Chapter 22

Oxygenation of Anandamide by Lipoxygenases

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Abstract

The endocannabinoids anandamide and 2-arachidonoylglycerol are not only metabolized by serine hydrolases, such as fatty acid amide hydrolase, monoacylglycerol lipase, and α,β-hydrolases 6 and 12, but they also serve as substrates for cyclooxygenases and lipoxygenases. These enzymes oxygenate the 1*Z*,4*Z*-pentadiene system of the arachidonic acid backbone of endocannabinoids, thereby giving rise to an entirely new array of bioactive lipids. Hereby, a protocol is provided for the enzymatic synthesis, purification, and characterization of various oxygenated metabolites of anandamide generated by lipoxygenases, which enables the biological study and detection of these metabolites.

Key words Lipoxygenase, Endocannabinoid, Anandamide, 2-Arachidonoylglycerol, Oxygenated metabolite

1 Introduction

The endocannabinoids anandamide (AEA, (5*Z*,8*Z*,11*Z*,14*Z*)-*N*-(2-hydroxyethyl)icosa-5,8,11,14-tetraenamide) and 2-arachidonoylglycerol (2-AG, 1,3-dihydroxypropan-2-yl (5*Z*,8*Z*,11*Z*,14*Z*)-icosa-5,8,11,14-tetraenoate) are signaling lipids that act on type 1 and type 2 (CB_1 and CB_2) cannabinoid receptors [1, 2]. They modulate a wide array of physiological functions. For example, in the brain they act as retrograde messengers that inhibit neurotransmitter release, whereas at the periphery they influence immune cell migration [3, 4]. The biological effect of endocannabinoids at CB_1 and CB_2 receptors depends on their life-span in the extracellular space. Both endocannabinoids serve as substrates for metabolic serine hydrolases that hydrolyze the amide bond of AEA or the ester bond of 2-AG, thereby releasing arachidonic acid and ethanolamine or glycerol, respectively. Fatty acid amide hydrolase is the main enzyme responsible for anandamide hydrolysis, whereas 2-AG is converted by monoacylglycerol lipase, α,β-hydrolase 6 and 12 [5]. Hydrolysis of the amide or ester bond results in termination of CB_1 and CB_2 receptor signaling [6].

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Both AEA and 2-AG contain an arachidonic acid backbone, which may serve as a substrate for enzymes such as cyclooxygenases and lipoxygenases [7–11]. Indeed, both enzyme classes convert endocannabinoids into oxygenated metabolites, such as prostamides, prostaglandin-glyceryl esters, and hydro(pero)xy-derivatives. These oxygenated products do not necessarily serve to terminate endocannabinoid signaling, but may exert biological actions of their own and/or modulate the pharmacology of endocannabinoids by interfering with fatty acid amide hydrolase activity. The action of cyclooxygenases on endocannabinoids has been studied in detail and specific receptors have been found for the prostamides, whereas the characterization of the biological presence and action of the lipoxygenase products is lacking behind [12].

There are three types of lipoxygenases (5-, 12-, and 15-lipoxygenases) that insert O_2 at C5, C12, or C15 of arachidonic acid, respectively. The same enzymes oxygenate in a regio- and stereospecific manner also AEA and 2-AG, adding O_2 at different positions of their arachidonic acid backbone (Fig. 1). The resulting hydroperoxide products are rapidly and nonenzymatically reduced in the cell to their corresponding hydroxyl derivatives.

12(*S*)-Hydroxy-AEA has been shown to bind to CB_1 receptor with comparable affinity as AEA, whereas the other metabolites showed reduced binding affinity [13]. Of note, all lipoxygenase products were found to be inhibitors of fatty acid amide hydrolase in the low to submicromolar range. In various physiological processes, action of lipoxygenase metabolites of endocannabinoids has been suggested, based on intervention studies with lipoxygenase inhibitors [14]. To interrogate the physiological relevance and function of lipoxygenase metabolites of endocannabinoids in more detail, it is important to have sufficient quantities of the oxygenated products. Here, we provide a protocol for the enzymatic synthesis, purification, and analysis of 5(*S*)-hydroxy-AEA, 11(*S*)-hydroxy-AEA, 12(*S*)-hydroxy-AEA, 15(*S*)-hydroxy-AEA, 5(*S*),15(*S*)-dihydroxy-AEA, and 8(*S*),15(*S*)-dihydroxy-AEA (*see* **Note 1**) [13–15].

2 Materials

2.1 Buffers and Solutions

1. 0.1 M Sodium borate buffer (pH 9.0): Weigh 6.18 g boric acid and transfer to a 1 l conical flask. Add 900 ml of water and dissolve boric acid under magnetic stirring. Adjust the pH to 9.0 with 1 M NaOH, and bring the volume to 1 l.
2. 0.1 M Sodium phosphate buffer (pH 7.0): Weigh 15.60 g sodium phosphate monobasic dihydrate and transfer to a 1 l conical flask. Add 900 ml of water and dissolve sodium phosphate under magnetic stirring. Adjust the pH to 7.0 with 1 M NaOH and bring the volume to 1 l.

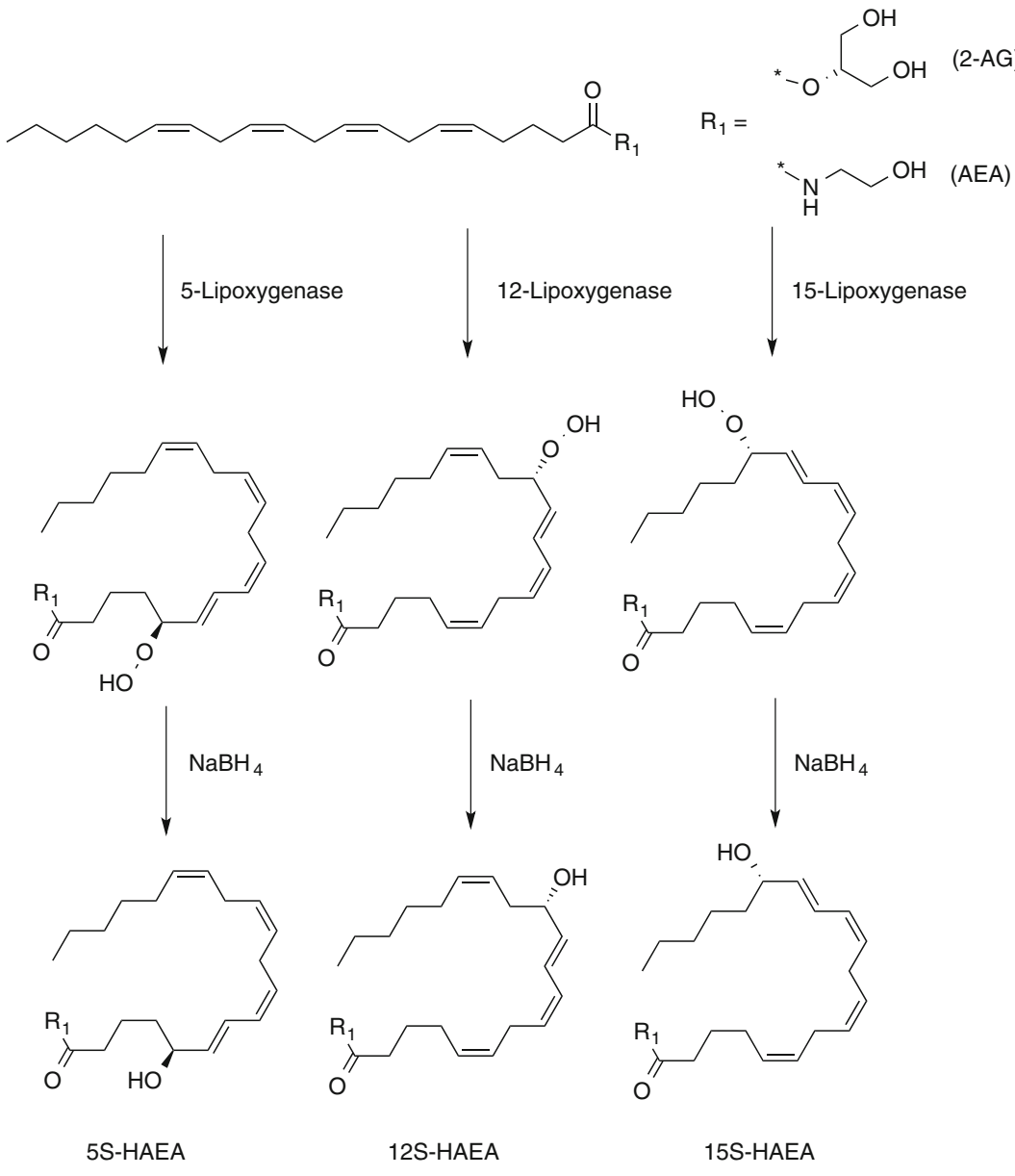

Fig. 1 Structures of oxygenated metabolites of anandamide produced by distinct lipoxygenases

3. PBS (pH 7.4): Weigh 8 g sodium chloride, 0.2 g potassium chloride, 1.80 g sodium phosphate dibasic dehydrate, and 0.24 g potassium phosphate monobasic anhydrous and transfer to a 1 l conical flask. Add 900 ml of water and dissolve the salts under magnetic stirring. Adjust the pH to 7.0 with 0.1 M HCl and bring the volume to 1 l.
4. Hypertonic PBS: Dissolve 2.7 g NaCl in 100 ml of PBS.

5. 0.05 M Sodium acetate buffer (pH 5.5), containing 134 g/l ammonium sulfate: Add 2.90 ml of glacial acetic acid to 700 ml of H_2O, and bring to pH 5.5 with 3 M NaOH. Add 134 g ammonium sulfate, dissolve, and bring volume to 1 l with H_2O.
6. 0.15 M Sodium citrate: Dissolve 22 g sodium citrate tribasic dehydrate in 500 ml of water.
7. 0.15 M Potassium phosphate (pH 6.0), containing 2 M ammonium sulfate: Dissolve 2 g potassium dihydrogen phosphate in 50 ml of water, and bring pH to 6.0 with 3 M KOH. Add 26.8 g ammonium sulfate, dissolve, and bring volume to 100 ml with H_2O.
8. 100 mM AEA: Weigh 35 mg AEA and dissolve in 1 ml of methanol in an autosampler vial.
9. Tetrahydrofuran/methanol/water/acetic acid mixture (25/30/45/0.1, vol/vol/vol/vol): Mix 250 ml of tetrahydrofuran, 300 ml of methanol, 450 ml of water, and 1 ml of glacial acetic acid. Degas the HPLC solvent just before use by purging helium through the solution for 15 min.
10. Methanol/water/acetic acid (75/25/0.1, vol/vol/vol): Mix 750 ml of methanol, 250 ml of water, and 1 ml of glacial acetic acid. Degas the HPLC solvent just before use by purging helium through the solution for 15 min.
11. Silylation reagent solution (pyridine/1,1,1,3,3,3-hexamethyldisilazane/trimethylchlorosilane, 5/1/1, vol/vol/vol): Mix 500 μl of anhydrous 99.8 % pyridine, 100 μl of 99.9 % 1,1,1,3,3,3-hexamethyldisilazane, and 100 μl of 99.0 % trimethylchlorosilane just before use in a 1 ml glass vial.

2.2 Preparation of Enzymes

Soybean lipoxygenase-1 (soyLOX, from Maple Glen cultivar) is a 15-lipoxygenase that can be obtained from Sigma-Aldrich. The protein concentration is estimated from the absorbance at 280 nm ($\epsilon_{280} = 1.6 \times 10^5\ \mathrm{l \times mol^{-1} \times cm^{-1}}$). The enzyme is stored at 4 °C at a concentration of 1.9 mg/ml (corresponding to 20 mM) in 0.05 M sodium acetate buffer (pH 5.5) containing 134 g/l ammonium sulfate. 5-Lipoxygenase, mostly isolated from potato, can be obtained from Cayman Chemicals. The enzyme is stored at 4 °C at a concentration of 1 mg/ml in 0.15 M potassium phosphate (pH 6.0) containing 2 M ammonium sulfate. Bovine lipoxygenase (bovLOX) is a 12-lipoxygenase isolated from bovine leukocytes, performing the entire purification at room temperature as follows. Bovine blood is collected in a vessel, containing 0.15 M sodium (100 ml per l) as an anticoagulant. The anticoagulated blood is centrifugated at $470 \times g$ for 20 min in a swing-out rotor, to separate blood cells from plasma: the lower part of the tube contains the red and white blood cells, and the upper part contains the platelet-rich plasma that is discarded. The leukocyte fraction is

separated from erythrocytes by lysis. To this end, 2 volumes of distilled water are added to the cell pellet and, after gently shaking for 30 s, 1 volume of hypertonic PBS is also added, to make the final suspension hypotonic. The mixture is centrifuged for 10 min at 470 × *g*, and the supernatant containing the lysed erythrocytes is removed. The pellet is resuspended in 10 ml of PBS, and the lysis procedure is repeated once more. The erythrocyte-free leukocyte pellet is suspended in 15 ml of PBS, and carefully layered on 14 ml of Ficoll-Paque plus (GE healthcare) in a 50 ml tube, and centrifuged at 400 × *g* for 40 min in a swing-out rotor. Leukocytes are found at the bottom of the tube, whereas the lymphocytes and monocytes are found on top of the Ficoll-Paque plus. Leukocytes are washed with PBS and centrifugated at 2000 × *g*, and the final cell pellet is resuspended in 10 ml of PBS.

3 Methods

3.1 Production of Hydroperoxy- AEA

1. Incubate for 45 min at room temperature 1 U enzyme (soyLOX, barLOX or bovLOX) with 5 μmol AEA (50 μl from a 100 mM stock) in 50 ml of rigorously stirred, air-saturated appropriate buffer: 0.1 M sodium borate buffer, pH 9.0 (for soyLOX), 0.1 M sodium phosphate buffer, pH 7.0 (for barLOX), or PBS, pH 7.4 (for bovLOX) (*see* **Notes 2–4**).
2. Stop reactions by acidifying the reaction mixtures to pH 4.0 with 3 M HCl.
3. Extract metabolites using 60 mg OASIS HLB (Waters) solid-phase extraction columns (or equivalent, like Bakerbond C18, J.T. Baker).Condition cartridge with 5 ml of methanol and 5 ml of H_2O, respectively. Do not allow the cartridge to run dry. Load reaction mixture into a 100 cc syringe connected to the SPE column via an SPE tube adaptor. Positive pressure is achieved by the plunger of the 100 cc syringe. Wash the column with 5 ml of H_2O until dryness, and elute the concentrated and purified reaction products with 2.5 ml of methanol.
4. Azeotropically evaporate residual water by co-evaporation with methanol under a gentle stream of nitrogen gas.
5. Dissolve products in 200 μl of methanol, and store at −25 °C until use.

3.2 Reduction of Hydroperoxy-AEA to Hydroxy-AEA

1. Reduce lipoxygenase products with a molar excess of $NaBH_4$ in 3 ml of methanol.
2. Stir reaction mixture for 30 min at 0 °C under a N_2 atmosphere.
3. Terminate reaction by adding 15 ml of water and acidify the mixture to pH 4.0 with 3 M HCl. Stir until gas production ends.

4. Extract and concentrate the reduced products with an SPE column, as described above.
5. Dissolve the metabolites in 100 µl of methanol.

3.3 Purification of Hydroxy-AEA

1. Purify the lipoxygenase metabolites with preparative HPLC on a Cosmosil 5C18-ARII (5 µm; 250×10 mm i.d.; Nacalai Tesque) column, using a tetrahydrofuran/methanol/water/acetic acid mixture (25/30/45/0.1, vol/vol/vol/vol) as the eluent, at a flow rate of 3 ml/min.
2. Dilute the collected fractions with a tenfold excess of water.
3. Concentrate the diluted fractions with an SPE column, as described above.
4. Dissolve the purified compounds in 200 µl of methanol.
5. Store compounds under N_2 at –25 °C.
6. Chiral separations of the purified sodium borohydride-reduced AEA metabolites can be carried out on a Chiralcel OD-R column (5 µm, 250×4.6 mm, Daicel) with methanol/water/acetic acid (75/25/0.1 vol/vol/vol) as the eluent, at a flow rate of 0.5 ml/min (*see* **Note 5**). Chirally pure AEA metabolites can be concentrated again with an SPE column as described above, and are stored under N_2 at –25 °C.

3.4 Analytical Characterization

1. Dry the hydroxyl-AEA derivatives under a stream of N_2 gas.
2. Dissolve them in $CHCl_3$ or $CDCl_3$, in order to perform FTIR- or ^{1}H-NMR measurements, respectively. Record IR spectra on an FTIR spectrometer from 4000 cm^{-1} to 400 cm^{-1} with a resolution of 4 cm^{-1} and a co-addition of 60 scans in a N_2 atmosphere at 20 °C. Correct the spectra with a co-addition of 60 blank scans and a manual baseline correction.
3. ^{1}H-NMR spectra are recorded in $CDCl_3$ with a Bruker AC 300 or 500 MHz spectrometer at 27 °C.
4. For GC-MS analysis of the hydroxyl position, dry aliquots of the purified hydroxy-AEA metabolites under a stream of N_2 gas.
   (a) Redissolve in 1 ml of hexane and hydrogenate the double bonds with a catalytic amount (5 %) of palladium on calcium carbonate under a gentle stream of H_2 gas.
   (b) After 30 min, remove the catalyst by filtration over a prewashed (hexane) piece of cotton wool an empty pasteur pipette.
   (c) Evaporate hexane under N_2.
   (d) Add 50 µl of freshly prepared silylation reagent solution.
   (e) After 30 min at room temperature, evaporate the silylation reagent under a stream of N_2 and redissolve the residue in 10 µl of hexane.

(f) Analyze aliquots by GC/MS, equipped with a CP-Sil 5 CB-MS (or equivalent) column (25 m × 0.25 mm × 0.25 μm). The column temperature is held at 200 °C for 1 min, increased in 13 min to 330 °C, and held at this temperature for 2 min. Mass spectra are recorded under electron impact with an ionization energy of 70 eV.

5. Record IR spectra on an FTIR spectrometer from 4000 to 400 cm^{-1} with a resolution of 4 cm^{-1} and a co-addition of 60 scans in a N_2 atmosphere at 20 °C. Correct the spectra with a co-addition of 60 blank scans and a manual baseline correction.
6. Record UV absorption spectra from 200 to 300 nm on 40 μM hydroxyl-AEA in methanol with a diode array spectrophotometer (*see* **Note 6**).
7. Record CD spectra (resolution of 1 nm and 20 scans, 10 nm/min) in a 1.0 cm cuvette at 20 °C on a CD spectrophotometer from 210 to 270 nm, using metabolites at a concentration of 40 μM in methanol (*see* **Note 7**).

3.5 Chemical Data of the Oxygenated Derivatives

5R/S-HAEA [5(R/S)-Hydroxy-eicosa-6E,8Z,11Z,14Z-tetraenoyl-N-(2-hydroxyethyl) amine]. Produced by barLOX: ^1H NMR ($CDCl_3$): δ 6.55 (dd, J = 13.6, 10.6 Hz 1H), 6.02 (t J = 10.5 1H), 5.71 (dd, J = 13.6 Hz, 1H), 5.39 (m, 5H), 4.16 (m, 1H), 3.72 (t, 2H), 3.42 (q, 2H), 2.97 (m, 2H), 2.81 (m, 2H), 2.22 (t, J = 7.8 Hz, 2H), 2.11 (m, 2H), 1.72 (q, J = 6.2 Hz 2H), 1.34 (m, 6H), 0.89 (t, J = 6.2 Hz, 3H). $NaBH_4$ and H_2 reduced, hydrogenated trimethyl silyl ether GC/MS m/z 515 [M^+], 500 [M^+–CH_3], 313 [$C_{16}H_{32}OTMS^+$], 304 [M^+–$C_{15}H_{31}$], 214 [304-TMSOH], 116 [$C_2H_3OTMS^+$], 73 [TMS^+].

11S-HAEA [11(S)-Hydroxy-eicosa-5Z,8Z,12E,14Z-tetraenoyl-N-(2-hydroxyethyl)amine]. Produced by barLOX: ^1H NMR ($CDCl_3$): δ 6.52 (dd, J = 11.0; 10.1 Hz 1H), 5.98 (t, J = 11.0 Hz 1H), 5.69 (dd, J = 15.1; 6.5 Hz 1H), 5.40 (m, 5H), 4.23 (q, 2H), 3.72 (t, J = 4.6 Hz, 2H), 3.42 (q J = 5.5 Hz; 2H), 2.81 (m, 2H), 2.32 (m, 2H), 2.22 (t, J = 7.4 Hz; 2H), 2.12 (m, 4H), 1.73 (q, 2H), 1.25 – 1.38 (m, 6H), 0.88 (t, J = 6.9 3H). $NaBH_4$ and H_2 reduced, hydrogenated trimethyl silyl ether GC/MS m/z 515 [M^+], 500 [M^+–CH_3], 388 [M^+–C_9H_{19}], 229 [$C_{10}H_{20}OTMS^+$], 116 [$C_2H_3OTMS^+$], 73 [TMS^+].

12S-HAEA [12(S)-Hydroxy-eicosa-5Z,8Z,10E,14Z-tetraenoyl-N-(2-hydroxyethyl)amine]. Produced by bovLOX: ^1H NMR ($CDCl_3$): δ 6.58 (dd, J = 14.2 Hz 1H), 5.99 (t, J = 10.5 Hz 1H), 5.74 (dd, J = 6.2 Hz 1H), 5.40 (m, 5H), 4.25 (q, 1H), 3.72 (t, J = 4.6 Hz 2H), 3.42 (q, J = 4.6 Hz 2H), 2.95 (m, 2H), 2.33 (m, 2H), 2.21 (t, J = 7.5 Hz 2H), 2.10 (m, 4H), 1.74 (q, J = 7.3 Hz, 2H), 1.28 (m, 6H), 0.89 (t, J = 6.9 Hz 3H). $NaBH_4$ and H_2 reduced, hydrogenated trimethyl silyl ether GC/MS m/z 515 [M^+], 500 [M^+–CH_3], 402

[M^+–C_8H_{17}+], 215 [$C_9H_{18}OTMS^+$], 116 [$C_2H_3OTMS^+$], 73 [TMS^+].

15S-HAEA [15(S)-Hydroxy-eicosa-5Z,8Z,11Z,13E-tetraenoyl-N-(2-hydroxyethyl)amine]. Produced by soyLOX: ^{1}H NMR ($CDCl_3$): δ 6.55 (dd, J=15.4;12,2 Hz 1H), 6.00 (t, J=10.7 Hz, 1H), 5.72 (dd, J=7.2 Hz, 1H), 5.40 (m, 5H), 4.12 (q, 1H), 3.72 (t, 2H) 3.42 (q, 2H), 2.97 (m, 2H), 2,82 (m, 2H), 2.22 (t, J=7.5 Hz, 2H), 2.11 (m, 2H), 1.72 (q, J=7.3 Hz, 2H), 1.56 (m, 2H), 1.31 (m, 6H), 0.89 (t, J=6.9 Hz, 3H). $NaBH_4$ and H_2 reduced, hydrogenated trimethyl silyl ether GC/MS m/z 515 [M^+], 500 [M^+–CH_3], 444 [M^+–C_5H_{11}], 173 [$C_6H_{11}OTMS^+$], 116 [$C_2H_3OTMS^+$], 73 [TMS^+].

5,15-diHAEA[5,15-Dihydroxy-eicosa-6E,8Z,11Z,13E-tetraenoyl-N-(2-hydroxyethyl) amine]. Produced by soyLOX: ^{1}H NMR ($CDCl_3$): δ 6.58 (m, 2H), 6.01 (m, 2H), 5.72 (m, 2H), 5.43 (m, 2H), 4.21 (m, 2H), 3.72 (t, J=4.6 Hz, 2H), 3.42 (q, J=4.6 Hz, 2H), 2.97 (m, 2H), 2.28 (t, J=6.9 Hz, 2H), 1.76 (m, 2H), 1.57 (m, 4H), 1.30 (m, 6H), 0.89 (t, 3H). $NaBH_4$ and H_2 reduced, hydrogenated trimethyl silyl ether GC/MS m/z 603 [M^+], 588 [M^+–CH_3], 532 [M^+–C_5H_{11}], 304 [M^+–$C_{15}H_{31}$], 214 [304-TMSOH], 116 [$C_2H_3OTMS^+$], 73 [TMS^+].

8,15-diHAEA[8,15-Dihydroxy-eicosa-5Z,9E,11Z,13E-tetraenoyl-N-(2-hydroxyethyl) amine]. Produced by soyLOX: ^{1}H NMR ($CDCl_3$): δ 6.70 (m, 2H), 5.97 (m, 2H) 5.74 (m, 2H), 5.46 (m,2H), 4.27 (m, 1H), 4.18 (m, 1H), 3.72 (t, J=4.6 Hz, 2H), 3.42 (q, J=4.6 Hz, 2H), 2.32 (m, 2H), 2.19 (t, J=6.9 Hz, 2H), 2.09 (q, 2H), 1.70 (m, 2H), 1.50 (m, 2H), 1.32 (m, 6H), 0.88 (t, 3H). $NaBH_4$ and H_2 reduced, hydrogenated trimethyl silyl ether GC/MS m/z 603 [M^+], 588 [M^+–CH_3], 532 [M^+–C_5H_{11}], 346 [M^+–$C_{12}H_{24}OTMS$], 173 [$C_6H_{11}OTMS^+$], 116 [$C_2H_3OTMS^+$], 73 [TMS^+].

4 Notes

1. This procedure is not suitable for the generation of lipoxygenase products of 2-AG, because of the chemical instability of this endocannabinoid that rapidly isomerizes to 1-AG during the incubation.
2. All solutions where organic solvents or reagents are involved should be in glass. Small beaker glasses (20–100 ml) or autosampler vials (1–20 ml) with PTFE septa are recommended.
3. Use 5 U soyLOX to produce 8(*S*),15(*S*)-dihydroxy AEA and 5(*S*),15(*S*)-dihydroxy-AEA.
4. Incubate 2 U barLOX with 5 μmol AEA in 50 ml of 0.1 M phosphate buffer (pH 7.4) for 60–90 min at 20 °C, to produce 5(*S*)- and 11(*S*)-hydroxy AEA.

5. Incubate 2 U bovLOX with 5 μmol AEA in 50 ml of 0.1 M phosphate buffer (pH 7.4) for 60–90 min at 20 °C, to produce 12S-hydroxy-AEA. Skip **step 2**, because the products are already reduced.
6. Alternatively, hydroxyl-AEAs can be separated with a 20-min linear gradient of methanol/water/acetic acid, from 60/40/0.1 (vol/vol/vol) to 95/5/0.1 (vol/vol/vol), at 1 ml/min on an analytical Cosmosil 5C18 ARII (5 μm, 250×4.6 mm) column.
7. A molar absorbance of 23,000 M^{-1} cm^{-1} at 236 nm is used to quantify hydroxy-AEAs. For 8,15- and 5,15-dihydroxy-AEAs, molar absorbances of 40,000 M^{-1} cm^{-1} at 269 nm and of 33,500 M^{-1} cm^{-1} at 243 nm are used, respectively.

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