Esters, Retroesters, and a Retroamide of Palmitic Acid: Pool for the First Selective Inhibitors of *N*-Palmitoylethanolamine-Selective Acid Amidase

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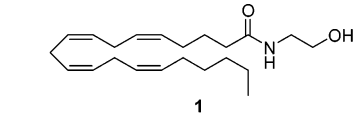
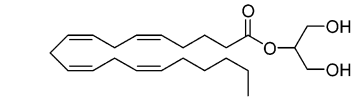
Abstract: Cyclohexyl hexadecanoate, hexadecyl propionate, and *N*-(3-hydroxypropionyl)pentadecanamide, respectively ester, retroester, and retroamide derivatives of *N*-palmitoylethanolamine, represent the first selective inhibitors of "*N*-palmitoylethanolamine hydrolase" described so far. These compounds are devoid of affinity for CB_1 and CB_2 receptors and characterized by high percentages of inhibition of *N*-palmitoylethanolamine-selective acid amidase (84.0, 70.5, and 76.7% inhibition at 100 μM, respectively) with much lower inhibitory effect on either fatty acid amide hydrolase or the uptake of anandamide.

Introduction. Endogenous fatty acid amides and esters are the subject of growing interest in pharmacology since the discovery of anandamide (Chart 1), the ethanolamide of arachidonic acid that has been proposed as an endogenous ligand of cannabinoid receptors.[1] Anandamide displays, as a result of its actions upon cannabinoid and vanilloid receptors, a number of interesting pharmacological properties including effects on nociception, memory processes, lung function, spasticity, appetite, and cell proliferation.[2] Among the endocannabinoids (i.e., the endogenous agonists of cannabinoid receptors), several polyunsaturated fatty acid ethanolamides have been described but also esters such as 2-arachidonoylglycerol (Chart 1). All these molecules are rapidly inactivated by uptake into cells followed by enzymatic hydrolysis. In the case of the polyunsaturated fatty acid ethanolamides, the enzyme responsible is fatty acid amide hydrolase (FAAH).[3] This key hydrolase is abundant in the central nervous system, the liver, and the kidney. FAAH activity is characterized by an optimal pH value at 8.5−10[4,5] and by high sensitivity to phenylmethylsulfonyl fluoride (PMSF)[6] and methyl arachidonoyl fluorophosphonate (MAFP).[7,8] In contrast to soluble hydrolases of the same family, FAAH is able to integrate into cell membranes and establish direct access to the bilayer membrane from its active site. Its 2.8 Å crystal structure was recently reported.[9]

Chart 1. Structures of Anandamide (**1**), 2-Arachidonoylglycerol (**2**), *N*-Oleoylethanolamine (**3**), and *N*-Palmitoylethanolamine (**4**)



1



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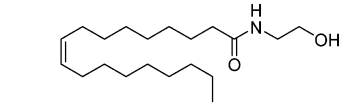

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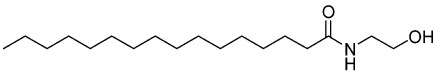

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Other related endogenous lipid derivatives such as oleamide, *N*-oleoylethanolamine (Chart 1), and *N*-palmitoylethanolamine (PEA, Chart 1) induce pharmacological responses[10] albeit without significant binding either to cannabinoid receptors[11] or to vanilloid receptors. Oleamide induces physiological sleep in animals, analgesia, and cannabinoid-like behavioral responses. *N*-Palmitoylethanolamine exhibits antiinflammatory and analgesic effects, both suppressed by the administration of the CB_2 cannabinoid receptor antagonist SR144528.[12] *N*-Oleoylethanolamine has been recently proposed as an endogenous anorexic lipid messenger acting peripherally in the control of appetite.[13] These three molecules are also substrates for FAAH,[14,15] although they are hydrolyzed at a lower rate than anandamide.[4,5,16,17]

Recently, another fatty acid amide hydrolyzing enzyme, which is catalytically distinct from the previously reported FAAH, was found in a human megakaryoblastic leukemia cell line (CMK) and rat tissues.[18,19] This enzyme is abundant in the lung, the spleen, and the small intestine, and it is characterized by an optimal pH value at 5. It is also much less sensitive to nonselective compounds such as PMSF and MAFP. Among the *N*-acylethanolamines, PEA is the most active substrate for this enzyme.[19] This enzyme, highly purified but not yet cloned,[19] is termed "*N*-palmitoylethanolamine-selective acid amidase (NPAA)" in this paper.

Characterization of the physiological role of NPAA would greatly be aided by the discovery of selective inhibitors of this enzyme. Analogues of *N*-palmitoylethanolamine, the most active substrate, would be a reasonable starting point. In this report, we describe a comparative study on the inhibitory potentials of some esters, retroesters, and a retroamide of palmitic acid, toward FAAH and NPAA. Affinity of the compounds for the CB_1 and CB_2 cannabinoid receptors was also evaluated by competitive binding experiments. Among the tested compounds, cyclohexyl hexadecanoate, hexadecyl propionate, and *N*-(3-hydroxypropionyl)pentadecana-

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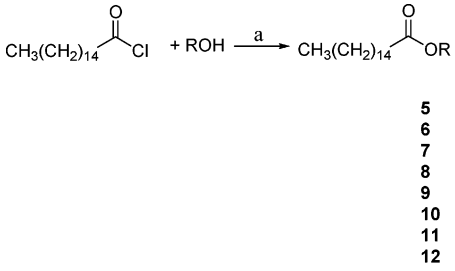
Scheme 1. Syntheses of Esters (**5**−**12**), Retroesters (**13** and **14**), and Retroamide **15**[a]



[a] Reagents: (a) room temperature, 2 h; (b) HCl (cat.), dichloromethane, reflux 4 h (75%); (c) dichloromethane, reflux 3 h (86%); (d) pyridine, mesyl chloride (2 equiv), room temperature 5 h; sodium azide (4 equiv), DMF, 90 °C overnight; (e) diethyl ether, lithium aluminum hydride (1 equiv), reflux 3 h; (f) dichloromethane, trimethylaluminum (1 equiv), β-propiolactone (1 equiv), reflux 6 h.

Table 1. FAAH, NPAA Inhibitory Potentials and Cannabinoid Receptor Recognition of Esters **5**−**12**, Retroesters **13** and **14**, Retroamide **15**, and Amides **4** and **16**−**18** of Palmitic Acid

	CB_1 receptor affinity[a] (% inhibition of specific binding of [^{3}H]-CP55,940)	CB_2 receptor affinity[b] (% inhibition of specific binding of [^{3}H]-Win55,212−2)	FAAH[c] % inhibition		NPAA[d] % inhibition
	10 μM	10 μM	10 μM	100 μM	100 μM
Esters of Palmitic Acid					
5, OCH_3	11 ± 0.3	14 ± 0.3	18 ± 7	69 ± 4	65 ± 4.
6, $OCH(CH_3)_2$	8 ± 0.4	0.5 ± 0.1	<0[e]	<0	44 ± 3
7, $OC(CH_3)_3$	14 ± 0.9	14 ± 0.3	<0[e]	<0	23 ± 3
8, $OCH_2CH{=}CH_2$	2 ± 0.1	1 ± 0.2	16 ± 0.4	36 ± 2	48 ± 0.4
9, OCH_2CH_2Cl	14 ± 0.5	6 ± 0.3	27 ± 4	45 ± 3	55 ± 0.3
10, OCH_2CH_2Br	2 ± 0.1	2 ± 0.1	19 ± 4	31 ± 3	45 ± 0.8
11, *O*-cyclohexyl	14 ± 0.4	6 ± 0.4	6 ± 5	36 ± 4	84 ± 0.1
12, *O*-benzyl	5 ± 0.3	5 ± 0.5	6 ± 2	24 ± 5	50 ± 2
Retroesters					
13, hexadecyl acetate	10 ± 0.2	9 ± 0.1	<0[e]	<0	68 ± 1
14, hexadecyl propionate	12 ± 0.2	2 ± 0.2	<0[e]	<0	71 ± 5
Retroamide					
15, *N*-(3-hydroxypropionyl)-pentadecanamide	4 ± 0.4	3 ± 0.3	<0	8 ± 8	77 ± 1
Amides of Palmitic Acid					
PEA, **4**, $NHCH_2CH_2OH$	24 ± 0.07[f]	14 ± 2[f]	49 ± 7[f]	68 ± 4[f]	34 ± 6
16, $NHCH_3$	3 ± 0.5[f]	8 ± 0.1[f]	35 ± 3[f]	68 ± 2[f]	30 ± 2
17, $NHCH(CH_3)_2$	25 ± 5[f]	12 ± 0.8[f]	30 ± 4[f]	52 ± 4[f]	25 ± 3
18, NH-cyclohexyl	34 ± 2[f]	8 ± 0.2[f]	8 ± 1[f]	11 ± 3[f]	23 ± 7

[a] Affinity for the CB_1 cannabinoid receptor was determined using CHO-hCB_1 transfected cell membranes and 1 nM [^{3}H]-CP55,940. Results are expressed as percentages of inhibition of specific binding of the radioligand. Nonspecific binding has been determined with 10 μM HU-210.[11] In our hands, anandamide has K_i values of 558 ± 16 nM (in the presence of PMSF 50 μM) and 583 ± 26 nM (in absence of PMSF) and 2-arachidonoylglycerol has K_i values of 481 ± 8 nM (in the presence of PMSF 50 μM) and 513 ± 15 nM (in absence of PMSF) on CHO−CB_1 cell membranes. [b] Affinity for the CB_2 cannabinoid receptor was determined using CHO-hCB_2 transfected cell membranes and 1 nM [^{3}H]-WIN55,212−2. Results are expressed as percentages of inhibition of specific binding of the radioligand. Nonspecific binding has been determined with 10 μM HU-210.[11] In our hands, anandamide has K_i values of 1880 ± 6 nM (in the presence of PMSF 50 μM) and 1891 ± 11 nM (in absence of PMSF) and 2-arachidonoylglycerol has K_i values of 1595 ± 34 nM (in the presence of PMSF 50 μM) and 1715 ± 28 nM (in absence of PMSF) on CHO−CB_2 cell membranes. [c] FAAH inhibition studies have been performed as described previously.[21] <0 indicates that the activity remaining was >100% at the concentration tested (e.g., 102 ± 3% and 105 ± 2% for 10 and 100 μM **7**, respectively). In our hands, this assay gives a pI_{50} value for the inhibition of FAAH by AM404 of 5.44 ± 0.02[21]. [d] NPAA enzyme (12 μg of protein) solubilized from the 12 000 x *g* pellet of rat lung homogenates was incubated with 100 μM [^{14}C]-*N*-palmitoylethanolamine (10 000 cpm) and 100 μM of test compounds at 37 °C for 30 min in 100 μL of 50 mM citrate−sodium phosphate (pH 5.0) containing 3 mM DTT and 0.1% Triton X-100. The reaction was terminated by addition of 0.35 mL of a mixture of diethyl ether/methanol/1 M citric acid (30:4:1 vv^{-1}). The ethereal extract was spotted on a Merck silica gel 60 F_{254} aluminum sheet and subjected to TLC with a mixture of chloroform/methanol/28% ammonium hydroxide (80:20:2 vv^{-1}) at 4 °C. Radioactivity on the sheet was scanned with a BAS 1500 bioimaging analyzer.[19] [e] A concentration of 20 μM rather than 10 μM is shown here. [f] These data have been published previously[21] and are included here for comparative purposes.

mide hardly have inhibitory effect on FAAH, but they produce marked inhibition of NPAA.

Results and Discussion. Three sets of analogues of PEA have been prepared (Scheme 1). The first set includes eight esters **5**−**12**, compounds differing by the nature of the alkyl groups. These compounds have been prepared from palmitoyl chloride and corresponding alcohols at room temperature. The second set contains retroesters **13** and **14** which were synthesized from hexadecanol and acetic acid or propionyl chloride, respectively. *N*-(3-Hydroxypropionyl)pentadecanamide christened "retro-PEA" **15** was prepared in three steps. Pentadecanol is treated by mesyl chloride followed by sodium azide in dimethylformamide to give azidopentadecane which is subsequently reduced into the corresponding amine by lithium aluminum hydride. Pentadecylamine reacts with β-propiolactone to give the retroamide **15**. The synthesis of palmitoylethanolamide **4** and reference amides **16**−**18** has been described previously.[11]

The compounds were assessed to evaluate their ability to interact with cannabinoid receptors in radiobinding assays using [^{3}H]-CP55,940 and [^{3}H]-WIN 55,212−2 in CHO cell homogenates either expressing the human CB_1 receptor or the human CB_2 receptor, respectively (results are shown in Table 1; the transfected CHO cells were kindly provided by Euroscreen s.a., Belgium). At the concentration of 10 μM, none of the compounds exhibit a specific radioligand displacement superior to 35% from the CB_1−CHO cell homogenates and to 15% from the CB_2−CHO cell homogenates. The retroamide **15**, compared to PEA, confirms the results obtained for "retro-anandamide"[20] compared to anandamide in the recognition of cannabinoid receptors. The inversion of the amide bond leads to decrease of affinity in both cases. Given that a K_i in the nM range can be considered a prerequisite for cannamimetic activity in vivo, these data indicate that active compounds toward NPAA and/or FAAH are not likely to have direct effects upon CB receptors.

The ability of esters, retroesters, and the retro-PEA to inhibit [^{3}H]-anandamide hydrolysis was investigated at an assay pH value of 7.6 using rat brain homogenates as the source of FAAH (results are shown in Table 1). The data shown in Table 1 are for concentrations of 10 and 100 μM (to allow comparison with the effects toward CB receptors and NPAA, respectively), although in most cases a concentration range from 0.5 to 100 μM was tested. Neither the retroesters **13**, **14** nor the retro-PEA **15** inhibited FAAH**.** The esters **5**, **8**, **9**, **10**, **12**, on the other hand, inhibited FAAH. Analysis of the full concentration range indicated that compound **5**, the methyl ester of palmitate, inhibited FAAH with an IC_{50} value (for the inhibitable component of the activity) of 18 μM (see refs 20, 21 for details of the analysis used). Regardless of the maximum inhibition attained, none of the compounds had IC_{50} values (for the inhibitable component of the activity) lower than that for PEA **4** (5 μM). However, compound **5** has a similar potency to the corresponding methylamide **16** (IC_{50} value 27 μM).

Inhibition of NPAA was evaluated using solubilized proteins from 12000 x *g* pellet of rat lung homogenates, an assay pH value of 5, and [^{14}C]-PEA as substrate (results are shown in Table 1). The amides of palmitic acid, inhibited NPAA to less extent than FAAH. More surprising was the result obtained with "retro-PEA" **15**. This compound was completely devoid of the inhibitory activity against FAAH, but inhibited NPAA. Thus, a single inversion of the amide bond of the endogenous substrate **4** for both enzymes results in a loss of affinity toward FAAH but not NPAA. Among the palmitoyl ester family, most of the compounds are moderate inhibitors of NPAA**.** Cyclohexyl palmitate **11** produced the greatest inhibition (84%). Its corresponding amide **18** is, however, almost completely inactive. This structural comparison raises the importance of the ester bond in the design of inhibitors of NPAA based upon the palmitoyl structure. Hexadecyl acetate **13** and hexadecyl propionate **14**, two representatives of the "retro-ester" family of PEA, also inhibited NPAA without affecting the activity of FAAH. Concentration−inhibition curves have been built for the three most potent inhibitors: **11**, **14**, and **15** exhibit pI_{50} values of 4.72 ± 0.06, 4.27 ± 0.05, and 4.50 ± 0.03, respectively.

The difference between the abilities of compounds **11**, **14**, and **15** to inhibit NPAA and FAAH might be due to differences in the assay conditions used, rather than to a true separation of effects upon NPAA and FAAH. pH and solubility issues may be of importance, since amide analogues of PEA produce more inhibition at pH 9 than at pH 6 (although the IC_{50} values for the inhibitable component of the activity are not affected).[22] Furthermore, the NPAA assays were undertaken in the presence of Triton X-100, whereas this was not the case for FAAH. To rule out these possibilities, the ability of **11**, **14**, and **15** to inhibit FAAH using different pH values and assay buffers was examined (Table 2). In addition, in view of the finding that some PEA analogues can inhibit the cellular uptake of AEA,[22] the potencies of these compounds were also tested in an uptake assay. As seen in Table 2, compounds **14** and **15** showed, at

Table 2. Assay pH and Buffer Dependency of FAAH Inhibition, and Inhibition of Anandamide Uptake into RBL-2H3 Cells by **11**, **14**, and **15**

	compd concn (μM)	% inhibition of FAAH[a]				% inhibition of [^{3}H]-anandamide uptake[b]
		buffer A		buffer B		
		pH 6	pH 9	pH 6	pH 9	
11	10	8 ± 3	5 ± 17	20 ± 11	12 ± 21	5 ± 4
	30	2 ± 3	17 ± 16	34 ± 10	12 ± 16	16 ± 1
	100	37 ± 10	12 ± 13	51 ± 9	46 ± 9	29 ± 0.4
14	10	2 ± 3	26 ± 4	5 ± 9	<0	11 ± 8
	30	<0	21 ± 24	2 ± 11	<0	12 ± 3
	100	<0	4 ± 3	<0	<0	15 ± 0.4
15	10	8 ± 1	20 ± 7	11 ± 2	6 ± 12	8 ± 2
	30	<0	5 ± 13	10 ± 6	<0	13 ± 2
	100	2 ± 0.5	7 ± 5	16 ± 12	<0	28 ± 6

[a] 10 μM [^{3}H]-ethanolamine-labeled anandamide was used as substrate, with 10 and 3 μg protein/assay at pH 6 and 9, respectively. Buffer A: 10 mM Tris HCl + 1 mM EDTA, assays containing fatty acid-free bovine serum albumin; Buffer B: 125 mM Tris, 1 mM EDTA, 0.2% glycerol, 0.02% Triton X-100, 0.4 mM HEPES, no fatty acid-free bovine serum albumin. The pHs were adjusted by addition of either NaOH or HCl. [b] Uptake was assayed essentially as described by Rakhshan et al.,[23] using RBL-2H3 cells (passage number 38), [^{3}H]-arachidonoyl-labeled anandamide (2 μM) and an incubation time with anandamide of 8 min after preincubation with the test compounds. Parallel wells were assayed in the absence of cells, and the difference in anandamide accumulation was defined here as the uptake. As a positive control, 200 μM of AM404 was included in all assays and found to produce 96 ± 0.8% inhibition of uptake.

best, modest effects toward FAAH regardless as to whether Triton X-100 was present in the assay and regardless of the assay pH. There was also little effect of **14** and **15** on the uptake of AEA into RBL-2H3 cells.

In conclusion, this study has demonstrated that the sensitivity of NPAA to inhibition by ester derivatives of PEA, as well as by retroamide and retroester compounds, is different from that for FAAH. Such compounds may be very useful in delineating the physiological role played by this enzyme.

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Supporting Information Available: Spectroscopic data (TLC, mp, ^{1}H NMR, ^{13}C NMR, and mass spectrometry) for compounds **5**−**15**. This material is available free of charge via the Internet at http://pubs.acs.org.

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