Two New Unsaturated Fatty Acid Ethanolamides in Brain That Bind to the Cannabinoid Receptor

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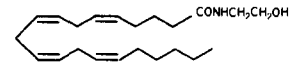
Arachidonic acid ethanolamide (anandamide) (1) was recently identified by us as a brain constituent that binds to the cannabinoid receptor and produces a concentration-dependent inhibition of the electrically evoked twitch response of the mouse vas deferens.[1] We have also shown that anandamide produces many of the pharmacological effects caused by Δ^9-tetrahydrocannabinol (Δ^9-THC) in mice, such as hypothermia, analgesia, and reduced activity in an immobility and in an open-field test.[2] Vogel et al. have shown that anandamide parallels Δ^9-THC in its specific interaction with the cannabinoid receptor and on inhibition of adenylate cyclase.[3] These observations indicate that anandamide is an endogenous ligand-agonist that may serve as a genuine mediator for the cannabinoid receptor.

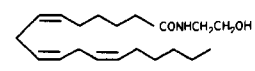
Many of the mediators in the body, in particular those derived from arachidonic acid, such as the prostaglandins and the leukotrienes, are present not as single entities but as large families of chemically related substances. It seemed reasonable to expect that anandamide may also be only the first identified representative of a class of unsaturated fatty acid-derived ethanolamides binding to the cannabinoid receptor. In view of the known existence in brain of the saturated palmitoylethanolamide (2)[4] (which however does not bind to the cannabinoid receptor in concentrations up to 1 μM)[1] and other acylethanolamines,[4,5] we expected that additional *polyunsaturated* fatty acids ethanolamides may be present in the brain. Indeed, in our original isolation of anandamide we noted that several chromatographic fractions, which did not contain anandamide, gave positive results in the ligand-binding assay. We report now the identification in porcine brain, the structural elucidation, and the synthesis of two novel fatty acid ethanolamides which bind to the cannabinoid receptor.

The classical approach for the isolation of active constituents from natural sources is based on chromatographic separation monitored by a suitable *in vivo* or *in vitro* test. This approach was indeed followed by us in the isolation of anandamide. However, as the large quantities and variety of lipids in the brain make the separation of individual lipid components a very tedious undertaking, we decided to approach the problem through the synthesis of specific unsaturated fatty acid ethanolamides which, on the basis of their biogenetic relationship to anandamide,[6] could be expected to exist in the brain. We could then look for their presence in fractions of porcine brain by thin layer chromatographic (TLC) and by gas chromatographic–mass spectrometric (GC–MS) comparisons with the synthetic compounds.

We prepared linoleylethanolamide (3), linolenylethanolamide (4), homo-γ-linolenylethanolamide (5), and 7,10,13,16-docosatetraenylethanolamide (6). Their synthesis followed the procedure described by us for anandamide (1), namely conversion of the respective acid into its acid chloride by reaction with oxalyl chloride, followed by addition of the chloride to excess ethanolamine.[1] As only the ethanolamides 5 and 6 were found to be present in brain (see below) only these two compounds are discussed. Both 5 and 6 are oily substances. Their NMR spectra[7] are very similar and resemble that recorded for anandamide,[1] except that in 5 only six vinyl protons are observed; in 6, eight such protons appear. There are four doubly allylic protons in 5 and six in 6. Anandamide (1) was compared to the ethanolamides 5 and 6 in both regular and reverse-phase TLC systems.[8] Anandamide (1) had a slightly higher R_f value than 5 and 6 in both systems described in ref 8.



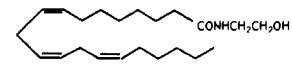
anandamide (1)



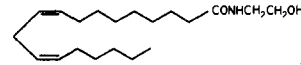
linolenyl ethanolamide (4)

$CH_3(CH_2)_{14}CONHCH_2CH_2OH$

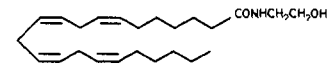
palmityl ethanolamide (2)



homo-γ-linolenylethanolamide (5)



linoleyl ethanolamide (3)



docosatetraenylethanolamide (6)

Anandamide (1), as well as the synthetic ethanolamides 5 and 6, when injected separately into the GC–MS,[9] form pairs of chromatographic peaks due to the original material and its dehydrated product. The latter is presumably a 2-oxazoline derivative.[1,10] The intensity ratio of the pair peaks varies, depending on the injection temperature, the injection volume, and the quantity of material injected. Under the specified GC conditions, the retention times for pure anandamide (1), homo-γ-linolenylethanolamide (5) and docosatetraenylethanolamide (6) were 7.30, 7.44, and 8.42 min, respectively. The dehydrated forms of these ethanolamide compounds were also completely separated, with retention times of 5.58, 6.04, and 6.49 min, respectively.

The EI spectra of dehydrated 5 and 6 shown in Figures 1 and 2 are quite similar to the published spectrum of dehydrated anandamide. In addition to the m/z $[M - H_2O]^+$ peaks observed at 331 and 357, respectively, of particular interest are the typical peaks at m/z 85 (McLaferty rearrangement ion) and m/z 98 (product of a γ-cleavage). Contrary to anandamide, an m/z 112 ion is formed due to δ-cleavage which is possible in 5 and 6, in which the first double bond is in position 8 and 7, respectively, but not in anandamide in which it is in position 5. In addition, the EI spectra demonstrate sequential fragmentation with m/z differences of 14 and 26 units representing gradual cleavage of $-CH_2-$ and $-CH{=}CH-$ (see Figures 1 and 2). Figures 3 and 4 depict the EI spectra of 5 and 6 (nondehydrated form). In

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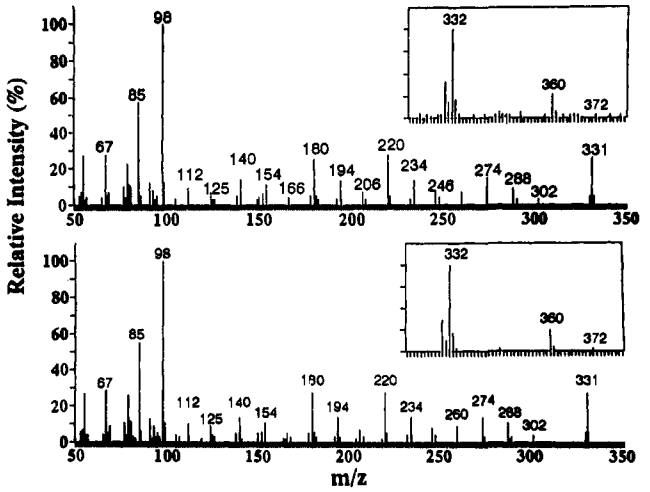

Figure 1. EI GC–MS spectra of dehydrated homo-γ-linolenylethanolamide (**5**). Top: identified in brain. Bottom: synthetic. Inserts: CI GC–MS spectra of these compounds. For details, see ref 9.

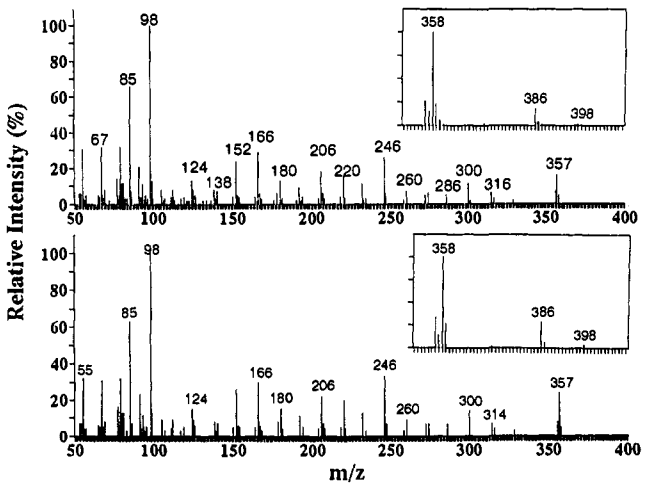

Figure 2. EI GC–MS spectra of dehydrated docosatetraenylethanolamide (**6**). Top: identified in brain. Bottom: synthetic. Inserts: CI GC–MS spectra of these compounds. For details, see ref 9.

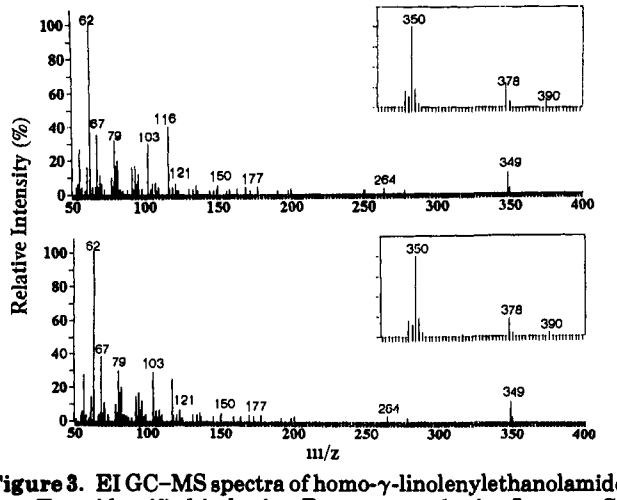

Figure 3. EI GC–MS spectra of homo-γ-linolenylethanolamide (**5**). Top: identified in brain. Bottom: synthetic. Inserts: CI GC–MS spectra.

addition to the molecular ions at m/z 349 and 375, respectively, the most prominent peak (m/z 62) corresponding to protonated ethanolamine was observed in both spectra.

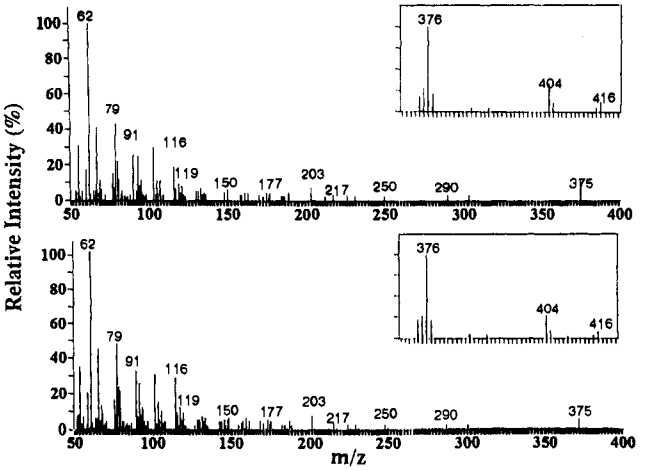

Figure 4. EI GC–MS spectra of docosatetraenylethanolamide (**6**). Top: identified in brain. Bottom: synthetic. Inserts: CI GC–MS spectra.

The corresponding CI spectra are presented as inserts in Figures 1–4. In addition to the $[M + 1]^+$ fragments at m/z 350 and 376 for the parent compounds **5** and **6** (Figures 3 and 4) and 332 and 358 for the dehydrated forms, (Figures 1, 2), $[M + 29]^+$ and $[M + 41]^+$ fragments were also observed that are due to the use of methane as a reactant gas.

Porcine brains were first extracted with methanol, and acetone was then added to the concentrated extract. Largely inactive materials precipitated. The solution was evaporated and partitioned between water and chloroform. Most of the activity (as measured by binding to the cannabinoid receptor) was found in the chloroform layer. Chromatography on a silica gel column led to active fractions eluted with chloroform/petroleum ether/methanol (80:12:1). All of these fractions, on TLC,[8] showed a prominent spot, with an R_f identical to or slightly smaller than that of anandamide. In several fractions this spot was essentially the only one observed with only traces of additional spots. In view of the lability of anandamide, and hence presumably that of related compounds, we refrained from additional column chromatographic separations but took the combined active fractions, which appeared as close spots on TLC slightly below that of anandamide, directly for comparative GC–MS analysis. In order to prevent contamination with the synthetic materials, a new GC column was employed and the synthetic materials **5** and **6** were injected *after* the GC–MS analysis of the brain extracts had been completed. The GC–MS of the mixture of **5** and **6** from brain exhibited the four chromatographic peaks corresponding to the parent and the dehydrated forms. The GC–MS analysis of each of these peaks (EI and CI spectra) showed complete identity with the respective peaks derived from the synthetic standard pure compounds regarding retention time (not shown), molecular peaks, and fragmentation pattern of their mass spectra (Figures 1–4).

Both ethanolamides **5** and **6** displace a radiolabeled cannabinoid probe in a centrifugation-based ligand binding assay which has been described in detail in previous publications.[1,11] The probe [^{3}H]HU-243 (11-hydroxyhexahydrocannabinol-3-yldimethylheptyl homolog) has a dissociation constant of 45 pM in rat synaptosomal membranes.[1,11] Anandamide (**1**), homo-γ-linolenylethanolamide (**5**), and docosatetraenylethanolamide (**6**) inhibited the specific binding of [^{3}H]HU-243 to synapto-

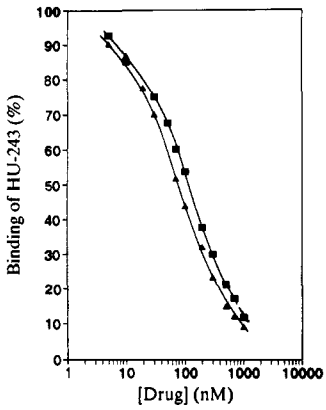

Figure 5. Competitive inhibition of [^{3}H]HU-243 binding by homo-γ-linolenylethanolamide (**5**) (squares) and docosatetraenylethanolamide (**6**) (triangles).

somal membranes in a manner typical of competitive ligands with inhibition constants (K_i values) (± standard error of mean, n = 3) of 52 ± 1.8, 53.4 ± 5.5, and 34.4 ± 3.2 nM, respectively (see Figure 5 for compounds **5** and **6**). In this system, the K_i of Δ^9-THC was 46 ± 3 nM.

The identification of homo-γ-linolenylethanolamide (**5**) and 7,10,13,16-docosatetraenylethanolamide (**6**) as constituents of porcine brain that bind to the cannabinoid receptor demonstrates that anandamide is not the sole representative of this class of potential mediators. So far we have identified three representatives, all of which are derivatives of the n-6 type of fatty acids.[6] Presumably derivatives of fatty acids of the n-3 type may also be present. Whether all unsaturated fatty acids ethanolamides that bind to the cannabinoid receptor exhibit biological activities of the same type or differ, as is the case with the prostaglandins and leukotrienes, remains to be determined.[12]

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Supplementary Material Available: Details of the GC–MS instrumentation and conditions used, TLC comparisons, synthesis, identification of homo-γ-linolenylethanolamide (**5**) and docosatetraenylethanolamide (**6**) in porcine brain and receptor binding (5 pages). Ordering information is given on any current masthead page.

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(6) Agranoff, B. W. In *Basic Neurochemistry*; Seigel, G., Agranoff, B., Albers, R. W., Molinoff, P., Eds.; Raven Press: New York, 1989; pp 91–107.
(7) The NMR spectra (in $CDCl_3$) were obtained on a Varian VXR 300s spectrometer. The chemical shifts are presented in parts per million.
(8) TLC: *Regular phase* (Kieselgel 60 F254, Merck), elution with chloroform/petroleum ether/methanol (40:6:4); the R_f's of 1, 5, and 6 are 0.36, 0.34, 0.34; *reverse phase* (RP-18, F254's Merck), elution (twice) with methanol/dichloromethane (8:1), R_f's of 1, 5, and 6 are 0.7, 0.69, 0.66. For additional TLC systems used, see the supplementary material.
(9) GC–MS analyses were carried out with a Finnigan SSQ 70 mass spectrometer coupled to a Varian 3400 gas chromatograph. Chromatographic separation were performed on a Rtx-1 column (Restek Corp.). Helium was used as carrier gas at a head pressure of 8 psi. The column temperature was programmed to increase from 130 °C to 270 °C at a rate of 20 °C per minute following 13 min of holding time. Injector and transfer line were kept at 250 °C and 275 °C, respectively. Mass spectra were obtained both in EI and CI (methane as the collision gas) modes with electron energy of 70 eV. The quadrupole was scanned in the m/z range of 50–500 (EI) and 60–500 (CI) in 1 s. Ion source temperature was 150 °C.
(10) Wenker, H. The Synthesis of Δ^2-oxazolines and Δ^2-thiazolines from N-acyl-2-aminoethanols. *J. Am. Chem. Soc.* **1935**, *57*, 1079–1080.
(11) Devane, W. A.; Breuer, A.; Sheskin, T.; Järbe, T. U. C.; Eisen, M.; Mechoulam, R. A Novel Probe for the Cannabinoid Receptor. *J. Med. Chem.* **1992**, *35*, 2065–2069.
(12) In view of the existence of numerous related unsaturated fatty acid ethanolamides which bind to the cannabinoid receptor, we suggest to name this group of compounds "anandamides" with each individual member identified with the parent fatty acid indicated (in parentheses) using the generally accepted fatty acid shorthand designation.[6] Thus the anandamide derived from arachidonic acid will be anandamide (20:4, n-6). According to this proposed nomenclature homo-γ-linolenylethanolamide (**5**) will be anandamide (20:3, n-6) and docosatetraenylethanolamide will be anandamide (22:4, n-6).