

TRIFLUOROMETHYL KETONE INHIBITORS OF FATTY ACID AMIDE HYDROLASE: A PROBE OF STRUCTURAL AND CONFORMATIONAL FEATURES CONTRIBUTING TO INHIBITION

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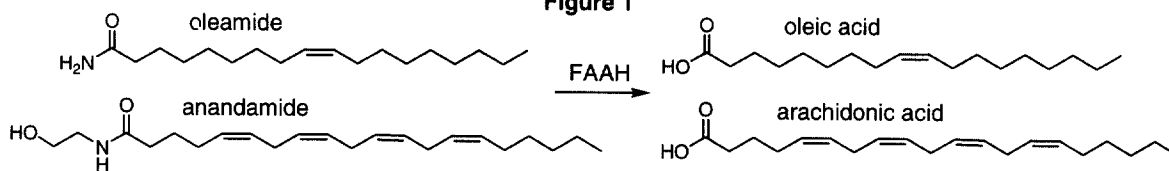
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Abstract. The examination of a series of trifluoromethyl ketone inhibitors of Fatty Acid Amide Hydrolase (FAAH, oleamide hydrolase, anandamide amidohydrolase) is detailed in efforts that define structural and conformational properties that contribute to enzyme inhibition and substrate binding. The results imply an extended bound conformation, highlight a role for the presence, position, and stereochemistry of a $\Delta^{9,10}$ *cis* double bond, and suggest little apparent role for C11–C18/C22 of the fatty acid amide substrates. © 1999 Elsevier Science Ltd. All rights reserved.

Oleamide,^{1,2} the prototypical member^{1–4} of a growing class of endogenous fatty acid primary amides,⁵ was found to accumulate in the cerebrospinal fluid under conditions of sleep deprivation^{2,6,7} and induce physiological sleep in animals.^{1,2} In a structurally specific manner, oleamide modulates the serotonergic system,^{8–10} benzodiazepine-sensitive GABA_A receptors,¹¹ blocks glial gap junction communication,^{12,13} and exhibits the characteristic in vivo analgesic and cannabinoid effects of anandamide in mice¹⁴ albeit without cannabinoid receptor binding. Anandamide^{3,4} binds to both the central CB1 and peripheral CB2 cannabinoid receptors^{3,4} through which it is thought to exhibit its analgesic and cannabinoid effects, blocks glial gap junction communication,^{12,13,15} differentially modulates the serotonergic system,^{10,16,17} and exhibits a number of additional in vitro and in vivo biological properties.³ Additional fatty acid primary amides beyond oleamide have been reported to possess biological properties including erucamide,¹⁸ an angiogenic factor stimulating new blood vessel formation and growth, and the biological role of fatty acid ethanolamides beyond anandamide have been studied.¹⁹ Notably, palmitoyl ethanolamide has been shown to bind selectively over anandamide to the peripheral CB2 cannabinoid receptor²⁰ indicating that perhaps the CB1 and CB2 receptors recognize distinct fatty acid amides as their respective endogenous ligands.²¹

Figure 1



Fatty acid amide hydrolase (FAAH, oleamide hydrolase, anandamide amidohydrolase) is an integral membrane protein that degrades and inactivates both fatty acid primary amides and ethanolamides including oleamide^{1,2,22,23} and anandamide (Figure 1).^{22–29} The distribution of FAAH in the CNS suggests that it is posed to degrade the neuromodulating fatty acid amides at their sites of action and therefore intimately related to the regulation of the effects evoked.³⁰ In addition, FAAH has also been shown to hydrolyze the related CB1 agonist 2-arachidonylglycerol fourfold faster than anandamide, as well as the related 1-arachidonylglycerol and 1-oleylglycerol, and the corresponding simple ester, methyl arachidonate^{31–33} illustrating its plurality of fatty acid amide or ester substrates.

Despite this emerging role of FAAH, only a select set of inhibitors of the enzyme have been disclosed to date.^{34–39} This includes the discovery of the endogenous potent inhibitor 2-octyl γ -bromoacetoacetate,³⁴ which had been previously disclosed as an endogenous sleep-inducing compound.³⁵ In addition to the examination of this inhibitor and its elaboration into more potent FAAH inhibitors,³⁴ the remaining classes are composed of reversible electrophilic carbonyl inhibitors^{36,37} (trifluoromethyl ketones, α -halo ketones, α -keto esters and amides, and aldehydes) or irreversible inhibitors^{2,38,39} (sulfonyl fluorides, fluorophosphonates) incorporated into the fatty acid structures. Herein, we detail the examination of a series of trifluoromethyl ketone inhibitors of FAAH representative of the class of electrophilic carbonyl inhibitors that explore the structural and conformational properties contributing to enzyme inhibition.

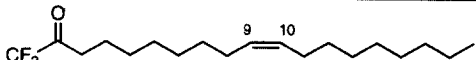
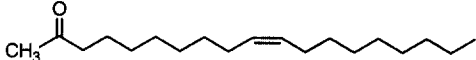
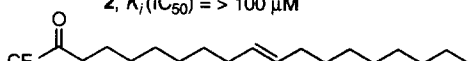
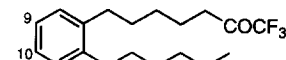
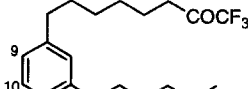
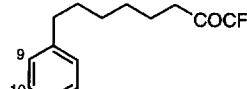
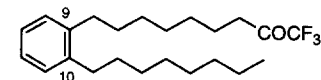
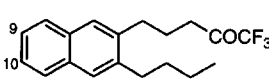
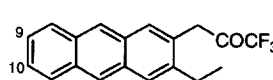
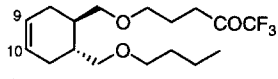
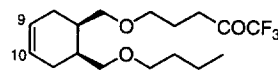
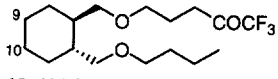
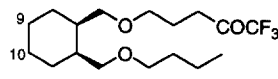
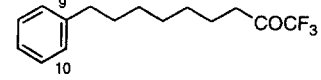
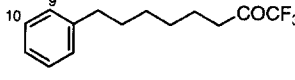
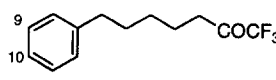
Inhibitor Synthesis. The trifluoromethyl ketones⁴⁰ **1–6** and **11–23** were prepared in a single operation from the corresponding carboxylic acids⁴¹ by first conversion to the respective acid chloride (3 equiv (COCl)₂, CH₂Cl₂, 25 °C, 3 h) and subsequent treatment with TFAA–pyridine⁴² (6 equiv/8 equiv, Et₂O, 1–2 h, 41–82%).⁴³ The remaining trifluoromethyl ketones **7–10** were synthesized from the corresponding ethyl esters by treatment with LDA and ethyl trifluoroacetate (1.5 equiv/2 equiv, THF, –78 to 25 °C, 26 h)⁴⁴ followed by refluxing 40% aq H₂SO₄ (94 h).

Inhibition Studies.⁴⁵ All enzyme assays were performed at 20–23 °C using a solubilized liver plasma membrane extract³⁷ in a reaction buffer of 125 mM Tris, 1 mM EDTA, 0.2% glycerol, 0.02% Triton X-100, 0.4 mM HEPES, pH 9.0 buffer.³⁴ The hydrolysis was monitored by following the breakdown of ¹⁴C-oleamide to oleic acid after extraction and separation of the TLC, and phosphorimaging as previously described.^{2,34} Linear least squares fits were used for all reaction progress curves and R² values were consistently >0.97. IC₅₀ values were determined from the inhibition observed at 3–4 different concentrations of inhibitor (from three or more independent trials at each inhibitor concentration) using the formula $IC_{50} = [I]/[v_o/v_i - 1]$,⁴⁶ where v_o is the control reaction rate without inhibitor and v_i is the rate with inhibitor at concentration [I]. K_i values were determined by the Dixon method (x-intercepts of weighted linear fits of [I] versus 1/rate plots at constant substrate concentration, which were converted to K_i values using the formula $K_i = -x_{int}/[1 + [S]/K_m]$) and the results are summarized in Table 1.

The relative abundance of the enzyme in rat liver allowed for a more facile preparation of FAAH than from

brain tissue. Previous work²² has demonstrated that the identical enzyme is isolated from both sources and that it is the single enzyme responsible for oleamide or anandamide hydrolysis. In addition, the rat and human enzyme²³ are very homologous (84%), exhibit near identical substrate specificities, and incorporate an identical amidase consensus sequence and SH3 binding domain suggesting the observations made with rat FAAH will be analogous to those of human FAAH.

Table 1

		R	K_i (IC_{50})
 1 , K_i (IC_{50}) = 0.082 μ M (0.46 μ M)	 2 , K_i (IC_{50}) = > 100 μ M	 3 , K_i (IC_{50}) = 0.17 μ M (0.78 μ M)	4 , (CH ₂) ₁₆ CH ₃ 0.24 μ M (1.2 μ M)
			5 , (CH ₂) ₁₂ CH ₃ 0.49 μ M (2.3 μ M)
			6 , (CH ₂) ₁₀ CH ₃ 0.14 μ M (0.72 μ M)
			7 , (CH ₂) ₈ CH ₃ 0.13 μ M (0.60 μ M)
			8 , (CH ₂) ₆ CH ₃ 1.2 μ M (6.6 μ M)
			9 , (CH ₂) ₅ CH ₃ 10.7 μ M (52.8 μ M)
			10 , (CH ₂) ₄ CH ₃ 26.9 μ M (185.2 μ M)
<hr/>			
 11 , K_i (IC_{50}) = 2.8 μ M (13.1 μ M)	 12 , K_i (IC_{50}) = 0.14 μ M (0.76 μ M)	 13 , K_i (IC_{50}) = 0.096 μ M (0.59 μ M)	
 14 , K_i (IC_{50}) = 0.32 μ M (2.5 μ M)	 15 , K_i (IC_{50}) = 4.4 μ M (30.1 μ M)	 16 , K_i (IC_{50}) = > 100 μ M	
 17 , K_i (IC_{50}) = 0.44 μ M (2.4 μ M)	 19 , K_i (IC_{50}) = 0.57 μ M (3.0 μ M)		
 18 , K_i (IC_{50}) = 1.2 μ M (9.7 μ M)	 20 , K_i (IC_{50}) = 0.78 μ M (5.7 μ M)		
 21 , K_i (IC_{50}) = 0.025 μ M (0.12 μ M)	 22 , K_i (IC_{50}) = 0.10 μ M (0.45 μ M)	 23 , K_i (IC_{50}) = 0.17 μ M (0.84 μ M)	

Discussion. The inhibitory potency of the simple straight chain aliphatic trifluoromethyl ketones followed a well-defined behavior. FAAH inhibition required the electrophilic carbonyl (**1** versus **2**), the incorporation of the oleamide $\Delta^{9,10}$ *cis* double bond with **1** versus the saturated trifluoromethyl ketone **4** increased the potency threefold, and its replacement with a *trans* double bond in **3** resulted in an analogous twofold reduction in potency in

agreement with prior observations.³⁷ Progressive shortening of the saturated aliphatic chain had little effect for the saturated C18–C10 trifluoromethyl ketones and all were within a fourfold range of potency with the C12 and C10 inhibitors being the most potent. However, further shortening of the saturated aliphatic chain below C10 led to a more pronounced and progressive loss of inhibitory potency. Notably, this progressive loss of potency starts once the inhibitor length is equal to or shorter than C8 which corresponds to the positional length of the oleamide $\Delta^{9,10}$ *cis* double bond.

Clear from the trends in examining **11–13**, the more extended conformation embodied in **13** was found to be essentially equipotent with **1** and **4**, and the inhibitor potency smoothly declined as the structures progressively approach the bent or hairpin conformation (**13** > **12** > **11** and **14**). Additional restrictions to the hairpin conformation resulted in a further progressive loss of activity (**11** > **15** > **16**). An analogous drop in inhibitor potency was observed with **17** and **19**, which also embody conformational characteristics of the hairpin conformation. The *trans* and *cis* isomers were essentially equipotent and both exhibited properties intermediate of those observed with **11** and **14**. Removal of the double bond from both **17** and **19** with **18** and **20** resulted in a small 1.5 to 3-fold decrease in potency analogous to that observed with **1** versus **4**.

Consistent with these observations, the examination of **21–23** and their comparison with **11–14** confirmed that the C11–C18 tail does not contribute productively to the inhibitor potency, that its incorporation into the restricted hairpin conformations (**21** versus **14**, **23** versus **11**) results in a tenfold reduction in inhibitor potency, and that its incorporation into the restricted bent conformation (**22** versus **12**) or restricted extended conformation (**22** versus **13**) has essentially no impact on the inhibitor potency. Notably, **21**, which lacks the C11–C18 tail, is the most potent trifluoromethyl ketone inhibitor of FAAH in the series being slightly more than threefold more potent than the oleyl trifluoromethyl ketone (**1**) itself.

Conclusions. Together these results imply a preferred extended bound conformation, highlight a role for the presence, position, and stereochemistry of the $\Delta^{9,10}$ *cis* double bond of oleamide, and suggest little apparent role for C11–C18/C22 of the fatty acid amide substrates. These observations, which define some of the more fundamental structural and conformational features contributing to active site binding, should prove useful as the continued design of potent FAAH inhibitors and serve to define gross overall features of the active site shape and size.

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References and Notes

1. Review: Boger, D. L.; Henriksen, S. J.; Cravatt, B. F. *Curr. Pharm. Design.* **1998**, *4*, 303.

2. Cravatt, B. F.; Prospero-Garcia, O.; Siuzdak, G.; Gilula, N. B.; Henriksen, S. J.; Boger, D. L.; Lerner, R. A. *Science* **1995**, *268*, 1506.
3. Review: Di Marzo, V.; Fontana, A. *Prostaglandins, Leukotrienes, Essen. Fatty Acids* **1995**, *53*, 1.
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. Arafat, E. S.; Trimble, J. W.; Andersen, R. N.; Dass, C.; Desiderio, D. M. *Life Sci.* **1989**, *45*, 1679.
6. Lerner, R. A.; Siuzdak, G.; Prospero-Garcia, O.; Henriksen, S. J.; Boger, D. L.; Cravatt, B. F. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 9505.
7. Cravatt, B. F.; Lerner, R. A.; Boger, D. L. *J. Am. Chem. Soc.* **1996**, *118*, 580.
8. Huidobro-Toro, J. P.; Harris, R. A. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 8078.
9. Thomas, E. A.; Carson, M. J.; Neal, M. J.; Sutcliffe, J. G. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 14115.
10. Boger, D. L.; Patterson, J. E.; Jin, Q. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 4102.
11. Yost, C. S.; Hampson, A. J.; Leonoudakis, D.; Koblin, D. D.; Bornheim, L. M.; Gray, A. T. *Anesth. Analg.* **1998**, *86*, 1294.
12. Guan, X.; Cravatt, B. F.; Ehring, G. R.; Hall, J. E.; Boger, D. L.; Lerner, R. A.; Gilula, N. B. *J. Cell Biol.* **1997**, *139*, 1785.
13. Boger, D. L.; Patterson, J. E.; Guan, X.; Cravatt, B. F.; Lerner, R. A.; Gilula, N. B. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 4810.
14. Mechoulam, R.; Frider, E.; Hanus, L.; Sheskin, T.; Bisogno, T.; Di Marzo, V.; Bayewitch, M.; Vogel, Z. *Nature* **1997**, *389*, 25.
15. Di Marzo, V.; Fontana, A.; Cadas, H.; Schinelli, S.; Cimino, G.; Schwartz, J.-C.; Piomelli, D. *Nature* **1994**, *372*, 686.
16. Fan, P. J. *Neurophysiol.* **1995**, *73*, 907.
17. Kimura, T.; Ohta, T.; Watanabe, K.; Yoshimura, H.; Yamamoto, I. *Biol. Pharm. Bull.* **1998**, *21*, 224.
18. Wakamatsu, K.; Masaki, T.; Itoh, F.; Kondo, K.; Sudo, K. *Biochem. Biophys. Res. Commun.* **1990**, *168*, 423.
19. Review: Schmid, H. H. O.; Schmid, P. C.; Natarajan, V. *Prog. Lipid Res.* **1990**, *29*, 1.
20. Barg, J.; Frider, E.; Hanus, L.; Levy, R.; Matus-Leibovitch, N.; Heldman, E.; Bayewitch, M.; Mechoulam, R.; Vogel, Z. *Eur. J. Pharmacol.* **1995**, *287*, 145.
21. Skaper, S. D.; Burianni, A.; Dal Toso, R.; Petrelli, L.; Romanello, S.; Facci, L.; Leon, A. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 3984.
22. Cravatt, B. F.; Giang, D. K.; Mayfield, S. P.; Boger, D. L.; Lerner, R. A.; Gilula, N. B. *Nature* **1996**, *384*, 83.
23. Giang, D. K.; Cravatt, B. F. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 2238.
24. Deutsch, D. G.; Chin, S. A. *Biochem. Pharmacol.* **1993**, *46*, 791.
25. Desarnaud, F.; Cadas, H.; Piomelli, D. *J. Biol. Chem.* **1995**, *270*, 6030.
26. Hillard, C. J.; Wilkison, D. M.; Edgmond, W. S.; Campbell, W. B. *Biochim. Biophys. Acta* **1995**, *1257*, 249.
27. Ueda, N.; Kurahashi, Y.; Yamamoto, S.; Tokunaga, T. *J. Biol. Chem.* **1995**, *270*, 23823.
28. Omeir, R. L.; Chin, S.; Hong, Y.; Ahern, D. G.; Deutsch, D. G. *Life Sci.* **1995**, *56*, 1999.
29. Maurelli, S.; Bisogno, T.; De Petrocellis, L.; Di Luccia, A.; Marino, G.; Di Marzo, V. *FEBS Lett.* **1995**, *377*, 82.
30. Thomas, E. A.; Cravatt, B. F.; Danielson, P. E.; Gilula, N. B.; Sutcliffe, J. G. *J. Neurosci. Res.* **1997**, *50*, 1047. See also refs 25 and 26.
31. Goparaju, S. K.; Ueda, N.; Yamaguchi, H.; Yamamoto, S. *FEBS Lett.* **1998**, *442*, 69.
32. Kurahashi, Y.; Ueda, N.; Suzuki, H.; Suzuki, M.; Yamamoto, S. *Biochem. Biophys. Res. Commun.* **1997**, *237*, 512.
33. Bisogno, T.; Sepe, N.; Melck, D.; Maurelli, S.; De Petrocellis, L.; Di Marzo, V. *Biochem. J.* **1997**, *322*, 671.
34. Di Marzo, V.; Bisogno, T.; Sugiura, T.; Melck, D.; De Petrocellis, L. *Biochem. J.* **1998**, *331*, 15.
35. Patricelli, M. P.; Patterson, J. E.; Boger, D. L.; Cravatt, B. F. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 613.
35. Torii, S.; Mitsumori, K.; Inubushi, S.; Yanagisawa, I. *Psychopharmacologia* **1973**, *29*, 65.

36. Koutek, B.; Prestwich, G. D.; Howlett, A. C.; Chin, S. A.; Salehani, D.; Akhavan, N.; Deutsch, D. G. *J. Biol. Chem.* **1994**, *269*, 22937.
37. Patterson, J. E.; Ollmann, I. R.; Cravatt, B. F.; Boger, D. L.; Wong, C.-H.; Lerner, R. A. *J. Am. Chem. Soc.* **1996**, *118*, 5938.
38. Deutsch, D. G.; Omeir, R.; Arreaza, G.; Salehani, D.; Prestwich, G. D.; Huang, Z.; Howlett, A. *Biochem. Pharmacol.* **1997**, *53*, 255. Deutsch, D. G.; Lin, S.; Hill, W. A. G.; Morse, K. L.; Salehani, D.; Arreaza, G.; Omeir, R. L.; Makriyannis, A. *Biochem. Biophys. Res. Commun.* **1997**, *231*, 217.
39. De Petrocellis, L.; Melck, D.; Ueda, N.; Maurelli, S.; Kurahashi, Y.; Yamamoto, S.; Marino, G.; Di Marzo, V. *Biochem. Biophys. Res. Commun.* **1997**, *231*, 82.
40. The trifluoromethylketones **1**, **3–4**^{36,37} have been disclosed previously and have been included for direct comparison under the present assay conditions.
41. For the carboxylic acids leading to **11–14**, **17**, and **19**: Patterson, J. E., Ph.D. thesis, The Scripps Research Institute, La Jolla, CA 1997; *Chem. Abstr.* **1998**, *129*, 90549v. The carboxylic acids leading to **15** and **16** were prepared by analogous procedures and **18** and **20** were prepared by hydrogenation (H_2 , Pd–C, CH_3OH , 23 h, 25 °C) of **17** and **19**. The carboxylic acids leading to **21–23** are commercially available.
42. Boivin, J.; El Kaim, L.; Zard, S. Z. *Tetrahedron Lett.* **1992**, *33*, 1285.
43. For **1** and **3** (ref. 37); for **2** (Besterman, H. J.; Stansky, W.; Vostrowsky, O.; Range, P. *Chem. Ber.* **1975**, *108*, 3582; for **4–7** (Parrilla, A.; Villuendas I.; Guerrero, A. *Bioorg. Med. Chem.* **1994**, *2*, 243); for **8** (Begue, J.-P.; Bonnet-Delpon, D.; Mesureur, D.; Nee, G.; Wu, S.-W. *J. Org. Chem.* **1992**, *57*, 3807); for **9** (Dishart, K. T.; Levine, R. *J. Am. Chem. Soc.* **1956**, *78*, 2268); for **10** (Barkley, L. B.; Levine, R. *J. Am. Chem. Soc.* **1953**, *75*, 2059). Full characterization of **11–23** can be supplied upon request.
44. Linderman, R. J.; Graves, D. M. *J. Org. Chem.* **1989**, *54*, 661.
45. Rat liver plasma membranes were isolated from 15 rat livers as described,³⁷ suspended in 80 mL of buffer (10% glycerol, 1% Triton X-100, 1 mM EDTA, 20 mM HEPES, pH 7.2) and dounce homogenized. The membrane solution was stirred at 4 °C for 2 h and then centrifuged at 27,000 rpm for 1 h (SW-28 rotor, Beckmann). The supernatant from this spin constitutes the solubilized liver plasma membranes. ^{14}C -oleamide (51 $\mu Ci/\mu M$) was diluted to 5 mM in EtOH and 4 μL was added to a vial. Subsequently, 5 μL of inhibitor (at 40-fold the desired concentration) was added in either DMSO or EtOH, followed by 91 μL of reaction buffer (125 mM Tris, 1 mM EDTA, pH 9.0). Care was taken not to allow the EtOH to evaporate prior to buffer addition as this facilitated dissolution of oleamide. A solution of 4 μL of the liver plasma membrane preparation in 96 μL of reaction buffer was added and the reaction mixtures were incubated at 20–23 °C. After 5, 10, and 15 min, 50 μL of the reaction mixture was removed and quenched in 600 μL of 0.07 N HCl, extracted with 1 mL EtOAc, and the organic layer was evaporated with a stream of N_2 . The products were resuspended in 8 μL EtOH, spotted on thin layer chromatography plates and developed in 50% EtOAc–hexanes. The developed plates were placed on a phosphorimaging film (2–12 h) and the conversion of oleamide to oleic acid was determined via a phosphorimager (Packard brand phosphorimager, model A431201).
46. Conde-Frieboes, K.; Reynolds, L. J.; Lio, Y.-C.; Hale, M. R.; Wasserman, H. H.; Dennis, E. A. *J. Am. Chem. Soc.* **1996**, *118*, 5519.