed by intracellular hydrolysis by specific enzymes. Inhibitors of endocannabinoid degradation could offer a rational therapeutic approach to a variety of diseases including pain, anxiety, cancer and neurodegenerative disorders in which the elevation of endocannabinoid levels represent an adaptive reaction to re-establish normal homeostasis when this is pathologically perturbed.[3]

The levels of the two most studied endocannabinoids, anandamide (AEA)[4] and 2-arachidonoylglycerol (2-AG),[5] appear to be regulated in different, and sometimes even opposing ways. AEA is assumed to be transported into the cell by a specific transporter and then rapidly hydrolyzed by the enzyme fatty acid amide hydrolase (FAAH),[6] whereas a monoacylglycerol lipase (MAGL)[7] is critical in degrading 2-AG.

To date, the existence of an AEA membrane transporter independent of FAAH activity remains, however, questionable and alternative hypotheses have been proposed, including passive diffusion, endocytosis and intracellular sequestration.[8] While the nature of the mechanism responsible for the AEA uptake is still a matter of debate, the recent individuation of a number of anandamide-based selective AEA transport inhibitors that do not appear to inhibit FAAH has stimulated further studies to distinguish between the relative contributions of the putative AEA transporter and FAAH in AEA removal.[8d,9] 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 drug-like characteristics.

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.[10] In 2006, Moore et al. described a potent, competitive carbamoyl tetrazole 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.[11] However, the same group subsequently reported that both LY2183240 (**1**) (Fig. 1), the parent compound of LY2318912, and a *N*-cyclopropyl analogue of SEP-0200228[12] do inhibit

Keywords: Endocannabinoids; Anandamide uptake; Anandamide uptake inhibitors; Tetrazoles.
* Corresponding author. Tel.: +39 6 49913612; fax: +39 6 491491; e-mail: giorgio.ortar@uniroma1.it

0960-894X/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2008.04.003

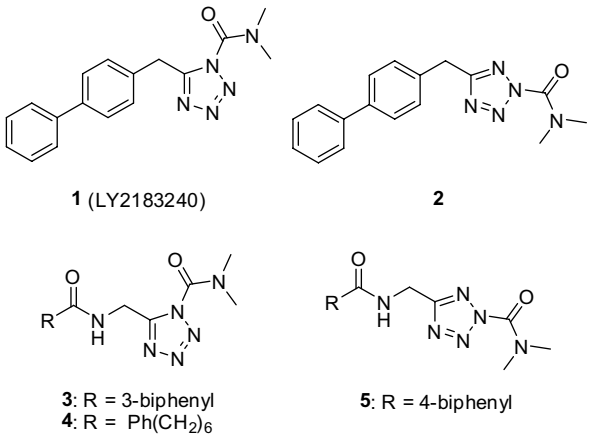


Figure 1. Structures of carbamoyl tetrazoles previously described as non-selective inhibitors of AEA cellular uptake.

enzyme activity in purified FAAH preparations,[13] and Alexander and Cravatt eventually demonstrated that **1** (actually a mixture of **1** and its 2,5-regioisomer **2**)[14] is a potent, covalent inhibitor not only of FAAH but also of several other serine proteases, including MAGL, via carbamylation of an active site catalytic serine.[15]

In order to gain further insight into the actions of the carbamoyl tetrazole class of compounds, we embarked recently on a structure-activity relationship (SAR) study focused on the nature of the 5-substitution and of the regiochemistry.[14] Five of the 18 compounds synthesized and evaluated for their activity on AEA uptake, FAAH, MAGL, and DAGL (diacylglycerol lipase catalyzing the biosynthesis of 2-AG)[16] were found to be active on AEA uptake, although all potently inhibited FAAH and some of them MAGL and DAGL as well (compounds **1**–**5**) (Fig. 1). With the aim of 'designing out' these cross-activities and attain a good selectivity towards the AEA uptake process, we have now investigated the effect of replacing the *N*,*N*-dimethylaminocarbonyl portion of previous tetrazolic ureas with analogous groups devoid of inherent carbamylating reactivity. We report here that this modification indeed results in the identification of some new tetrazole-based selective AEA uptake inhibitors.

From the set of compounds **1**–**5**, the most promising 4-biphenyl and biphenyl-4-carboxamido moieties were selected, since **1** and **5** were already lacking inhibitory activity against MAGL and DAGL. The tetrazoles listed in Table 1 were prepared by the alkylation of 5-[(biphenyl-4-yl)methyl]-1*H*-tetrazole (**16**)[14] or of *N*-[(1*H*-tetrazol-5-yl)methyl]biphenyl-4-carboxamide (**17**)[14] (Scheme 1).[17] From the mixture of regioisomers **11a** + **11b**, only 2,5-regioisomer **11b** could be isolated in a pure form. The structures of the regioisomeric tetrazoles were assigned on the basis of their ^{1}H and ^{13}C NMR spectra.[18] NMR studies of disubstituted tetrazoles, included those of our previous paper, have in fact shown that protons of the CH_2 group attached to N^1 and CN_4 carbon atom of 1,5-disubstituted tetrazoles are more shielded than the corresponding atoms of the 2,5-disubstituted tetrazoles.[19]

The effects of tetrazoles **6**–**15** on (a) [^{14}C]anandamide hydrolysis by rat brain membranes (which express FAAH as the only AEA hydrolyzing enzyme); (b) [^{14}C]anandamide uptake by intact RBL-2H3 cells (where a putative AEA transporter has been characterized pharmacologically); (c) [^{14}C]2-AG hydrolysis by COS cell cytosolic fractions (which express MAGL); (d) *sn*-1-[^{14}C]oleoyl-2-arachidonoyl-glycerol (DAG) conversion into 2-AG and [^{14}C]oleic acid by COS cells membranes overexpressing the human recombinant DAGLα; (e) CB_1 and CB_2 receptors in transfected human COS cells; and (f) intracellular Ca^{2+} elevation mediated by transient receptor potential ankyrin type 1 (TRPA1) and vanilloid type 1 (TRPV1) channels overexpressed in HEK-293 cells, are shown in Table 1.[20] The effects on these two channels were studied since TRPV1, like cannabinoid CB_1 and CB_2 receptors, is activated by AEA and can be activated indirectly also following FAAH inhibition and the subsequent elevation of AEA levels,[21] whereas TRPA1 has been reported to be activated by the widely used FAAH inhibitor URB-597.[22]

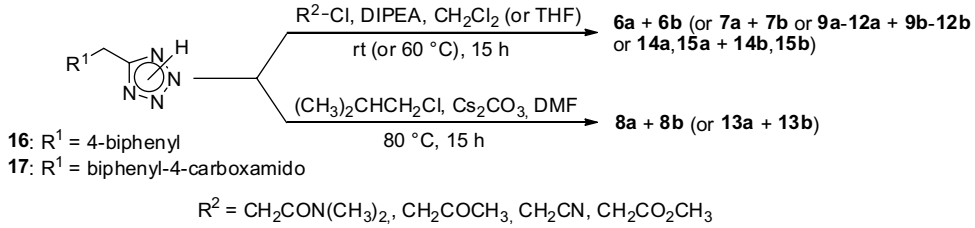
None of the compounds inhibited significantly FAAH, MAGL and DAGL. Thus, the substitution of the carbamylating *N*,*N*-dimethylaminocarbonyl group proved to be detrimental to the inhibitory potency on the three serine hydrolases, despite the fact that some of them could still have acted by way of their electrophilic group interacting with the catalytic triad to produce a covalent intermediate, as assumed for α-ketoheterocycle-based inhibitors[23] and some bis-arylimidazole esters.[24] However, compounds **6a**, **7a**, **9b**, **10a** and **11b** retained the ability to inhibit efficaciously the uptake process with IC_{50} values in the low micromolar range (2.3–5.1 μM), a result which seems to represent yet another piece of evidence in favour of the existence of a FAAH-independent mechanism for AEA removal from the extracellular milieu. Consistent with the previous observations,[14] 1,5-isomers are generally significantly more potent inhibitors than the corresponding 2,5 counterparts (**9b** representing the only exception to this rule). The most potent inhibitor (**11b**), however, belongs to the latter class of regioisomers. Unfortunately, as mentioned before, the 1,5-regioisomer **11a**, which might have been even more potent, could not be evaluated since it was formed in a very low yield and could not be isolated in a pure form. The inhibitory activity seems to depend on the presence of a functional group that might conceivably reinforce the interaction with the protein(s) involved in membrane transport, as compounds lacking any functionality at N^1 (**8a**, **b** and **13a**, **b**) were unable to inhibit AEA uptake process. Overall, the putative AEA membrane transporter appears to display a weak preference for the 4-biphenyl over the biphenyl-4-carboxamido moieties. All compounds exhibited weak or no affinity for CB_1, CB_2, TRPA1, and TRPV1 receptors. In particular, of the five most active compounds on AEA uptake, only one (**9b**) exhibited measurable activity at CB_2 and TRPA1 receptors. When at all active on cannabinoid receptors, the new compounds preferred CB_2 over CB_1 receptors, with only one exception (**8b**). Opposite to what observed for AEA uptake, the 2,5-iso-

Table 1. Effect of tetrazoles **6**–**15** on [^{14}C]anandamide hydrolysis by rat brain membranes, [^{14}C]anandamide uptake by intact RBL-2H3 cells, [^{14}C]2-AG hydrolysis by COS cell cytosolic fractions, *sn*-1-[^{14}C]oleoyl-2-arachidonoyl-glycerol (DAG) conversion into 2-AG and [^{14}C]oleic acid by COS cells membranes, cannabinoid CB_1 and CB_2 receptors in transfected human COS cells, and transient receptor potential TRPA1 and TRPV1 receptors transfected in HEK-293 cells[a]

6: R^1 = 4-biphenyl; R^2 = $CH_2CON(CH_3)_2$
7: R^1 = 4-biphenyl; R^2 = CH_2COCH_3
8: R^1 = 4-biphenyl; R^2 = $CH_2CH(CH_3)_2$
9: R^1 = 4-biphenyl; R^2 = CH_2CN
10: R^1 = 4-biphenyl; R^2 = $CH_2CO_2CH_3$
11: R^1 = 4-biphenyl-4-carboxamido; R^2 = $CH_2CON(CH_3)_2$
12: R^1 = 4-biphenyl-4-carboxamido; R^2 = CH_2COCH_3
13: R^1 = 4-biphenyl-4-carboxamido; R^2 = $CH_2CH(CH_3)_2$
14: R^1 = 4-biphenyl-4-carboxamido; R^2 = CH_2CN
15: R^1 = 4-biphenyl-4-carboxamido; R^2 = $CH_2CO_2CH_3$

Compound	AEA hydrolysis (IC_{50}, μM)	AEA uptake (IC_{50}, μM)	2-AG hydrolysis (IC_{50}, μM)	DAG hydrolysis to 2-AG (IC_{50}, μM)	CB_1 (K_i, μM)	CB_2 (K_i, μM)	TRPA1 (EC_{50}, μM)	TRPA1 (efficacy)[b]	TRPV1 (EC_{50}, μM)	TRPV1 (efficacy)[c]
6a	>50	5.1	>10	>10	>10	>10	>100	0	>100	4.0
6b	>50	>25	>10	>10	>10	>10	>100	0	>100	5.3
7a	>50	5.0	>10	>10	>10	>10	>100	11.4	>100	0
7b	>50	>25	>10	>10	>10	5.2	>100	61.4	>100	0
8a	>50	>25	>10	>10	>10	7.7	>100	50.8	>100	4.7
8b	>50	>25	>10	>10	1.6	4.7	26.3	136.0	10.0	20.7
9a	>50	24.8	>10	>10	>10	>10	26.3	83.2	>100	5.4
9b	>50	5.1	>10	>10	>10	6.2	16.2	101.7	>100	0
10a	>50	4.9	>10	>10	>10	>10	>100	0	>100	0
10b	>50	>25	>10	>10	>10	8.1	>100	36.0	>100	0
11b	>50	2.3	>10	>10	>10	>10	>100	0	>100	1.2
12a	>50	12.5	>10	>10	>10	>10	>100	0	>100	7.7
12b	>50	>25	>10	>10	>10	>10	>100	14.0	>100	0
13a	>50	>25	>10	>10	>10	>10	>100	26.1	15.0	9.1
13b	>50	>25	>10	>10	>10	>10	>100	0	53.5	10.2
14a	>50	>25	>10	>10	>10	>10	>100	0	>100	6.3
14b	>50	>25	>10	>10	>10	>10	>100	0	>100	2.6
15a	>50	>25	>10	>10	>10	>10	>100	0	29.6	8.6
15b	>50	>25	>10	>10	>10	>10	>100	0	>100	2.2

[a] Data are means of $n = 4$ separate determinations. Standard errors are not shown for the sake of clarity and were never higher than 10% of the means.
[b] Expressed the effect of a 100 μM concentration of each compound as percent of the effect of 100 μM mustard oil.
[c] Expressed as the effect of a 10 μM concentration of each compound as percent of the effect on intracellular Ca^{2+} of 4 μM ionomycin.



Scheme 1. Synthesis of tetrazoles **6**–**15**.

mers were more potent than the 1,5 isomers on both CB_2 and TRPA1. All compounds exhibited very weak, and often hardly measurable, efficacy at TRPV1 receptors, and therefore the EC_{50} values calculated for this type of activity have little pharmacological meaning. Interestingly, compounds **8b**, **9a** and **9b** were as efficacious as the mustard oil (allyl isothiocyanate) ($EC_{50} = 2.5 \pm 0.7$ μM, efficacy 100%) on TRPA1-mediated increase of intracellular Ca^{2+}, albeit less potent. This suggests that, like previously shown for the carbamoyl-derivative, URB-597,[22] tetrazole-containing derivatives also can interact with this ion channel, which has been involved in pain transduction by peripheral sensory neurons. Since these latter compounds, unlike URB-597, lack the capability of covalently binding to nucleophilic groups like serine hydroxyl groups or, in the case of TRPA1, cysteine sulphydryl groups, this finding also supports the concept that not all TRPA1 agonists possess sulphydryl reacting moieties in their chemical structure.[25]

In conclusion, we have shown here that it is possible to develop, through appropriate modifications of the compounds introduced by Eli Lilly and subsequently studied and extended by us, selective and non-fatty acid-based AEA uptake inhibitors that might be useful in the elucidation of the 'vexed question of the AEA transporter protein'.[1]

References and notes

1. (a) Alexander, S. P. H.; Kendall, D. A. *Br. J. Pharmacol.* **2007**, *152*, 602; (b) Lambert, D. M.; Fowler, C. J. *J. Med. Chem.* **2005**, *48*, 5059; (c) Howlett, A. C. *Prostaglandins Other Lipid Mediat.* **2002**, *68*, 619; (d) Sugiura, T.; Waku, K. *J. Biochem.* **2002**, *132*, 7.
2. (a) Di Marzo, V.; De Petrocellis, L. *Ann. Rev. Med.* **2006**, *57*, 553; (b) Jonsson, K. O.; Holt, S.; Fowler, C. J. *Basic Clin. Pharmacol. Toxicol.* **2006**, *98*, 124; (c) Mackie, K. *Ann. Rev. Pharmacol. Toxicol.* **2006**, *46*, 101.
3. Di Marzo, V.; Petrosino, S. *Curr. Opin. Lipidol.* **2007**, *18*, 129.
4. Devane, W. A.; Hanus, L.; Breuer, A.; Pertwee, R. G.; Stevenson, L. A.; Griffin, G.; Gibson, D.; Mandelbaum, A.; Etinger, A.; Mechoulam, R. *Science* **1992**, *258*, 1946.
5. (a) Mechoulam, R.; Ben-Shabat, S.; Hanus, L.; Ligumsky, M.; Kaminski, N. E.; Schatz, A. R.; Gopher, A.; Almog, S.; Martin, B. R.; Compton, D. R.; Pertwee, R. G.; Griffin, G.; Bayewitch, M.; Barg, J.; Vogel, Z. *Biochem. Pharmacol.* **1995**, *50*, 83; (b) Sugiura, T.; Kondo, S.; Sukagawa, A.; Nakane, S.; Shinoda, A.; Itoh, K. *Biochem. Biophys. Res. Commun.* **1995**, *215*, 89.
6. (a) Bracey, M. H.; Hanson, M. A.; Masuda, K. R.; Stevens, R. C.; Cravatt, B. F. *Science* **2002**, *298*, 1793; (b) Cravatt, B. F.; Giang, D. K.; Mayfield, S. P.; Boger, D. L.; Lerner, R. A.; Gilula, N. B. *Nature* **1996**, *384*, 83; (c) Giang, D. K.; Cravatt, B. F. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 2238; (d) Patricelli, M. P.; Cravatt, B. F. *Vitam. Horm.* **2001**, *62*, 95.
7. Dinh, T. P.; Carpenter, D.; Leslie, F. M.; Freund, T. F.; Katona, I.; Sensi, S. L.; Kathuria, S.; Piomelli, D. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 10819.
8. (a) Glaser, S. T.; Kaczocha, M.; Deutsch, D. G. *Life Sci.* **2005**, *77*, 1584; (b) Oddi, S.; Bari, M.; Battista, N.; Barsacchi, D.; Cozzani, I.; Maccarrone, M. *Cell. Mol. Life Sci.* **2005**, *62*, 386; (c) Ligresti, A.; Morera, E.; Van Der Stelt, M.; Monory, K.; Lutz, B.; Ortar, G.; Di Marzo, V. *Biochem. J.* **2004**, *380*, 265; (d) Fegley, D.; Kathuria, S.; Mercier, R.; Li, C.; Goutopoulos, A.; Makriyannis, A.; Piomelli, D. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 8756; (e) McFarland, M. J.; Barker, E. L. *Pharmacol. Ther.* **2004**, *104*, 117; (f) Glaser, S. T.; Abumrad, N. A.; Fatade, F.; Kaczocha, M.; Studholme, K. M.; Deutsch, D. G. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 4269; (g) Hillard, C. J.; Jarrahian, A. *Br. J. Pharmacol.* **2003**, *140*, 802.
9. (a) López-Rodríguez, M. L.; Viso, A.; Ortega-Gutiérrez, S.; Fowler, C. J.; Tiger, G.; de Lago, E.; Fernández-Ruiz, J.; Ramos, J. A. *J. Med. Chem.* **2003**, *46*, 1512; (b) Ortar, G.; Ligresti, A.; De Petrocellis, L.; Morera, E.; Di Marzo, V. *Biochem. Pharmacol.* **2003**, *65*, 1473.
10. Hopkins, S. C.; Wang, F. *Symposium on the Canabinoids*; International Cannabinoid Research Society: Burlington, VT, 2004, p. 103.
11. Moore, S. A.; Nomikos, G. G.; Dickason-Chesterfield, A. K.; Schober, D. A.; Schaus, J. M.; Ying, B.-P.; Xu, Y.-C.; Phebus, L.; Simmons, R. M. A.; Li, D.; Iyengar, S.; Felder, C. C. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 17852.
12. Aquila, B. A.; Hopkins, S.; Lockshin, C. A.; Wang, F. WO Patent 03097573, 2003.
13. Dickason-Chesterfield, A. K.; Kidd, S. R.; Moore, S. A.; Schaus, J. M.; Liu, B.; Nomikos, G. G.; Felder, C. C. *Cell. Mol. Neurobiol.* **2006**, *26*, 407.
14. Ortar, G.; Cascio, M. G.; Schiano Moriello, A.; Camalli, M.; Morera, E.; Nalli, M.; Di Marzo, V. *Eur. J. Med. Chem.* **2008**, *43*, 62.
15. Alexander, J. P.; Cravatt, B. F. *J. Am. Chem. Soc.* **2006**, *128*, 9699.
16. Di Marzo, V.; Bifulco, M.; De Petrocellis, L. *Nat. Rev. Drug Discov.* **2004**, *3*, 771.
17. General procedure for the synthesis of compounds **6**–**15**. To a stirred solution of tetrazole **16**[14] or **17**[14] and *N*,*N*-diisopropylethylamine (3–6 equiv) in CH_2Cl_2 (3 mL/mmol of tetrazole), the appropriate chloro derivative (1.5–3 equiv) was added and the mixture was stirred at room temperature overnight (in the case of compounds **14**, the reaction was carried out at 60 °C in THF, while in the case of compounds **8** and **13** the reaction was run at 80 °C in

DMF with Cs_2CO_3 as the base). 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 purified by column chromatography on silica gel using hexane/AcOEt or CH_2Cl_2/AcOEt mixtures as eluents, the less polar 2,5-disubstituted tetrazole (16–70% yields) eluting prior to the 1,5-disubstituted regioisomer (14–53% yields).

18. Data for selected compounds: Compound **6a**: yield 53%; mp 181–182 °C (from $CHCl_3$); IR ($CHCl_3$) 3034, 2932, 1675, 1602, 1488, 1406, 1264, 1150 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 2.95 (3H, s), 2.99 (3H, s), 4.40 (2H, s), 4.90 (2H, s), 7.23–7.58 (9H, m); ^{13}C NMR (75 MHz, $CDCl_3$) δ 29.54, 36.00, 36.52, 48.39, 126.99, 127.64, 127.72, 128.92, 129.31, 132.72, 140.23, 140.75, 154.89, 163.50. Anal. ($C_{18}H_{19}N_5O$) C, H, N.
Compound **7a**: yield 23%; mp 145–146 °C (from hexane/CH_2Cl_2); IR (KBr) 3031, 2974, 1731, 1517, 1484, 1459, 1445, 1352, 1250, 1176, 1128 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 2.13 (3H, s), 4.30 (2H, s), 4.94 (2H, s), 7.18–7.58 (9H, m); ^{13}C NMR (75 MHz, $CDCl_3$) δ 26.98, 29.40, 55.80, 127.00, 127.71, 127.91, 128.92, 129.13, 132.23, 140.07, 140.98, 154.52, 197.24. Anal. ($C_{17}H_{16}N_4O$) C, H, N.
Compound **9b**: yield 46%; mp 86–87 °C (from hexane/CH_2Cl_2); IR (KBr) 3033, 2997, 2360, 1487, 1428, 1407, 1366, 1204, 1148, 1074, 1016 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 4.31 (2H, s), 5.46 (2H, s), 7.32–7.58 (9H, m); ^{13}C NMR (75 MHz, $CDCl_3$) δ 31.49, 39.80, 111.18, 127.06, 127.36, 127.56, 128.79, 129.31, 134.83, 140.26, 140.63, 167.16. Anal. ($C_{16}H_{13}N_5$) C, H, N.
Compound **10a**: yield 51%; mp 115 °C (from hexane/CH_2Cl_2); IR (KBr) 3004, 2958, 1737, 1513, 1488, 1438, 1408, 1230, 1114 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 3.67 (3H, s), 4.35 (2H, s), 4.95 (2H, s), 7.22–7.57 (9H, m); ^{13}C NMR (75 MHz, $CDCl_3$) δ 29.26, 47.94, 53.18, 126.98, 127.64, 127.84, 128.89, 129.12, 132.09, 140.16, 140.89, 154.42, 165.46. Anal. ($C_{17}H_{16}N_4O_2$) C, H, N.
Compound **11b**: yield 36%; mp 185–187 °C (from MeOH); IR (KBr) 3319, 3054, 2932, 1664, 1646, 1609, 1540, 1506, 1485, 1407, 1307, 1155 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ 2.72 (3H, s), 3.05 (3H, s), 4.77 (2H, d, J = 5.6 Hz), 5.65 (2H, s), 7.40–7.98 (9H, m), 9.29 (1H, m); ^{13}C NMR (75 MHz, DMSO-d_6) δ 32.16, 35.10, 35.77, 48.36, 126.49, 126.81, 127.99, 128.09, 128.99, 131.93, 138.95, 143.13, 154.22, 164.38, 166.17. Anal. ($C_{19}H_{20}N_6O_2$) C, H, N.
Compound **12a**: yield 30%; mp 196–198 °C (from MeOH); IR (KBr) 3306, 2947, 1728, 1640, 1542, 1485,1462, 1416, 1324, 1180, 1129 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ 2.27 (3H, s), 4.72 (2H, d, J = 5.6 Hz), 5.75 (2H, s), 7.40–7.97 (9H, m), 9.32 (1H, m); ^{13}C NMR (75 MHz, DMSO-d_6) δ 26.82, 31.89, 55.78, 126.53, 126.80, 127.96, 128.06, 128.96, 131.90, 138.96, 143.18, 153.98, 166.34, 199.80. Anal. ($C_{18}H_{17}N_5O_2$) C, H, N.
19. (a) May, B. C. H.; Abell, A. D. *Tetrahedron Lett.* **2001**, *42*, 5641; (b) Bethel, P. A.; Hill, M. S.; Mahon, M. F.; Molloy, K. C. *J. Chem. Soc. Perkin Trans. 1* **1999**, 3507; (c) Byard, S. J.; Herbert, J. M. *Tetrahedron* **1999**, *55*, 5931.
20. Detailed procedures for assays of AEA cellular uptake by rat basophilic RBL-2H3 cells, and of rat FAAH, human DAGL, MAGL, CB_1, CB_2, and TRPV1 activities were reported (see Ref. 14 and: Di Marzo, V.; Ligresti, A.; Morera, E.; Nalli, M.; Ortar, G. *Bioorg. Med. Chem.* **2004**, *12*, 5161). Assays for activity at TRPA1 channels were carried out as follows. HEK-293 cells stably transfected with the cDNA encoding for the rat TRPA1 receptor were plated on 10 cm diameter Petri dishes and after 3 days were loaded for 1 h at room temperature with the cytoplasmic calcium indicator Fluo4-AM 4 μM (Molecular Probes) containing Pluronic (0.02%, Molecular Probes). Cells were washed twice in Tyrode's buffer pH 7.4 (NaCl 145 mM; KCl 2.5 mM; $CaCl_2$ 1.5 mM; $MgCl_2$ 1.2 mM; D-Glucose 10 mM; HEPES 10 mM pH 7.4), resuspended in Tyrode's buffer and transferred to the quartz cuvette of the spectrofluorimeter (λ_{ex} = 488 nm; λ_{em} = 516 nm). (Perkin-Elmer LS50B) under continuous stirring. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was determined before and after the addition of various concentrations of test compounds. Potency was expressed as the concentration exerting a half-maximal agonist effect (i.e. half-maximal increases in $[Ca^{2+}]_i$) (EC_{50}), calculated by using GraphPad®. The efficacy of the agonists was determined by normalizing their effect to the maximal effect on $[Ca^{2+}]_i$ observed with 100 μM mustard oil.
21. De Petrocellis, L.; Bisogno, T.; Maccarrone, M.; Davis, J. B.; Finazzi-Agrò, A.; Di Marzo, V. *J. Biol. Chem.* **2001**, *276*, 12856.
22. Niforatos, W.; Zhang, X. F.; Lake, M. R.; Walter, K. A.; Neelands, T.; Holzman, T. F.; Scott, V. E.; Faltynek, C. R.; Moreland, R. B.; Chen, J. *Mol. Pharmacol.* **2007**, *71*, 1209.
23. Hardouin, C.; Kelso, M. J.; Romero, F. A.; Rayl, T. J.; Leung, D.; Hwang, I.; Cravatt, B. F.; Boger, D. L. *J. Med. Chem.* **2007**, *50*, 3359, and references therein..
24. Sit, S. Y.; Conway, C.; Bertekap, R.; Xie, K.; Bourin, C.; Burris, K.; Deng, H. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 3287.
25. (a) Hinman, A.; Chuang, H. H.; Bautista, D. M.; Julius, D. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 19564; (b) Macpherson, L. J.; Dubin, A. E.; Evans, M. J.; Marr, F.; Schultz, P. G.; Cravatt, B. F.; Patapoutian, A. *Nature* **2007**, *445*, 541.