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Synthesis and cannabinoid receptor binding activity of conjugated triene anandamide, a novel eicosanoid

M. L. Wise[a], K. Soderstrom[b], T. F. Murray[b], and W. H. Gerwick[a, b, *]

[a]*Department of Biochemistry and Biophysics, and* [b]*College of Pharmacy, Oregon State University, Corvallis (Oregon 97331, USA)*

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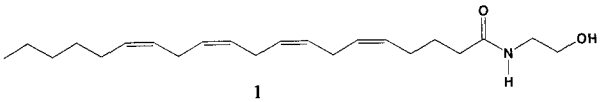
Abstract. A polyenoic fatty-acid isomerase (PFI) from a red marine alga was used to convert anandamide (5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenoyl-N-ethanolamide) to the 5*Z*,7*E*,9*E*,14*Z*-eicosatetraenoyl-N-ethanolamide isomer. This novel eicosanoid, termed conjugated triene anandamide (CTA), was assessed for its ability to bind to the cannabinoid receptor in rat brain membrane preparations. CTA is a high affinity cannabimimetic substance whose novel structure provides new insight into structure-activity relationships of cannabinoid receptor ligands. These experiments illustrate the utility of enzymes isolated from marine organisms in the development of pharmacological probes.

Key words. Cannabinoid receptor; polyenoic fatty-acid isomerase; marine enzyme; anandamide; red algae.

In 1992 Devane and Mechoulam[1] described the isolation of a candidate endogenous ligand for the cannabinoid receptor from porcine brain tissue. This compound, the N-ethanolamide of arachidonic acid, was termed anandamide (**1**) and represented the first high affinity endogenous ligand for the cannabinoid receptor[2] to be isolated from mammalian brain tissue. Its discovery has marshaled an intense effort to characterize structure-activity relationships in this new class of physiologically-active substance as well as a search for other endogenous ligands or pharmacologically-useful synthetic analogs.



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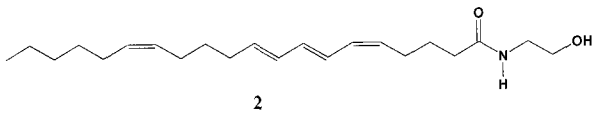

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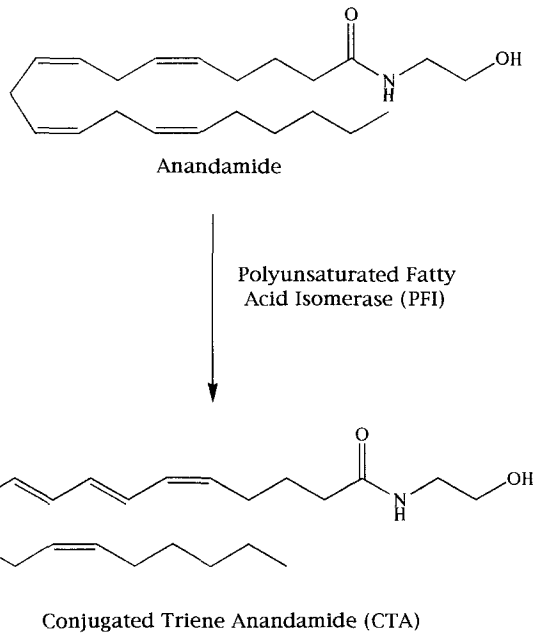
Recently a red algal enzyme, polyenoic fatty-acid isomerase (PFI), has been isolated from the marine Rhodophyte *Ptilota filicina*[3]. Characterization of this enzyme has shown it to be capable of isomerizing the methylene interrupted olefins of a wide range of polyenoic fatty acids into a conjugated triene functionality. Because anandamide contains the arachidonate moiety and PFI has shown such a broad substrate specificity for PUFA's, including arachidonate, it was of interest to attempt to react this compound with the enzyme (scheme I). In so doing we hoped to synthesize a novel cannabimimetic eicosanoid and to expand our understanding of the molecular characteristics of the binding site in this algal enzyme. Here, we describe the enzymatic transformation of anandamide to conjugated triene anandamide (CTA, **2**) and report the cannabinoid receptor affinity of this novel cannabimimetic substance. An important corollary of this work is that the cannabinoid receptor, as indicated through the retention of high affinity binding by this structurally-distinct CTA derivative, is shown to accept a broad range of molecular architectures in the arachidonate portion.



Scheme I. Enzymatic conversion of anandamide to CTA using polyenoic fatty-acid isomerase (PFI).

* Author for correspondence: Pharmacy Building 203, College of Pharmacy, Oregon State University, Corvallis, OR 97331-3507, USA. Fax +1 543 737 3999.

Materials and methods

Polyenoic fatty-acid isomerase was isolated and purified as described previously[3]. Briefly, the frozen tissue of *P. filicina* was extracted by a combination of grinding in liquid N_2 and then homogenization using Ultraturrex and Potter-Elvestrom homogenizers in 100 mM NaH_2PO_4 buffer, pH 6.5. This extract was subject to low and high speed centrifugation ($>100{,}000 \times g$) and the supernatant precipitated with ammonium sulfate (55–90%). The precipitant was resuspended in NaH_2PO_4 buffer and passed down a Sephacryl size exclusion column; the active fractions pooled, concentrated and subject to preparative isoelectric focusing (IEF, pI = 4.5).

Anandamide was obtained from Cayman Chemicals, Ann Arbor (Michigan, USA) and Research Biochemicals Inc, Wayland, (Massachusetts, USA). Gas chromatography/mass spectrometry was performed on a Hewlett Packard 5890 Series II GC with a HP5971A quadrupole mass selective detector interfaced with a Hewlett Packard Chemstation using G1034B software for data analysis. A Waters M-6000A pump and a Waters Lambda Max 480 UV detector were used for HPLC. NMR experiments were conducted on a Bruker ACP 300 instrument. Ultraviolet spectra were obtained on a Hewlett Packard 8452A diode array spectrophotometer.

Lipid extraction of reaction products was performed by quenching the reaction with five volumes of MeOH and then diluting with H_2O to a final ratio of 1:1 MeOH/H_2O. The MeOH/H_2O phase was extracted with an equal volume of diethyl ether three times. The lipids partitioning into the ether phase were dried under vacuum using a rotary evaporator and then resuspended in 100% EtOH.

Separation of CTA from unreacted anandamide was accomplished by HPLC using isocratic conditions [4% MeOH/$CHCl_3$, $2 \times 3.9 \times 300$ mm μPorasil (10 μ)]. The sample was prepared for HPLC by passage down a small silica column using 10% MeOH in $CHCl_3$. Trimethylsilane derivatization of the alcohol group of anandamide and CTA was accomplished using equal quantities of 1,1,1,3,3,3-hexamethyldisilazane and trimethylsilane chloride in pyridine[4].

Conjugated triene anandamide (CTA, 2). UV (EtOH) $\lambda_{max} = 262$, 272, 282 nm; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.35 (1H, dd, J = 11.5, 13.7, H-7), 6.17 (1H, m, H-8), 6.09 (1H, m, H-9), 6.05 (1H, m, H-6), 5.72 (1H, dt, J = 7.1, 14.3, H-10), 5.3–5.4 (3H, m, H-5, 14, 15), 3.73 (2H, t, J = 5.0, CH_2-O), 3.42, (2H, dt, J = 5.0, 5.0, N-CH_2), 2.19–2.28 (4H, m, H-2, 4), 2.12 (2H, dt, J = 7.8, 7.8, H-11), 2.02 (4H, m, H-13, 16), 1.77 (2H, tt, J = 7.4, 7.4 Hz, H-3), 1.45 (2H, tt, J = 7.6, 7.6 Hz, H-12), 1.25–1.36 (6H, m, H-17, 18, 19), 0.90 (3H, t, J = 6.81 Hz, H-20); ^{13}C DEPT ($CDCl_3$, 300 MHz, assignments based on model compounds, see ref. 7) δ135.23 (C-10; CH), 133.21 (C-15; CH), 130.41 (C-5, C-8, C-9; CHs), 130.25, 130.24, 129.62 (C-14; CH), 129.03 (C-6; CH), 125.48 (C-7; CH), 62.53 (CH_2-OH), 42.33 (N-CH_2), 35.45 (C-2; CH_2), 32.20, (C-11; CH_2), 31.33 (C-12; CH_2), 29.14 (CH_2), 27.02 (CH_2), 26.82 (CH_2), 25.52 (CH_2), 25.10 (CH_2), 24.85 (CH_2), 22.48 (C-19; CH_2), 13.89 (C-20, CH_3); LR EIMS m/z (rel. intensity) obs. [M^+] m/z 419 (11), 404 (20), 258 (15), 218 (13), 214 (12), 204 (15), 200 (10), 188 (18), 176 (13), 172 (11), 160 (14), 159 (12), 135 (11), 134 (41), 133 (20), 132 (45), 131 (18), 119 (23), 118 (22), 117 (33), 116 (100), 107 (10), 106 (14), 105 (24), 103 (19), 102 (11), 101 (20), 98 (23), 95 (12), 93 (21), 92 (20), 91 (64), 86 (13), 85 (89), 81 (16), 80 (11), 79 (32), 78 (12), 77 (17), 75 (19), 73 (50), 69 (11), 67 (22), 55 (23).

Equilibrium competitive binding experiments. [^{3}H]CP-55940 (131.5 Ci/mmol) was purchased from DuPont NEN, Boston (Massachusetts, USA). CP-55940 is a novel bicyclic cannabinoid which retains the three essential features required of cannabinoid compounds for interaction with the cannabinoid receptor; namely, an equatorial alcohol, a phenol, and the C-3 side chain (dimethylheptyl)[5]. Phenylmethanesulfonyl fluoride (PMSF) was purchased from Boehringer Mannheim Inc., Indianapolis (Indiana, USA). CTA was purified by HPLC and characterized as described above. Frozen rat brains were purchased from Pel-Freez, Rogers, (Arkansas, USA).

A washed P2 synaptosomal membrane preparation was prepared as follows. Rat brains, thawed on ice, were homogenized using a Potter-Elvehjem homogenizer in 40 volumes of a buffered sucrose solution (0.32 M sucrose, 5 mM HEPES, pH 7.45) and centrifuged at $1000 \times g$ for 10 min. The pellet was discarded and the supernatant was centrifuged at $30{,}000 \times g$ for 30 min. This pellet was snap-frozen and resuspended in buffer A (25 mM HEPES, 10 mM EDTA, 6 μg/ml bacitracin, pH 7.4), allowed to incubate for 3 h on ice, and centrifuged at $30{,}000 \times g$ for 30 min. The pellet was resuspended in buffer B (25 mM HEPES, pH 7.4) and centrifuged at $30{,}000 \times g$ for 30 min. This pellet was stored at −70 °C until used. When needed for binding assays, pellets were resuspended in 40 volumes of assay buffer (25 mM HEPES, 10 mM $MgCl_2$, pH 7.4).

Equilibrium binding experiments were carried out at 30 °C for 90 min. Incubations were conducted in a final volume of 500 μl containing: 100 μM PMSF, 20 mM HEPES, 8 mM $MgCl_2$, 1% BSA, 0.2% ethanol and 40 to 80 μg membrane protein. Incubations were terminated by rapid filtration over GF/C filters using a Brandel Cell Harvester, Gaithersburg (Maryland, USA). Filter trapped radioactivity was quantified with a Beckman LS 6000SC scintillation counter. Specific binding was defined as total binding minus that occurring in the presence of 10 μM levonantradol. Protein concentrations were determined using the method described by Lowry[6].

Results

Production of conjugated triene anandamide. An initial experiment in which 1.6×10^{-3} units of purified PFI was incubated with 0.1 mg of anandamide in 1 ml of reaction buffer (288 μM final concentration) resulted in the production of a conjugated triene metabolite. Spectra were obtained at timed intervals which showed the development of the distinctive three peaked chromophore identical to that obtained from incubation of the enzyme with arachidonic acid[3].

A larger scale incubation was performed to obtain sufficient product for spectral characterization and to conduct receptor binding studies. In a 10 ml Erlenmeyer flask, 5 mg of anandamide (288 μM) was incubated with 6.3×10^{-2} units of PFI in 5 ml of 100 mM NaH_2PO_4 buffer, pH 6.5 with 0.02% Tween-20. The progress of the reaction was monitored by periodic spectra of 1:10 dilutions of the reaction mixture. After 10.5 h the production of triene reached a plateau with an absorbance at 276 nm of 5.2 A.U., representing just under 33% conversion of substrate to product. A control reaction identical to the test solution but without enzyme showed a final absorbance at 276 nm of 0.01, i.e. no triene was formed. The enzyme reaction was quenched with 5 volumes of MeOH and the lipids extracted. By UV spectral analysis a total of 1.16 mg of CTA was obtained (assuming $\varepsilon = 57{,}000$ L mol^{-1} cm^{-1} (ref. 7).

Characterization of conjugated triene anandamide. The lipid extract from the above preparative-scale incubation was reduced under vacuum, resuspended in 4% MeOH in $CHCl_3$ and subjected to HPLC. Compounds showing significant UV absorption at 280 nm eluted in one major peak centered at 28.8 ml and two minor peaks centered at 7.8 ml and 35.7 ml. The major peak contained both the anandamide and the CTA (the minor peaks were not further analyzed). By fractionating this peak into two approximately equal portions, it was possible to separate anandamide, eluting first, from CTA.

GLC/MS analysis of anandamide typically results in a substantial proportion of the material dehydrating resulting in two GC peaks, one with a $M^+ = 347$ and another with a $M^+ = 329$ ($M^+ - 18$). To resolve this problem, we found that the trimethylsilyl (TMS) derivatives of anandamide and CTA were stable to GLC/MS. The TMS derivative of anandamide eluted in a single sharp peak ($R_t = 13.3$) whereas the TMS derivative of CTA eluted as dual peaks ($R_t = 15.6$ and 15.8 minutes). As both showed identical fragmentation patterns, they most likely represent thermal isomerization of the *ctt* isomer to the *ttt* isomer of CTA, a phenomenon commonly observed with conjugated polyenoic fatty acids[8].

The HPLC fraction eluting from 28.0 to 31.2 ml contained the characteristic triene UV chromophore and showed, following derivatization to TMS-ethers, a peak by GC/MS with an $R_t = 15.6$ min and a M^+ at $m/z = 419$. By UV measurement at 272 nm, 0.23 mg of pure CTA were isolated.

With the exception of a small up-field shift of the H_2-2 and H_2-4 protons (due to the amide linkage) and the signals from the N-ethanolamide functionality, the NMR characteristics of CTA were essentially identical to those for the methyl ester of 5*Z*,7*E*,9*E*,14*Z*-eicosatetraenoate, the major metabolite isolated from reaction of arachidonate with PFI (ref. 3). To firmly establish the position of the conjugated triene functionality in the fatty acid chain, a proton decoupling experiment was performed. By irradiating the 4H signal at 2.23 ppm the pentet at 1.77 ppm collapsed to a singlet and the multiplet at 6.05 ppm showed a distinct sharpening. These results clearly demonstrate that the signal at 2.23 ppm represents the H2 and H4 methylenes surrounding the H3 methylene (1.77 ppm) and that there is allylic coupling between the H4 methylene protons and the H6 olefinic proton (6.05 ppm). The relative stereochemistry of the C14–15 olefinic group could not be unequivocally determined; it is assumed to be unchanged from the substrate since this portion of the molecule is not affected by the enzymatic isomerization[3].

Evaluation of CTA as a ligand to the cannabinoid receptor. The ability of HPLC-purified CTA and anandamide to displace specific binding of the high-affinity cannabinoid agonist [^{3}H]CP-55940 to rat brain membranes was assessed. Binding of [^{3}H]CP-55940 was inhibited in a concentration-dependent manner by CTA and anandamide (fig. 1). The potency of CTA ($K_i = 607 \pm 44$ nM) as an inhibitor of [^{3}H]CP-55940 binding was only six-fold lower than that of anandamide ($K_i = 97 \pm 7$ nM). These binding experiments were conducted in the presence of PMSF in the incu-

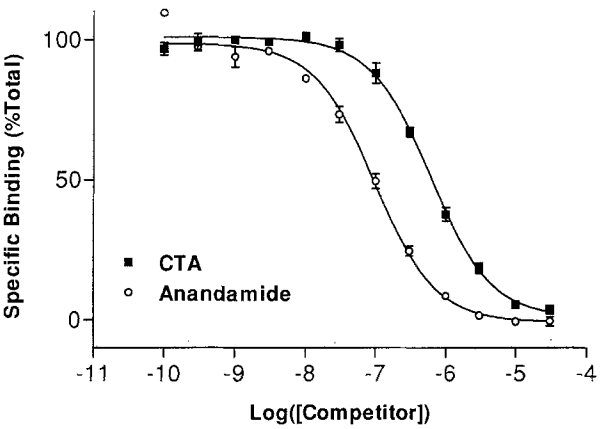


Figure 1. CTA and anandamide inhibition of the specific binding of [^{3}H]CP-55940 (100 pM) to rat neuronal membranes (40–70 μg of protein). Each data point is the mean ±SEM of two independent experiments performed in duplicate.

bation media (100 μM). This serine-protease inhibitor has been reported to prevent degradation of anandamide, presumably through inhibition of an amidase activity[9–11].

Discussion

Structure-activity studies on several analogs of anandamide have shown that a number of polyunsaturated fatty acid ethanolamides exhibit cannabinoid agonist behavior. Felder et al.[12] evaluated five fatty acid analogs [dihomo-γ-linolenic acid (8*Z*,11*Z*,14*Z*-eicosatrienoic acid); adrenic acid (7*Z*,10*Z*,13*Z*,16*Z*-docosatetraenoic acid); docosahexaenoic acid (4*Z*,7*Z*,10*Z*, 13*Z*,16*Z*,19*Z*-docosahexaenoic acid); γ-linolenic acid (6*Z*,9*Z*,12*Z* octadecatrienoic acid); palmitic acid (hexadecanoic acid)] as well as four new amide derivatives of anandamide [3-amino-1-propanol: R,S-2-amino-1-propanol; R,S-1-amino-2-propanol; arachidonamide] for their affinity for the cannabinoid receptor. Of these, the adrenic acid, dihomo-γ-linolenic acid and the 3-amino-1-propanol analogs showed K_is for binding and IC_{50}s for inhibition of cAMP accumulation comparable to anandamide ($K_i = 543 \pm 83$ nM and $IC_{50} = 160 \pm 13$ nM($\pm$SEM)). The other fatty acid analogs showed K_i values > 12.2 μM and IC_{50} values > 6.0 μM; the palmitic acid derivative showed no activity up to 1 mM. The three other ethanolamide derivatives had K_is ranging from 1.3 to 9.6 μM and IC_{50}s ranging from 507 nM to 10.1 μM. It is interesting to note that both adrenic acid and the dihomo-γ-linolenic acid ethanolamides have recently been isolated from porcine brain tissue and shown to be competitive inhibitors of [^{3}H]HU-243 (11-hydroxyhexahydrocannabinol-3-yldimethylheptyl homolog) binding to rat synaptosomal membranes with K_is comparable to Δ^9-THC[13].

From the above results, we speculate that a terminal aliphatic pentyl chain in the fatty acid portion is necessary, though not sufficient, for high affinity receptor binding and that an olefinic group (possibly the ω-6 double bond as all of the known high affinity anandamide-type derivatives, including CTA, possess this structural feature; see fig. 2) also plays a role in binding affinity, as evidenced by the failure of palmitoyl ethanolamide to bind. However, to our knowledge the critical compound needed for evaluation of this proposal, 5*Z*,8*Z*,11*Z*-eicosatrienoyl-N-ethanolamide, has not been evaluated in this regard. The relatively low activity of the γ-linolenate congener also indicates that there are other structural requirements, possibly a minimum chain length of the fatty acid.

In another study, the binding affinity of leukotriene B_4 dimethylamide (LTB_4DMA) as well as a number of prostaglandin amides were assessed for their ability to specifically bind the cannabinoid receptor in P_2 membranes prepared from mouse brains[11]. The

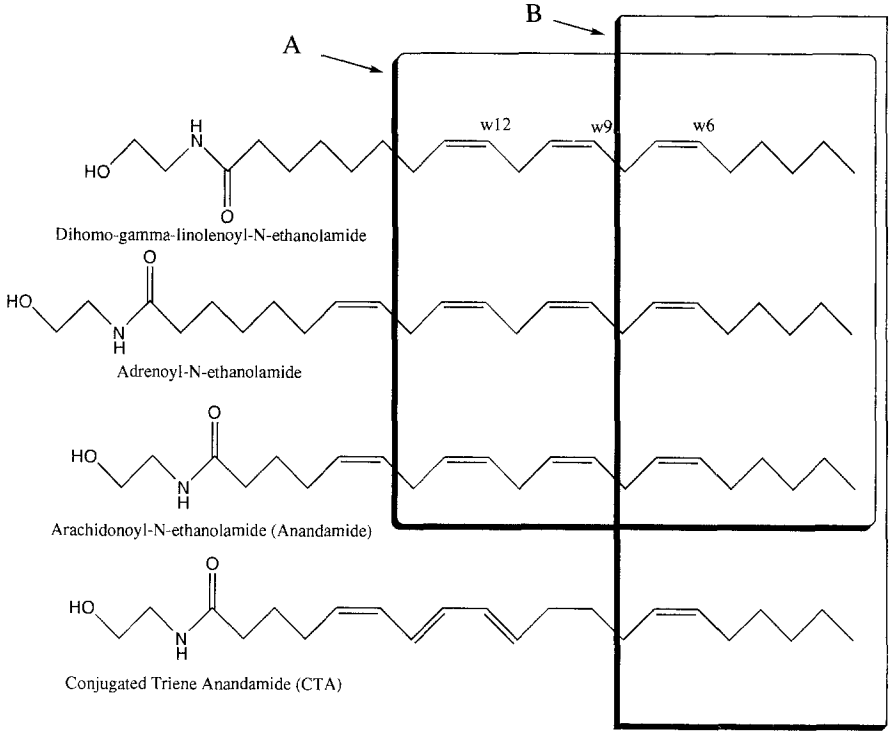


Figure 2. Comparison of the four known high affinity fatty acid ethanolamides. Set *A* identifies the common structural feature (ω6, ω9, and ω12 unsaturations) in those high affinity ligands known prior to the current study, while set *B* identifies the common structural feature (ω6 unsaturation) of all four high affinity ligands.

prostaglandin analogs failed to alter [^{3}H]CP-55940 binding at concentrations up to 100 μM and the LTB_4DMA had an $IC_{50} > 3$ μM.

The results from the binding studies with CTA clearly demonstrate that a degree of structural rigidity (i.e. that imposed by a conjugated triene between C5 and C10) in the anandamide fatty acid chain is permissible for receptor binding. This is particularly significant in view of the results obtained in experiments with LTB_4DMA (cf. ref. 11). It is evident from the results reported here that some aspect of LTB_4DMA other than the conjugated triene functionality prevents its binding to the cannabinoid receptor. Whether it is due to the hydroxyl groups, the dimethylamine or possibly its solution conformation will require further evaluation. Although it is possible to model the low energy conformations of CTA, without some knowledge of the solvent/protein/ligand interaction, these are of dubious value.

The results from these experiments offer a number of conclusions in several areas. First, they demonstrate the utility of marine enzymes in the synthetic production of novel compounds for use as pharmacological probes. CTA is a novel cannabimimetic substance whose activity offers new insight into the structure-activity relationships in this chemical class. They also present the possibility that new and pharmacologically-useful cannabimimetics may be present in nature, in particular in the marine environment. Finally, they provide some insight into the substrate binding characteristics (i.e. a terminal carboxylate is not required for substrate binding) of the novel enzyme polyenoic fatty-acid isomerase which was used in the synthesis of CTA.

Note added in proof. A recent report has shown that the ethanolamide derivative of 5*Z*,8*Z*,11*Z*-eicosatrienoic acid (mead acid) is also a high affinity agonist for the CB1 and CB2 cannabinoid receptors, a finding which is apparently inconsistent with our model presented in figure 2. [Priller, J., Briley, E. M., Mansouri, J., Devane, W. A., Mackie, K., and Felder, C. C., Molec. Pharmac. *48* (1995) 288.]

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