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Supplementary data

Anandamide and Analogous Endocannabinoids:

A Lipid Self-Assembly Study

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Anandamide and Anandamide Analogue Lipid Synthesis

Arachidonoyl chloride.

3 g of Arachidonic acid (>98.5% purity; Fluka) was mixed into 30 ml of dry dichloromethane (DCM) with a drop of dimethylformate (DMF) and cooled under argon to below 0°C. 2 eq. of oxalyl chloride (2.502 g) made to 2M solution in dry DCM was added drop wise within 15 min, keeping the temp at -5°C and stirring was continued for the next 4 hours, after which time NMR of an aliquot showed some unreacted acid still present, therefore the mixture was left in the freezer overnight. At this stage the reaction mixture was a pale yellow clear solution. The next day it was allowed to come to RT and the solvent was evaporated off (no heating applied). The residue, a red-yellow oily liquid had some visible polymeric type material. It was taken up in dry petroleum spirit 30/50, filtered off from impurities and evaporated again resulting in 3.04 g of a light yellow mobile liquid; yield 95.5%.

^{1}H NMR ($CDCl_3$, 200 MHz) δ 5.48-5.30, m, 8H, =CH; 2.78-2.93, m, 8H, =CHC$\underline{H_2}$CH= & CH_2CO; 2.01-2.17, m, 4H, C$\underline{H_2}$CH=; 1.75-1.86, m, 2H, C$\underline{H_2}CH_2CO$; 1.25-1.40, m, 6H, $(C\underline{H_2})_3CH_3$; 0.87-0.92, m, 3H, CH_3.

Anandamide (AE). Ethanolamine (Aldrich, 99+% pure), 10 eq. (6.01 g; 98.3 mmols) was diluted with 30 ml of dry dichloromethane and placed in a 100 ml 2-neck flask equipped with a magnetic stirrer, a thermometer and a pressure equalizing dropping funnel and cooled under argon atmosphere to -5°C. Both, the reaction flask and the funnel were covered with aluminium foil. 1 eq. of arachidonoyl chloride (3.81 g; 9.81 mmols) diluted with 25 ml of dry DCM was added drop wise, over 2.5 hours, keeping the temperature below 0°C. One hour later NMR of an aliquot showed the completion of the acylation and the reaction mixture was worked up as follows: 50 ml of cold 10% brine was added, the phases separated and the organic layer was washed again with 50 ml and 25 ml of 10% brine, then the aqueous phase was backwashed with 30 ml DCM. The combined DCM solution (yellow colour) was then washed twice with 10 ml portions of 4% citric acid and finally with 25 ml of 10% brine, dried over molecular sieves and evaporated. 2.7 g of yellow mobile oil was obtained, that was flashed with N_2 and placed in a freezer. At low temperature it solidified to a yellowish-white product. Yield 78%. HPLC Purity 99.15%

^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.90, bs, 1H, NH; 5.30-5.43, m, 8H, =CH; 3.72, bs, 2H, CH_2OH; 3.40-3.44, m, 2H, CH_2NH; 2.79-2.85, m, 6H, =CHC$\underline{H_2}$CH=; 2.62, bs, 1H, OH;

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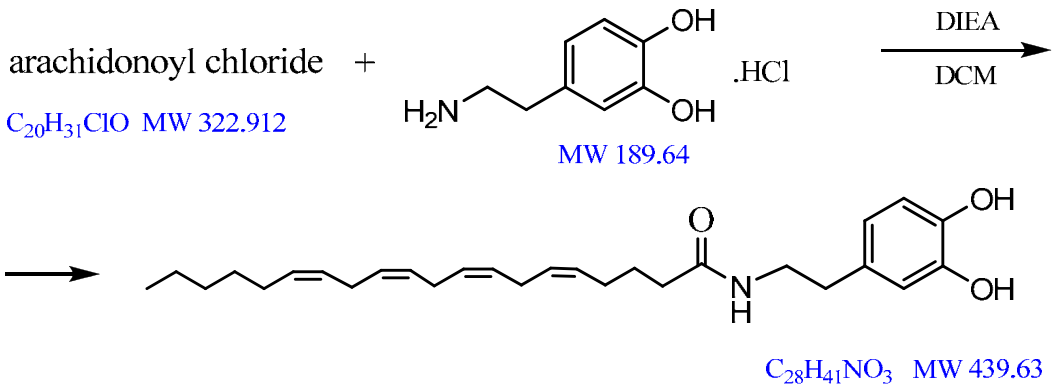
2.19-2.23, m, 2H, CH_2CO; 2.12, q, *J* 6.8 Hz, 2H, CH_2CH=; 2.05, q, *J* 6.9 Hz, 2H, CH_2CH=;1.69-1.77, m, 2H, $C\underline{H}_2CH_2CO$; 1.25-1.39, m, 6H, $(C\underline{H}_2)_3CH_3$; 0.89, t, *J* 6.9 Hz, 3H, CH_3.

Arachidonyl Dopamine



Icosa-5,8,11,14-tetraenoic acid [2-(3,4-dihydroxy-phenyl)-ethyl]-amide

Dopamine hydrochloride 98% purity, Sigma (3.84 g, 6 eq.) was dissolved in 40 ml of dry DMF; keeping the reaction flask under argon. 3.053 g (7 eq) of DIEA was added to it and the solution was cooled to -20 °C.

1.09 g (I eq.) of arachidonoyl chloride was diluted with 20 ml of dry DCM and added drop wise, over 45 min to the amine solution. After the addition the mixture was further kept in a cooling bath for ~2 hours, during which time the temperature, reached 0°C.

The crude product was purified on a silica column; chloroform was used for elution. HPLC showed 98.43% purity.

2-(Arachidonoyloxymethyl)oxirane

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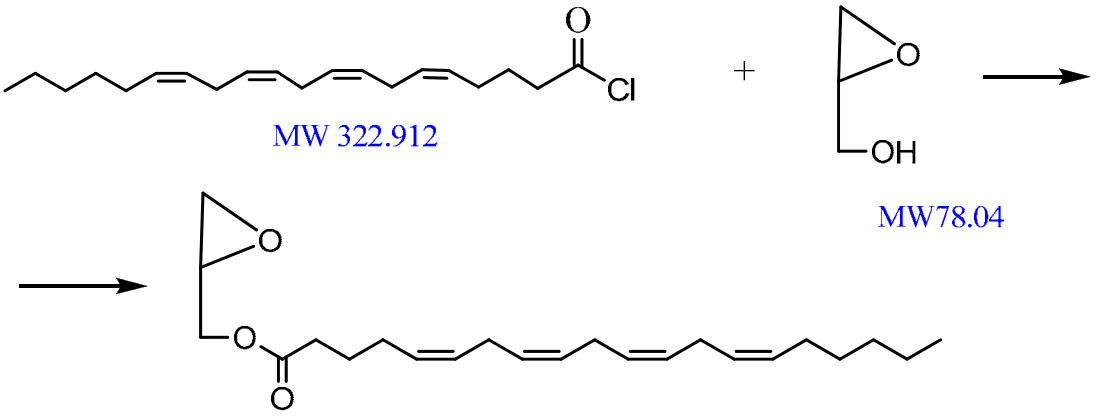

$C_{23}H_{36}O_3$ MW 360.54

1.84 g (5.698 mmol) of arachidonoyl chloride diluted with 10 ml of dry DCM was added to a solution of glycidol (0.351 g; 4.7484 mmol) and 4-DMAP (0.580 g; 4.7484mmol) in 10 ml of dry DCM at room temperature. The dropping funnel and the reaction flask were covered with aluminium foil and maintained under argon atmosphere, and the reaction flask was slightly cooled during the addition.. After 30 minutes, the reaction flask was protected from moisture and left overnight in the refrigerator. The reaction mixture, a clear yellow solution, was brought to room temperature (under argon) and subsequently filtered through a pad of silica (5 g), under nitrogen. 150 g of DCM was used to flash the silica. Evaporation at RT resulted in 1.96 g of yellow mobile oil. The crude product was purified by column chromatography (eluent: pentane –ethyl acetate 90 : 10 v/v) resulting in 0.866 g of a colourless clear oil (the target compound).

^{1}H NMR ($CDCl_3$, 400 MHz) 7.61, br.s, 1H (O**H**); 6.78, d, *J* 8.0 Hz, 1H (**H**27); 6.72, d, *J* 1.5 Hz, 1H (**H**24); 6.54, dd, *J* 8.0, 1.5 Hz, 1H (**H**28); 5.99, br.s, 1H (O**H**); 5.61, m, 1H (N**H**); 5.41-5.28, m, 8H (**H**5, 6, 8, 9, 11, 12, 14, 15); 4.46, dt, *J* 7.1, 5.6 Hz, 2H (**H**21); 2.86-2.73, m, 6H (**H**7, 10, 13); 2.67, t, *J* 7.1 Hz, 2H (**H**22); 2.15, m, 2H (**H**2); 2.08-2.00, m, 4H (**H**4, 16); 1.70-1.62, m, 2H (**H**3); 1.37-1.21, m, 6H (**H**17, 18, 19); 0.86, t, *J* 6.8 Hz, 3H (**H**20).

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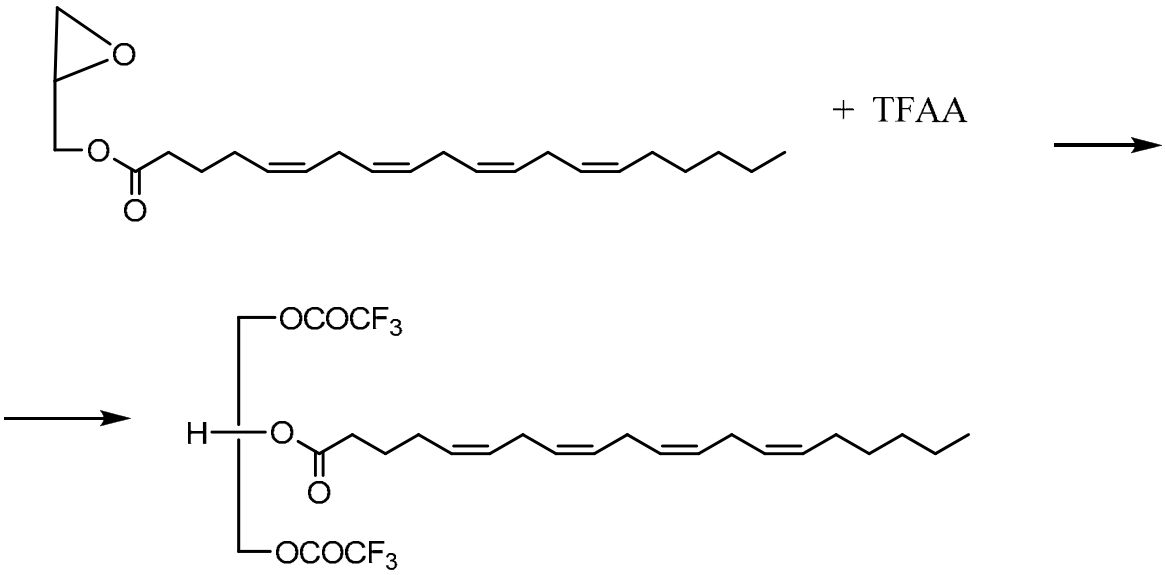
2-Arachidonoyl-1,3-bis(trifluoroacetyl)glycerol



$C_{27}H_{36}F_6O_6$ MW 570.57

To a solution of glycidyl ester (0.866 g. 2.4019 mmol) in 8 ml of DCM, TFAA (4 eq., 2.0178g) in 8 ml DCM was added at -20 °C over 0.5 h, and the reaction mixture was brought to room temperature. Evaporation at room temperature resulted in 1.47 g of a mobile yellow liquid crude material, which was filtered through 5 g of silica, using DCM for elution. 1.1 g of the purified material was obtained.

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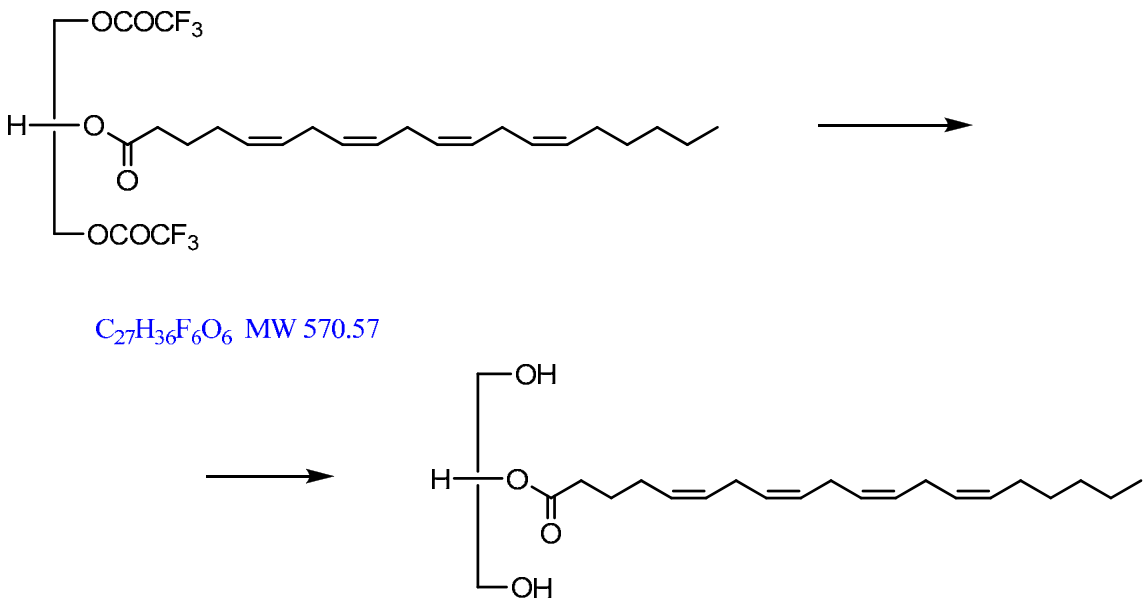
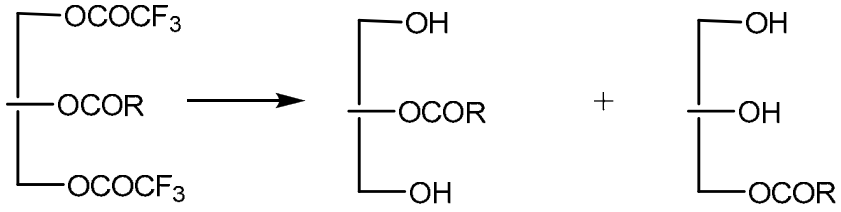
Scheme 4: 2-Arachidonoyl glycerol (2AG)



2-arachidonoyl-1,3-bis(trifluoroacetyl)glycerol is readily hydrolysed to the unprotected glycerol derivative in contact with an aqueous solvent. Additionally, the aqueous environment accelerates isomerisation of 2-acylglycerol to 1-acylglycerol.

Scheme 5: Isomerisation of acylglycerol



The two isomers can be resolved with an analytical column resolves as isomer 2 has a shorter retention time. However, prep HPLC requires very careful cutting of fractions.

0.525 g of 2-arachidonoyl-1,3-bis(trifluoroacetyl)glycerol (0.92 mmol) was dissolved under argon in 7 ml pentane – DCM 3:1 v/v mixture. The solution was cooled to -30°C, and a mixture of 0.71g (10eq) pyridine and 0.4806 g methanol (15 eq) in 5 ml of pentane was added via syringe at the above temperature. Due to a very strong exothermic effect, the reaction mixture temperature increased to -20°C and was maintained that this temperature for 1/2h after which it was allowed to come to room temperature. The solvent was evaporated off in a 40 °C water bath resulting in 400 mg of yellow oil,

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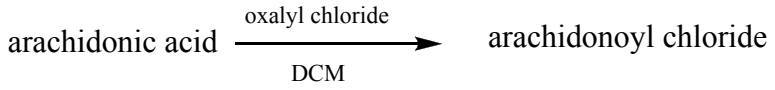
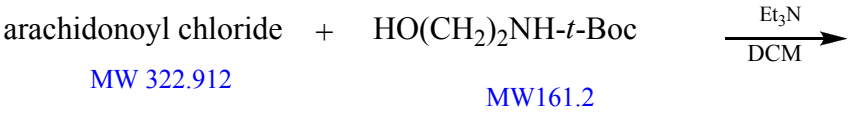
which was the purified by HPLC. Purification resulted in the following: 61 mg of isomer 2; 8 mg of isomer 1; 25 mg of a mixture of both isomers.

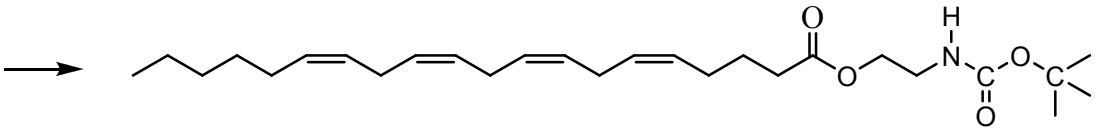
^{1}H NMR ($CDCl_3$, 400 MHz) 5.41-5.31, m, 8H (**H**5, 6, 8, 9, 11, 12, 14, 15); 4.93-4.88, m, 1H (**H**21); 3.84-3.87, m, 4H (**H**22, 23); 2.85-2.77, m, 6H (**H**7, 10, 13); 2.37, t, *J* 7.5 Hz, 2H (**H**2); 2.14-1.99, m, 4H (**H**4, 16); 1.75-1.68, m, 2H (**H**3); 1.38-1.26, m, 6H (**H**17, 18, 19); 0.87, t, *J* 6.8 Hz, 3H (**H**20).

Scheme 6: Virodhamine

Step 1







MW 448.112

Arachidonoyl chloride 1.21 g (3.747 mmol) diluted with 10 ml of dry DCM was placed in a pressure equalizing funnel, protected from light with Al foil. Boc protected ethanolamine [tert-butyl N-(2-hydroxyethyl)carbamate] 0.7248 g (4.4964 mmol), triethylamine 0.7583g (7.494 mmol) and DMAP 0.0457 g (0.3747 mmol) were dissolved in 10 ml of dry DMF in a flask covered with Al foil and equipped with a magnetic bar and a thermometer, and cooled under argon to -50 ^{o}C. Addition of the acid chloride was done drop wise, over 1 hour, keeping the temp ≤ -50 ^{o}C. Stirring in the cooling bath was continued for the next 3 hours, over which time the temperature reached 0 ^{o}C. The reaction mixture was then left in the refrigerator overnight.

The reaction mixture was diluted with DCM and washed 3 times with 10% NaCl followed by 10% citric acid and then the organic layer was filtered through a 1 cm silica pad. The solution was then evaporated and additionally dried on a vacuum pump. 1.57 g of yellow oil was obtained; crude product yield 93.5%.

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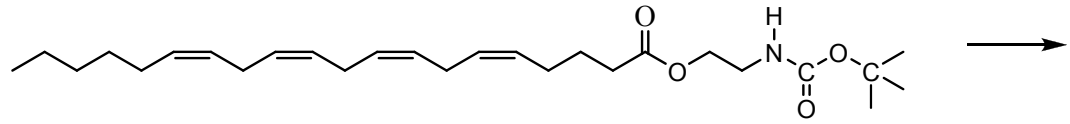
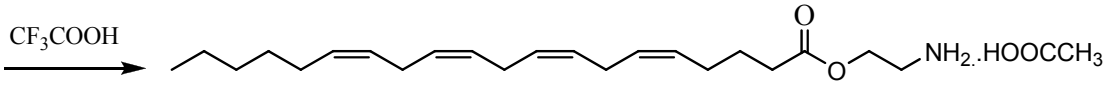
As Virodhamine is highly unstable, it was converted to the chemically stable form, Virodhamine trifluoroacetate for storage purposes.

Scheme 7: Virodhamine trifluoroacetate



MW 448.112



$C_{24}H_{38}F_3NO$ MW 461.558

1.57 g of Boc- protected Virodhamine was dissolved in 8 ml DCM in argon atmosphere, cooled to -5 ^{o}C, and 4 ml of trifluoroacetic acid was added drop wise over 10 min. It was then brought to room temperature, with continuous stirring for another hour. Excess of trifluoroacetate (TFA) and DCM were evaporated off; the resulting in a dark oil. Toluene was added to co-distill off the residual TFA HPLC analysis indicated 78.77% purity. The crude material was then purified by HPLC under the following conditions; mobile phase used: 80% aq. Acetonitrile (can) with TFA addition. Two fractions were obtained (0.644 g with 99.25% purity and 0.309 g with 98.43% purity).

Treatment of the virodhamine salt with alkali hydroxide, carbonate or hydrogen carbonate with simultaneous extraction into organic solvents was produces free Virodhamine. The compound undergoes rapid rearrangement in part to anandamide and /or self-promoted decomposition, thus any physicochemical characterization of this compound was done very rapidly in an attempt to avoid degradation/rearrangement.

^{1}H NMR ($CDCl_3$, 400 MHz) 8.09, br.s, 3H (N**H**$_3^+$); 5.42-5.26, m, 8H (**H**5, 6, 8, 9, 11, 12, 14, 15); 4.35-4.29, m, 2H (**H**21); 3.27-3.21, m, 2H (**H**22); 2.85-2.72, m, 6H (**H**7, 10, 13); 2.35, t, *J* 7.5 Hz, 2H (**H**2); 2.11-2.00, m, 4H (**H**4, 16); 1.66, tt, *J* 7.8, 7.5 Hz, 2H (**H**3); 1.37-1.22, m, 6H (**H**17, 18, 19); 0.86, t, *J* 6.8 Hz, 3H (**H**20).