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Research Article

Synthesis of ^{15}N and ^{13}C selectively labeled anandamide

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Summary

Anandamide (Figure 1a) (arachidonyl ethanolamide, AEA), (5Z,8Z,11Z, 14Z)-N-(2-hydroxyethyl)-5,8,11,14-Eicosatetraenamide, is an endogenous cannabinoid ligand possessing important biological activity. The conformation of AEA in its native receptor binding environment is particularly of interest for pharmaceutical research and biochemistry in general. Here we report a novel synthetic pathway, which selectively introduces ^{15}N and ^{13}C isotopes into the anandamide molecule. This isotopically labeled AEA can be studied conformationally in its native binding condition via solid state NMR. These synthetic procedures can also be adapted to produce radioactive ligands for receptor binding assays and other structural studies. Copyright © 2002 John Wiley & Sons, Ltd.

Key Words: anandamide; stable isotope labeled; NMR; cannabinoid ligand

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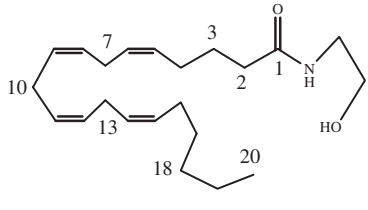
Introduction

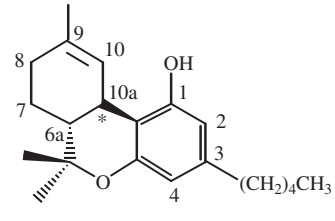
One of the most significant advances in recent cannabinoid (CB) research is the discovery of anandamide (arachidonyl ethanolamide, AEA, Figure 1a) as the first cannabinoid endogenous ligand, and the confirmation of the existence of the cannabinoid system in mammals.[1] It is known that AEA binds to the cannabinoid receptor and induces cannabimimetic activity comparable to (-)-Δ^9-tetrahydrocannabinol (Δ^9-THC, Figure 1b), the active constituent in marijuana.

Questions have been raised as to how such a structurally different molecule has similar pharmacological effects as that of Δ^9-THC. Also of interest is its native binding conformation(s), as well as the pharmacological implications of AEA as a lead compound for novel CB ligand development programs. Thus, intensive efforts[2,3] have been made to understand AEA's biological properties and its interaction with cannabinoid receptors. Although various computer-generated AEA conformations have been reported,[4–7] the *in vivo* conformation of receptor-bound AEA still remains unknown. Furthermore, these theoretical models are considered to be inconclusive due to the lack of experimental evidence.

Xie et al.[8] have recently reported the extended 'pseudo-helical shape' conformation of AEA based on the NMR Comparative Molecular Field Analysis (CoMFA) studies. Despite such efforts, solution NMR provided only limited conformational data due to the flexible nature of AEA and its rapid chain motion in solution. However, these limitations of liquid phase NMR can be overcome by using solid state NMR techniques[9] with a combination of isotopically labeled ligands.



(a) AEA; Anandamide (Endogenous ligands)



(b) Δ^9-THC; (-)-Δ^9tetrahydrocannabinol (Active constituent in marijuana)

Figure 1. Structures of anandamide (AEA) and (-)-Δ^9-tetrahydrocannabinol (Δ^9-THC)

REDOR (rotational echo double resonance) experiments are designed to observe isotopically labeled (e.g. ^{13}C, ^{15}N, or ^{2}H, as necessary) nuclei, and to allow direct distance measurements between strategically labeled positions to obtain structural information at the molecular level.

In this article, we report the synthesis of double-labeled AEA, with ^{15}N at the amine head group, and ^{13}C in the terminal methyl group. This labeled compound is designed for solid state NMR spectroscopy in order to obtain experimental data on AEA in both free and membrane-bound states. These data are then used to verify computer predicted models. Furthermore, this synthesis can be modified to produce radioactive ligands for receptor affinity binding assays or other similar structural studies.

Results and discussion

The synthetic pathway of double-labeled AEA is summarized in Figure 2. The synthesis started from the preparation of aldehyde **4** by a previously reported method.[10,11] In the preparation of the epoxide **2**, an ethereal solution of hydrogen peroxide (ether/H_2O_2) was prepared based on the Seltzman method.[10] From experimental trials (unpublished data) we found that the amount of sodium sulfate added affects the concentration of H_2O_2 and the subsequent epoxidation, since an excess of sodium sulfate tends to form complexes with H_2O_2.[12] Therefore, the amount of sodium sulfate added should be carefully controlled in order to obtain reproducible yields.

5-Bromopentanol was prepared by the cleavage of tetrahydropyran **5** with boron tribromide, and the hydroxyl group was protected using tetrahydropyranyl mixed acetyl function,[13] yielding bromide **6**. The Grignard reagent, the organocopper (I) intermediate, was prepared from **6**, and coupled with ^{13}C iodomethane in the presence of dilithium tetrachlorocuprate in THF at 0°C, to give 2-(6′-^{13}C-hexyloxy) tetrahydropyran **7**.[14] Treatment of compound **7** with dibromotriphenylphosphorane yielded the bromide **8**,[15] which was then refluxed with triphenylphosphine in xylenes to produce the phosphonium bromide **9**.

Coupling of compounds **4** and **9** was achieved by the ylide formation of **9** with *n*-butyllithium/hexanes in THF at −78°C, then linkage with aldehyde **4** through a Wittig reaction to produce 20-^{13}C labeled methyl

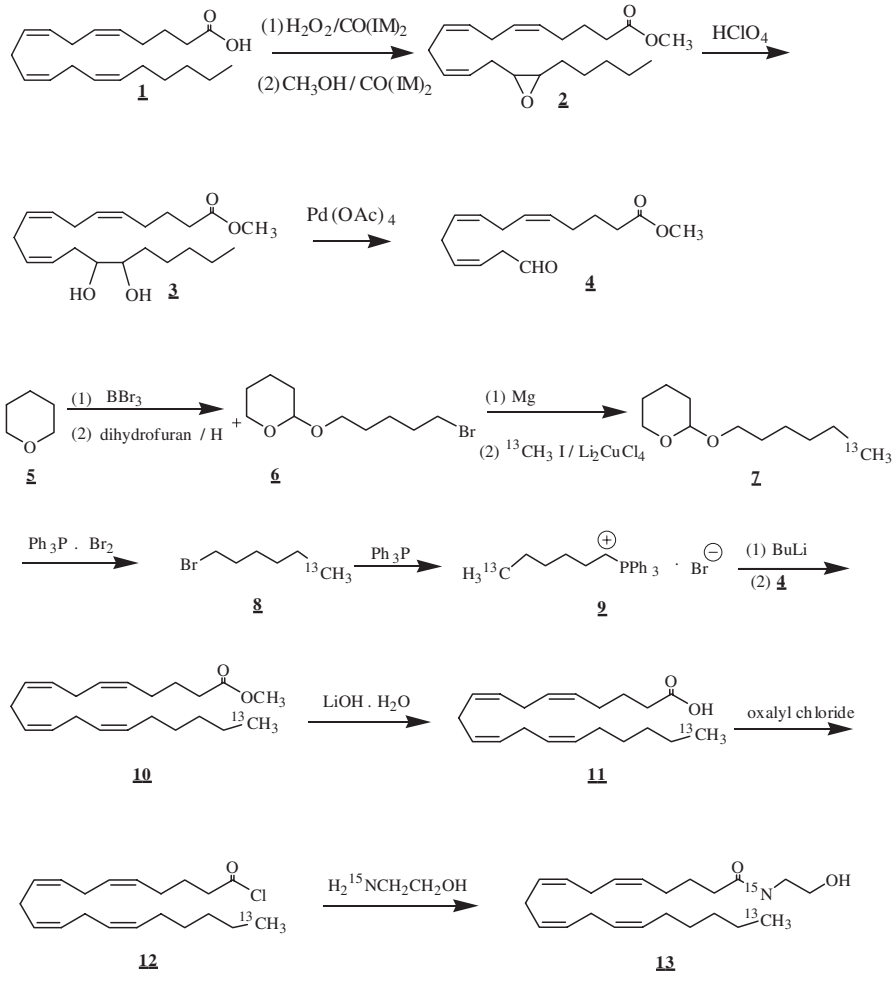


Figure 2. Procedures for the synthesis of the 20-^{13}C, ^{15}N-labeled AEA molecule

arachidonate **10**. Ester **10** was then hydrolyzed to give 20-^{13}C arachidonic acid, **11**, and further transformed to its acid chloride, **12**. Finally, the double-labeled ^{13}C, ^{15}N AEA **13** was obtained by reacting compound **12** with excess ^{15}N ethanolamine, prepared according to a previously reported procedure.[16]

NMR analysis of the end product (Figure 3) confirmed the ^{15}N and ^{13}C enrichment at the ethanolamine head group and terminal methyl group, respectively. Figure 3 shows a stacked plot of the isotope labeled AEA (Figure 3A) and the unlabeled AEA (Figure 3B). The labeled AEA shows a doublet-of-triplet peak pattern for the ^{15}NH group at

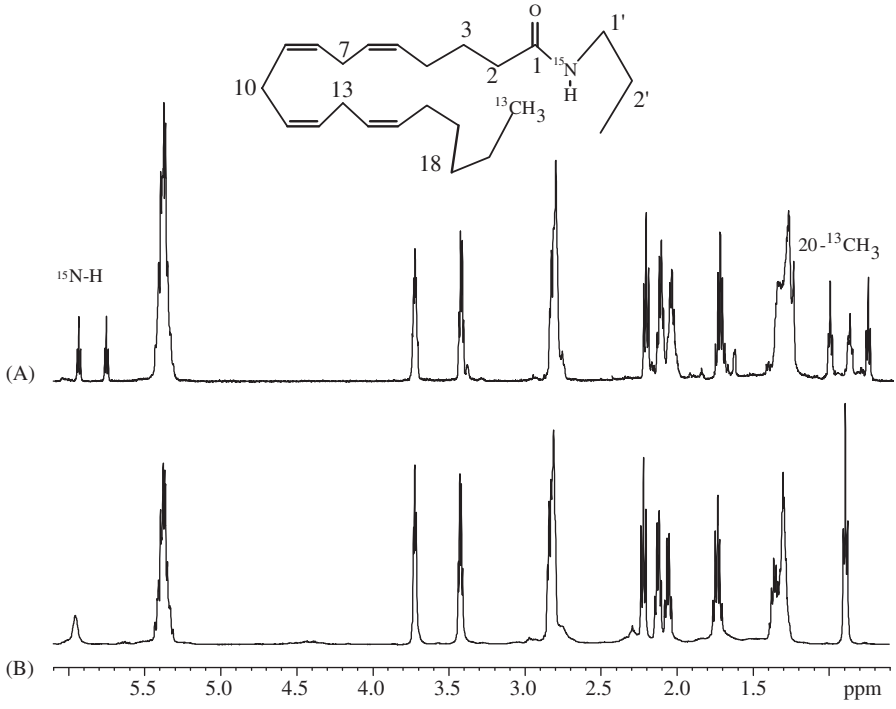


Figure 3. A stacked plot of high-resolution 500 MHz ^{1}H NMR spectra of the ^{15}N, ^{13}C-labeled AEA molecule (A), and the unlabeled compound (B)

5.84 ppm (dt, 2H, J ^{15}N-^{1}H = 90 Hz, J ^{1}H-^{1}H = 6 Hz). The NH chemical shift difference between the enriched and non-enriched AEA compounds is due to the concentration-dependent property of the NH proton. The methyl group was not fully ^{13}C labeled, thus showing a triplet-of-triplet peak pattern instead of the expected doublet-of-triplet peak pattern (dt, 3H, J ^{13}C-^{1}H = 124 Hz, J ^{1}H-^{1}H = 7 Hz). This is attributed to deterioration of the ^{13}C-enriched reagent CH_3I, which showed a similar NMR spectral pattern for the methyl peak (data not shown).

The synthetic procedure reported above can also be adapted to produce the corresponding ^{3}H or ^{14}C radioactive ligands for receptor binding assays. Radioactive (^{14}C and ^{3}H) forms of AEA or 2-arachidonylglycerol (2-AG) have been used as radioactive substrates, e.g. [1,2-^{14}C]-AEA[17] and [5,6,8,9,11,12,14,15-^{3}H]-AEA,[18] for a variety of radioenzymic assays, occurrence and metabolism studies,[19] transport and hydrolysis investigations,[20] and cellular uptake and enzymatic hydrolysis work.[21,22] The labeling approach reported here provides an

alternate approach to the labeled endogenous cannabinoid ligand for use in biophysical studies.

Experimental

Arachidonic acid was purchased from Nu-Chek Prep, Inc. (Elysian, MN). All other chemicals and chromatographic materials were purchased from Aldrich Chemical Co. (Milwaukee, WI). Solution NMR spectra were obtained on a Bruker AVANCE DMX500 Spectrometer using tetramethylsilane as an internal reference. All chemical reactions involved were conducted under dry N_2 gas to exclude moisture and prevent possible oxidation.

The double-labeled AEA **13** was prepared as illustrated in Figure 2, following literature procedures for the synthesis of the unlabeled material, with appropriate modification.

Methyl 14-oxotetradeca-cis-5,8,11-trienoate **4**

The synthesis started from arachidonic acid **1**, and involved three steps, as described by Seltzman.[10] In the preparation of the epoxide **2**, an ethereal solution of hydrogen peroxide (ether/H_2O_2) was prepared based on the reported method,[10] and sodium sulfate was used to remove the water in the ethereal solution. Since sodium sulfate tends to form complexes with H_2O_2,[12] the amount of sodium sulfate added affects the concentration of H_2O_2 and the subsequent epoxidation. Therefore, the proper amount (ethereal H_2O_2/Na_2SO_4, 100:6 v/w) of sodium sulfate was experimentally determined to obtain reproducible TLC results for the aldehyde conversion. The aldehyde product **4** was immediately used in the following reaction.

2-(6′-^{13}C-hexyloxy)tetrahydropyran **7**

The preparation of Grignard reagent is as follows: A solution of 2-(5′-bromopentyloxy)tetrahydropyran **6** (2 g, 7.97 mmol) in dry THF (10 ml) was added dropwise under gentle reflux conditions to a mixture of magnesium turnings (0.243 g, 10.0 mmol), dry THF (5 ml), a crystal of iodine and **6** (0.51 g, 2.03 mmol). Thereafter the reaction mixture was heated under reflux for 5 h until most of the magnesium had dissolved. The reaction was stopped and cooled to room temperature.

^{13}C Labeled iodomethane (1.42 g, 10.0 mmol), the Grignard solution prepared above (equivalent to 10.0 mmol) and anhydrous THF (20 ml) were combined in a 100 ml round-bottomed flask which was flushed with nitrogen and sealed with a rubber serum cap. The solution was cooled in an ice bath. A solution of dilithium tetrachlorocuprate in THF (0.1 M, 0.5 ml) was added. The reaction mixture was stirred for 3 h at 0°C, then poured into 100 g of crushed ice and extracted with hexanes (3 × 10 ml). The extract was dried over Na_2SO_4. Removal of the solvent in vacuo gave 1.2 g of the crude product, which was purified by silica gel column chromatography (30 g SiO_2, 2% ethyl acetate/hexane) to obtain 0.98 g of pure product in 52% yield. ^{1}H NMR ($CDCl_3$): δ 0.91 (dt, 3H, J ^{13}C-^{1}H = 124 Hz, J^{1}H-^{1}H = 7 Hz); 1.29–1.34 (m, 6H); 1.53–1.61 (m, 6H); 1.77 (m, 2H); 2.45 (m, 2H); 2.78 (m, 2H); 4.58 (t, 1H).

6-^{13}C-bromohexane **8**

2-(6′-^{13}C) Hexyloxytetrahydropyran **7** (1 g, 5.35 mmol) was dissolved in anhydrous CH_2Cl_2 (20 ml) and dibromotriphenylphosphorane (2.30 g, 5.45 mmol) was added, followed by 3 ml of CH_2Cl_2 for rinsing. The mixture was stirred at room temperature for 20 h, washed with 5% sodium bicarbonate (20 ml) and brine (2 × 20 ml), and dried over Na_2SO_4. After evaporation of the solvent in vacuo, the residue was extracted with hexanes (3 × 20 ml). The extract was concentrated on a rotary evaporator. Further purification of the crude product was accomplished by column chromatography (15 g SiO_2, petroleum ether) to afford 380 mg of **8** in 43% yield. ^{1}H NMR ($CDCl_3$): δ 0.92 (dt, 3H, J ^{13}C-^{1}H = 124 Hz, J ^{1}H-^{1}H = 7 Hz); 1.28–1.33 (m, 4H); 1.43 (m, 2H); 1.86 (m, 2H); 3.41 (t, 3H).

6-^{13}C-hexyltriphenylphosphonium bromide **9**

A solution of 6-^{13}C-bromohexane **8** (187 mg, 1.1 mmol) and triphenylphosphine (288 mg, 1.1 mmol) in xylenes (3 ml) was heated at reflux overnight. While the reaction was still warm, the condenser was removed and a stream of nitrogen was blown over the reaction until the solvent evaporated. After cooling, the excess triphenylphosphine was removed by repeated washing and decanting with hexanes/benzene (1/1, 5 ml). The solvent was decanted and the procedure was repeated. The remaining solvent was removed in vacuo and the residue was heated in a vacuum oven overnight at 85–90°C to give 385 mg of pale yellow gum in

82% yield. The material was used directly in the Wittig reaction, without characterization.

20-^{13}C-methyl arachidonate ***10***

A solution of the Wittig reagent was prepared by the dropwise addition of a solution of *n*-butyllithium in hexanes (2.5 M, 0.5 ml) to a solution of **9** in THF (5 ml) at –78°C and stirred at that temperature for 0.5 h. The aldehyde **4** (prepared from 383 mg of the diol **3**) dissolved in dry THF (5 ml) was added dropwise to the above Wittig reagent. The reaction mixture was stirred, allowing it to warm to room temperature over 2.5 h. The reaction was quenched with brine, extracted with ethyl acetate (3×10 ml) and dried over Na_2SO_4, evaporated in vacuo to give crude product which was then chromatographed on silica gel (10 g SiO_2, hexanes/ethyl acetate 5:1) to produce 198 mg of the pure product in 58% yield (based on the diol **4** used). ^{1}H NMR: δ 0.90 (dt, 3H, J ^{13}C-^{1}H = 124 Hz, J^{1}H-^{1}H = 7 Hz); 1.26–1.37 (m, 6H); 1.71 (m, 2H); 2.02–2.10 (m, 4 H); 2.31 (t, 2H); 2.77–2.84 (m, 6H); 3.67 (s, 3H); 5.36–5.42 (m, 8H).

20-^{13}C-arachidonic acid ***11***

To a solution of 20-^{13}C-methyl arachidonate **10** (198 mg, 0.62 mmol) in methanol (18 ml) and water (6 ml) was added $LiOH \cdot H_2O$ (100 mg, 2.4 mmol) and the reaction was heated at 60°C overnight. After cooling, the reaction was diluted with H_2O (20 ml) and ether (20 ml). The layers were separated and the aqueous layer was acidified with 1 N HCl and saturated with NaCl. The aqueous layer was extracted with ether (3×10 ml), and the combined extract was washed with brine (5 ml) and dried over Na_2SO_4. The ether was removed in vacuo to afford 148 mg of oil in 78% yield. The product was treated twice with fresh benzene (5×2 ml) and evaporated to remove moisture, and used directly in the following reactions.

^{15}N, 20-^{13}C-anandamide ***13***

To a solution of 20-^{13}C-arachidonic acid **11** (98 mg, 0.32 mmol) in anhydrous benzene (10 ml) cooled to 0°C was added oxalyl chloride (0.06 ml, 0.69 mmol) and a drop of DMF. The reaction mixture was slowly brought to room temperature while stirring for 2 h. The solvent

was removed in vacuo and the residue was treated twice with fresh benzene (2 × 10 ml) and evaporated to remove moisture. The crude acid chloride **12** was used in the following reaction without further purification.

A solution of ^{15}N labeled ethanolamine was prepared by treating ^{15}N ethanolamine hydrochloride (337 mg, 3.42 mmol) with triethylamine (0.48 ml, 3.45 mmol) at 0°C in anhydrous CH_2Cl_2 (20 ml) and stirred for 30 min.

The crude acid chloride **12** was dissolved in anhydrous CH_2Cl_2 (6 ml) and was added dropwise to the solution of ^{15}N ethanolamine at 0°C. The reaction was stirred at 0°C for 30 min, brine (20 ml) was added and then CH_2Cl_2 (20 ml). The aqueous layer was extracted with CH_2Cl_2 (3 × 10 ml). The organic phase was combined and dried over Na_2SO_4. After evaporation of the solvent in vacuo, the residue was chromatographed on a column (SiO_2 5 g, hexanes/ethyl acetate 1:5) to afford 46 mg of **13** in 41% yield. ^1H NMR: δ 0.90 (dt, 3H, J ^{13}C-^1H = 124 Hz, J ^1H-^1H = 7 Hz); 1.25–1.37 (m, 6H); 1.73 (m, 2H); 2.05 (m, 2H); 2.12 (m, 2H); 2.81–2.85 (m, 6H); 3.43 (m, 2H); 3.72 (m, 2H); 5.33–5.41 (m, 8H); 5.84 (dt, 2H, J ^{15}N-^1H = 90 Hz, J ^1H-^1H = 6 Hz).

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