Chemistry and Physics of Lipids 165 (2012) 705–711

Contents lists available at SciVerse ScienceDirect

Chemistry and Physics of Lipids

journal homepage: www.elsevier.com/locate/chemphyslip

Optimized synthesis and characterization of N-acylethanolamines and O-acylethanolamines, important family of lipid-signalling molecules

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ARTICLE INFO

Article history:
Received 31 May 2012
Received in revised form 29 June 2012
Accepted 30 June 2012
Available online 28 July 2012

Keywords:
Endocannabinoids
N-acylethanolamines
O-acylethanolamines
O,N-acyl migrations
N,O-acyl migrations

ABSTRACT

The endocannabinoid anandamide (N-arachidonoylethanolamine, AEA), a physiologically occurring bioactive compound on CB1 and CB2 receptors, has multiple physiological functions.

Since the discovery of AEA additional non-cannabinoid endogenous compounds such as N-palmitoylethanolamine (PEA), and N-oleoylethanolamine (OEA) have been identified from mammalian tissues. Virodhamine (O-arachidonoylethanolamine, VA) is the only identified new member of the endocannabinoid family that is characterised by an ester linkage between acylic acid and ethanolamine instead of the amide linkage found in AEA and others non-cannabinoid N-acylethanolamines. It has been reported, as a cautionary note for lipid analyses, that VA can be produced nonenzymatically from AEA (and vice versa) as consequence of O,N-acyl migrations. O,N-acyl migrations are well documented in synthetic organic chemistry literature, but are not well described or recognized with regard to methods in lipid isolation or lipid enzyme studies.

We here report an economical and effective protocol for large scale synthesis and characterization of some N- and O-acylethanolamines that could be useful as reference standards in order to investigate their possible formation in biological membranes, with potentially interesting biological properties.

1. Introduction

Endocannabinoids (ECs) including N-acylethanolamines (NAEs) are implicated in many physiological and disease states and elicit their activities via the cannabinoid receptors. Anandamide (N-arachidonoylethanolamine, AEA, **1a**) is the most characterized endocannabinoid and has been detected in many tissues and biofluids including human plasma and central nervous system. The endocannabinoid-like NAEs, N-palmitoylethanolamine (PEA), N-oleoylethanolamine (OEA) and N-linoleoylethanolamine (LEA) are described as entourage compounds because they elicit physiological effects similar to AEA but have little or no affinity for cannabinoid receptors. Although the pharmacology and bioactivity of these molecules have been extensively studied during the past decade, many basic questions remain unanswered.

During the last years, new members of the endocannabinoid family have been identified. Virodhamine (O-arachidonoylethanolamine, VA, **2a**) was at first isolated from rat brain and identified as a CB2 agonist (Porter et al., 2002) and it was also found in several rat peripheral tissues including skin, spleen, kidney and heart. VA is similar to AEA, in being formed from arachidonic acid and ethanolamine, but contains an ester bond rather than amide linkage. Information about VA biosynthesis, storage and release is still lacking and its pharmacology is poorly understood.

VA can also be produced nonenzymatically from **1a** as consequence of O,N-acyl migration. Markey et al. (2000) have shown that O-acylethanolamines (OAEs) are converted to N-acylethanolamines (NAEs) investigating the acid- and base-catalysed production of AEA in chromatographic fractions of rat brain extracts not containing AEA. OAEs are converted to NAEs through a cyclic intermediate that is also formed during chemical reactions commonly used for derivatization of acylethanolamines in a *facile* manner under mildly basic conditions, whereas the reverse reaction – formation of OAEs from NAEs – is catalysed by acid.

Studies on the endogenous brain levels of AEA and its biosynthesis have led several investigators to use basic conditions (incubations at pH 9, reaction quenching with strong base, ammonia containing thin-layer chromatographic solvents). These conditions could result in O-to-N acyl migration of VA, present in tissue, to AEA (Bachur and Udenfriend, 1966; Deutsch and Chin, 1993; Devane and Axelrod, 1994). These observations suggested the possible presence of OAEs as minor constituents in membranes

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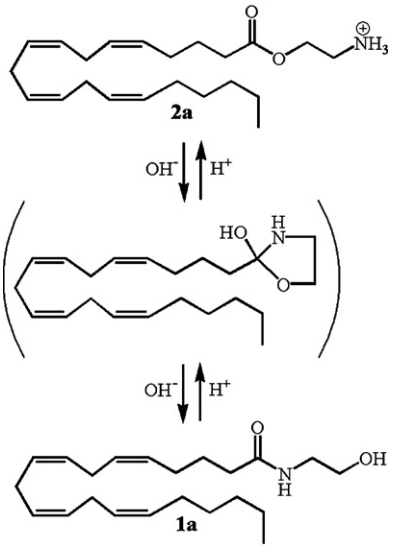
http://dx.doi.org/10.1016/j.chemphyslip.2012.06.010



Scheme 1. Interconversion of OEA **2a** to AEA **1a** and vice versa.

as the environment of endosomes and ischemic tissues are acidic in nature (Murphy et al., 1984; Rybak and Murphy, 1998; Boomer and Thompson, 1999).

It is likely that these minor constituents of membranes might have escaped detection for a long time due to their conversion to NAEs under conditions employed in the extraction, purification or detection (e.g., TLC using solvents containing ammonia) (Scheme 1). These observations indicate that OAEs containing other fatty acyl moieties may also be present in biological membranes, with potentially interesting biological properties.

More recently (Ferreri et al., 2008) it has been also reported that in the synthesis of AEA **1a** starting from methyl arachidonate in the presence of ethanolamine the expected compound **1a** was accompanied by a side-product that, after isolation and characterization, was identified as VA **2a**. This compound can be formed by an intramolecular nucleophilic attack, a process that is known to occur in carboxylic acid derivatives (Bender, 1960). By a careful examination of the reaction conditions and work-up procedure, the authors concluded that the rearrangement could occur during the acidification step required to eliminate the amine excess. Indeed, this rearrangement also occurred by leaving the deuterated chloroform solution of AEA prepared for the NMR analysis at room temperature for 10 h, reaching a 50% conversion (Ferreri et al., 2008).

Finally useful tandem mass spectrometry-based analytical techniques are studied most frequently to quantify ECs in biological fluids and tissues (Zoerner et al., 2011). Although tandem mass spectrometry by the nature of the technique has extremely good selectivity, the detection of AEA can be complicated by the presence of VA (Richardson et al., 2007), which has an identical molecular weight and which fragments to a daughter ion of comparable *m*/*z*.

Thereby the characterization and determination of biological formation of either N- or O-acylethanolamines requires awareness and consideration of the chemistry of this class of molecules and their proclivity to undergo N-to-O and O-to-N acyl rearrangements.

In this regard it is of considerable interest the availability of OAEs to be compared with corresponding NAEs. In the present study we report an economical and effective protocol for synthesis of a series of NAEs (**1a–1g**) and corresponding OAEs (**2a–2g**) (Fig. 1). The synthesized molecules could be used as reference compounds for comparison of properties of OAEs and NAEs or as standards in MS spectroscopy. A detailed NMR spectroscopic characterization has allowed the assignment of all proton signals. The complete NMR data of the NAEs and OAEs can be useful for the identification of

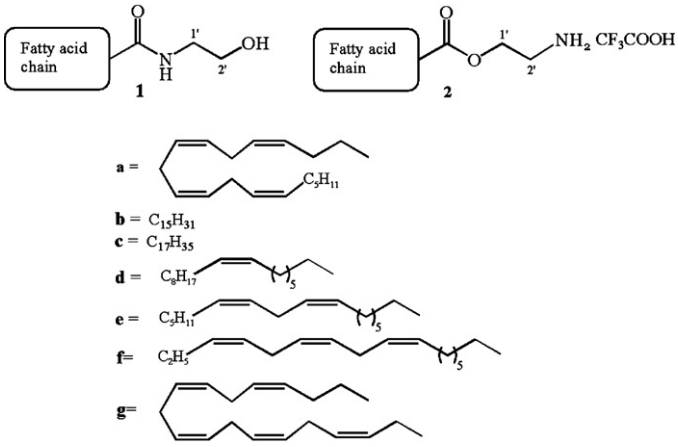


Fig. 1. Structure and numbering of the NAEs (**1a–1g**) and OAEs (**2a–2g**).

newly isolated or synthesized derivatives. Moreover NMR studies performed in acidic and basic environments allowed to define migration conditions.

2. Experimental

2.1. Materials

Column chromatography was performed on Silica Gel 60 (70–230 mesh) using the specified eluents. The progress of the reactions was monitored by analytical thin-layer chromatography (TLC) on pre-coated glass plates (Silica Gel 60 F254-plate-Merck, Darmstadt, Germany) and the products were visualized by UV light.

Purity of all compounds (≥99%) was verified by thin layer chromatography and NMR measurements. Elemental analyses were obtained for all intermediates and are within ±0.4% of theoretical values.

The chemicals and solvents were obtained from Sigma–Aldrich and used without further purification.

2.2. Instruments

Melting points were determined with a Stuart Scientific SMP3 melting point apparatus and are uncorrected.

^{1}H NMR spectra were recorded in $CDCl_3$ (isotopic enrichment 99.95%) or pyridine-d_5 (isotopic enrichment 99.98%) solutions at 300 K using a Bruker AVANCE 500 instrument (500.13 MHz for ^{1}H) using 5 mm inverse detection broadband probes and deuterium lock. Chemical shifts (δ) are given as parts per million relative to the residual solvent peak (7.26 ppm for ^{1}H) and coupling constants (J) are in Hertz. The experimental error in the measured ^{1}H–^{1}H coupling constants is ±0.5 Hz. The splitting pattern abbreviations are as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet, and bs, broad peak. For two-dimensional experiments, Bruker microprograms using gradient selection (gs) were applied.

2.3. Synthesis of NAEs (*1a–1g*)

2.3.1. General method

These compounds were synthesized according to the procedure described previously with slight modifications (El-Faham and Albericio, 2010)

The appropriate acid (0.15 mmol), (1-cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholino-carbenium hexafluoro phosphate (COMU, 64.2 mg, 0.15 mmol), and DIPEA (0.05 ml, 0.30 mmol) were dissolved in anhydrous CH_2Cl_2 (0.5 ml) and CH_3CN (2.5 ml) and the resulting orange-red solution was

stirred at rt for 10 min under a nitrogen atmosphere. Ethanolamine (**3**) (0.15 mmol) in CH_3CN (0.2 ml) was then injected into the reaction mixture and vigorous stirring at rt was continued until TLC (CH_2Cl_2/MeOH 98:2) confirmed the completion of the reaction (3–6 h). The reaction mixture was diluted with CH_2Cl_2 (3 ml) and the resulting mixture was washed with 5% HCl, saturated $NaHCO_3$ and brine. The organic layer was collected, dried over anhydrous Na_2SO_4, filtered. The solvent was evaporated under reduced pressure and crude purified by flash chromatography.

*2.3.2. N-arachidonoylethanolamine (AEA; **1a**)*

The title amide **1a** was prepared from arachidonic acid (30 mg) as colourless oil with isolated yield 29 mg (85%). *Rf* 0.35 (CH_2Cl_2/MeOH 95:5). ^{1}H NMR δ: 0.91 (3H, t, J = 7.6, 20-CH_3), 1.28–1.41 (6H, m, 17–19 CH_2), 1.75 (2H, tt, J = 7.0, 7.6, 3-CH_2), 2.08 (2H, dq, J = 7.6, 7.6, 16-CH_2), 2.14 (2H, dt, J = 7.0, 7.4, 4-CH_2), 2.24 (2H, t, J = 7.0, 2-CH_2), 2.83–2.86 (6H, m, 7- 10- and 13-CH_2), 3.44 (2H, dt, J = 5.1, 5.4, 1′-CH_2), 3.74 (2H, dt, J = 4.8, 5.5, 2′-CH_2), 5.33–5.44 (8H, m, 5- 6- 8- 9- 11- 12- 14- and 15-CH), 6.00 (1H, bs, NH), the OH signal was too broad to be detected. Physical and spectroscopic data were in accordance with those reported (Wyffels et al., 2009).

*2.3.3. N-palmitoylethanolamine (PEA; **1b**)*

The title amide **1b** was prepared from palmitic acid (50 mg) as white solid with isolated yield 53 mg (90%). *Rf* 0.52 (CH_2Cl_2/MeOH 95:5). Mp 99–100 °C. ^{1}H NMR δ: 0.91 (3H, t, J = 6.5, 16-CH_3), 1.22–1.34 (24 H, m, 4–15 CH_2), 1.65 (2H, tt, J = 7.0, 7.0, 3-CH_2), 2.22 (2H, t, J = 7.0, 2-CH_2), 2.65 (1H, bs, OH), 3.45 (2H, dt, J = 5.1, 5.4, 1′-CH_2), 3.74 (2H, dt, J = 4.8, 5.1, 2′-CH_2), 6.02 (1H, bs, NH). Physical and spectroscopic data were in accordance with those reported (Guan et al., 2009).

*2.3.4. N-stearoylethanolamine (SEA; **1c**)*

The title amide **1c** was prepared from stearic acid (50 mg) as white solid with isolated yield 52 mg (90%). *Rf* 0.52 (CH_2Cl_2/MeOH 95:5). Mp 94–95 °C. ^{1}H NMR δ: 0.90 (3H, t, J = 6.8, 18-CH_3), 1.24–1.35 (28 H, m, 4–17 CH_2), 1.66 (2H, tt, J = 6.8, 7.0, 3-CH_2), 2.23 (2H, t, J = 6.8, 2-CH_2), 2.61 (1H, bs, OH), 3.45 (2H, dt, J = 5.1, 5.4, 1′-CH_2), 3.75 (2H, dt, J = 4.8, 5.1, 2′-CH_2), 5.96 (1H, bs, NH). Physical and spectroscopic data were in accordance with those reported (Morales-Sanfrutos et al., 2011).

*2.3.5. N-oleoylethanolamine (OEA; **1d**)*

The title amide **1d** was prepared from oleic acid (50 mg) as white solid with isolated yield 51 mg (89%). Mp 75–76 °C. *Rf* 0.50 (CH_2Cl_2/MeOH 95:5). ^{1}H NMR δ: 0.90 (3H, J = t, 6.5, 18-CH_3), 1.26–1.39 (20 H, m, 4–7 and 12–17 CH_2), 1.66 (2H, tt, J = 7.0, 7.4, 3-CH_2), 2.01–2.05 (4H, m, 8- and 11-CH_2), 2.23 (2H, t, J = 7.4, 2-CH_2), 3.45 (2H, dt, J = 4.5, 5.5, 1′-CH_2), 3.75 (2H, dt, J = 4.8, 5.5, 2′-CH_2), 5.33–5.40 (2H, m, 9- and 10-CH), 6.00 (1H, bs, NH), the OH signal was too broad to be detected. Physical and spectroscopic data were in accordance with those reported (Plastina et al., 2009).

*2.3.6. N-linoleoylethanolamine (LEA; **1e**)*

The title amide **1e** was prepared from linoleic acid (30 mg) as amorphous solid with isolated yield 30 mg (88%). *Rf* 0.42 (CH_2Cl_2/MeOH 95:5). Mp 40–41 °C. ^{1}H NMR δ: 0.92 (3H, t, J = 6.9, 18-CH_3), 1.27–1.41 (14 H, m, 4–7 and 15–17 CH_2), 1.60–1.67 (2H, m, 3-CH_2), 2.07 (4H, dt, J = 6.4, 7.6, 8-CH_2 and 14-CH_2), 2.23 (2H, t, J = 7.4, 2-CH_2), 2.63 (1H, bs, OH), 2.79 (2H, t, J = 6.2, 11-CH_2), 3.45 (2H, dt, J = 4.8, 5.5, 1′-CH_2), 3.75 (2H, dt, J = 4.8, 4.8, 2′-CH_2), 5.30–5.45 (4H, m, 9- 10- 12- and 13-CH), 5.96 (1H, bs, NH). Physical and spectroscopic data were in accordance with those reported (Plastina et al., 2009).

*2.3.7. N-α-linolenoylethanolamine (ALEA; **1f**)*

The title amide **1f** was prepared from α-linolenic acid (30 mg) as colourless oil with isolated yield 30 mg (86%). *Rf* 0.40 (CH_2Cl_2/MeOH 95:5). ^{1}H NMR δ: 0.94 (3H, t, J = 6.9, 18-CH_3), 1.24–1.35 (8H, m, 4–7 CH_2), 1.57–1.63 (2H, m, 3-CH_2), 2.04 (2H, dt, J = 7.6, 7.6, 8-CH_2), 2.07 (2H, dq, J = 7.0, 7.4, 17-CH_2), 2.20 (2H, t, J = 7.6, 2-CH_2), 2.77–2.83 (4H, m, 11- and 14-CH_2), 2.92 (1H, bs, OH), 3.44 (2H, dt, J = 5.1, 5.4, 1′-CH_2), 3.74 (2H, dt, J = 4.8, 5.1, 2′-CH_2), 5.26–5.39 (6H, m, 9- 10- 12- 13- 15- and 16-CH), 6.04 (1H, bs, NH). Physical and spectroscopic data were in accordance with those reported (Plastina et al., 2009).

*2.3.8. N-eicosapentaenoylethanolamine (EPEA; **1g**)*

The title amide **1g** was prepared from eicosapentaenoic acid (30 mg) as colourless oil with isolated yield 30 mg (85%). *Rf* 0.36 (CH_2Cl_2/MeOH 95:5). ^{1}H NMR δ: 0.99 (3H, t, J = 7.6, 20-CH_3), 1.75 (2H, tt, J = 6.9, 7.6, 3-CH_2), 2.10 (2H, dq, J = 7.6, 7.6, 19-CH_2), 2.13 (2H, dt, J = 6.9, 7.6, 4-CH_2), 2.24 (2H, t, J = 6.9, 2-CH_2), 2.80–2.87 (8H, m, 7- 10- 13- and 16-CH_2), 3.45 (2H, dt, J = 4.8, 5.5, 1′-CH_2), 3.74 (2H, dt, J = 4.8, 4.8, 2′-CH_2), 5.31–5.45 (10H, m, 5- 6- 8- 9- 11- 12- 14- 15- 17- and 18-CH), 6.01 (1H, bs, NH), the OH signal was too broad to be detected. Physical and spectroscopic data were in accordance with those reported (Plastina et al., 2009).

*2.4. Synthesis of OAEs (**2a–2g**)*

*2.4.1. Synthesis of tert-butyl 2-hydroxyethylcarbamate (**4**)*

To a solution of ethanolamine **3** (0.611 g, 10 mmol) in anhydrous CH_2Cl_2 (10 ml) was added dropwise di-*tert*-butyl dicarbonate ($(Boc)_2O$; 1.82 g, 8.32 mmol) in anhydrous CH_2Cl_2 (3 ml) and the mixture was stirred at rt for 1 h. After additional 12 h at the same temperature without stirring, the reaction mixture was washed with saturated solution of $NaHCO_3$ (3 ml × 10 ml) and then the organic phases were dried over anhydrous Na_2SO_4. The solvent was removed in vacuum to provide **4** as a colourless oil in quantitative yield. ^{1}H NMR δ: 1.48 (9H, s, 3 × CH_3), 1.95 (1H, bs, OH), 3.32 (t, J = 4.8, CH_2NH), 3.73 (t, J = 4.8, CH_2OH), 4.93 (1H, bs, NH); NMR data were in accordance with those reported (Mattingly, 1990).

*2.4.2. O-ethanolamine-N-Boc derivatives **5a–5g**. General method*

The appropriate acid (0.15 mmol), COMU (64.2 mg, 0.15 mmol), and DIPEA (0.05 ml, 0.30 mmol) were dissolved in anhydrous CH_2Cl_2 (0.5 ml) and CH_3CN (2.5 ml) and the resulting orange-red solution was stirred at rt for 10 min under a nitrogen atmosphere. The *t*-butyl 2-hydroxyethylcarbamate (**4**) (0.15 mmol) in CH_3CN (0.2 ml) was then injected into the reaction mixture and vigorous stirring at rt was continued until TLC confirmed the completion of the reaction (3–6 h). The reaction mixture was diluted with CH_2Cl_2 (3 ml) and the resulting mixture was washed with 5% HCl, saturated $NaHCO_3$ and brine. The organic layer was collected, dried over anhydrous Na_2SO_4, filtered and concentrated to give crude products as colourless amorphous solids or oils.

2.4.2.1. **5a**. ^{1}H NMR δ: 0.91 (3H, t, J = 7.4, 20-CH_3), 1.27–1.41 (6 H, m, 17–19 CH_2), 1.47 (9H, s, C(CH_3)$_3$), 1.73 (2H, tt, J = 7.0, 7.6, 3-CH_2), 2.08 (2H, dq, J = 7.6, 7.6, 16-CH_2), 2.14 (2H, dt, J = 7.0, 7.4, 4-CH_2), 2.35 (2H, t, J = 7.0, 2-CH_2), 2.83–2.86 (6H, m, 7- 10- and 13-CH_2), 3.41 (2H, dt, J = 4.8, 5.0, 2′-CH_2), 4.15 (2H, t, J = 4.8, 1′-CH_2), 4.79 (1H, bs, NH), 5.33–5.45 (8H, m, 5- 6- 8- 9- 11- 12- 14- and 15-CH).

2.4.2.2. **5b**. ^{1}H NMR δ: 0.90 (3H, t, J = 6.5, 16-CH_3), 1.25–1.32 (24 H, m, 4–15 CH_2), 1.47 (9H, s, C(CH_3)$_3$), 1.64 (2H, tt, J = 7.0, 7.0, 3-CH_2), 2.34 (2H, t, J = 7.0, 2-CH_2), 3.42 (2H, dt, J = 4.8, 4.8, 2′-CH_2), 4.15 (2H, t, J = 4.8, 1′-CH_2), 4.77 (1H, bs, NH).

2.4.2.3. **5c**. ^{1}H NMR δ: 0.91 (3H, t, J = 7.0, 18-CH_3), 1.26–1.34 (28 H, m, 4–17 CH_2), 1.47 (9H, s, C(CH_3)$_3$), 1.65 (2H, tt, J = 6.8, 7.0,

3-CH_2), 2.34 (2H, t, J = 6.8, 2-CH_2), 3.42 (2H, dt, J = 4.8, 5.0, 2′-CH_2), 4.16 (2H, t, J = 5.0, 1′-CH_2), 4.77 (1H, bs, NH).

2.4.2.4. **5d**. ^{1}H NMR δ: 0.90 (3H, J = t, 6.8, 18-CH_3), 1.24–1.36 (20 H, m, 4–7 and 12–17 CH_2), 1.47 (9H, s, C(CH_3)$_3$), 1.61–1.68 (2H, m, 3-CH_2), 2.01–2.05 (4H, m, 8- and 11-CH_2), 2.35 (2H, t, J = 7.2, 2-CH_2), 3.41 (2H, dt, J = 4.8, 4.8, 2′-CH_2), 4.15 (2H, t, J = 4.8, 1′-CH_2), 4.79 (1H, bs, NH), 5.33–5.40 (2H, m, 9- and 10-CH).

2.4.2.5. **5e**. ^{1}H NMR δ: 0.91 (3H, t, J = 7.0, 18-CH_3), 1.30–1.39 (14 H, m, 4–7 and 15–17 CH_2), 1.46 (9H, s, C(CH_3)$_3$), 1.59–1.67 (2H, m, 3-CH_2), 2.08 (4H, dt, J = 6.9, 6.9, 8-CH_2 and 14-CH_2), 2.33 (2H, t, J = 7.6, 2-CH_2), 2.79 (2H, t, J = 6.2, 11-CH_2), 3.41 (2H, dt, J = 4.8, 5.0, 2′-CH_2), 4.15 (2H, t, J = 5.0, 1′-CH_2), 4.78 (1H, bs, NH), 5.32–5.43 (4H, m, 9- 10- 12- and 13-CH).

2.4.2.6. **5f**. ^{1}H NMR δ: 1.00 (3H, t, J = 6.9, 18-CH_3), 1.31–1.39 (8H, m, 4–7 CH_2), 1.47 (9H, s, C(CH_3)$_3$), 1.62–1.67 (2H, m, 3-CH_2), 2.06–2.13 (4H, m, 8-CH_2 and 17-CH_2), 2.34 (2H, t, J = 7.6, 2-CH_2), 2.81–2.84 (4H, m, 11- and 14-CH_2), 3.41 (2H, dt, J = 4.9, 4.7, 2′-CH_2), 4.15 (2H, t, J = 4.9, 1′-CH_2), 4.77 (1H, bs, NH), 5.31–5.45 (6H, m, 9- 10- 12- 13- 15- and 16-CH).

2.4.2.7. **5g**. ^{1}H NMR δ: 1.00 (3H, t, J = 7.4, 20-CH_3), 1.47 (9H, s, C(CH_3)$_3$), 1.73 (2H, tt, J = 6.9, 7.6, 3-CH_2), 2.10 (2H, dq, J = 7.6, 7.6, 19-CH_2), 2.13 (2H, dt, J = 6.9, 7.6, 4-CH_2), 2.35 (2H, t, J = 7.6, 2-CH_2), 2.82–2.88 (8H, m, 7- 10- 13- and 16-CH_2), 3.41 (2H, dt, J = 5.0, 5.0, 2′-CH_2), 4.15 (2H, t, J = 4.9, 1′-CH_2), 4.78 (1H, bs, NH),5.32–5.45 (10H, m, 5- 6- 8- 9- 11- 12- 14- 15- 17- and 18-CH).

2.4.3. Deprotection of compounds **5a–5g**. General method

The appropriate crude Boc-derivative (0.15 mmol) was dissolved in TFA 5% in CH_2Cl_2 (3 ml) and stirred overnight. The solvent was removed and crude was purified by column chromatography (CH_2Cl_2/MeOH 95:5 containing 0.5% TFA) to give the desired compound.

2.4.4. O-arachidonoylethanolamine-N-trifluoroacetate salt (**2a**)

The title compound **2a** was obtained from arachidonic acid as colourless oil; isolated overall yield 55.4 mg (80%). *Rf* 0.18 (CH_2Cl_2/MeOH 95:5). NMR δ: 0.91 (3H, t, J = 7.4, 20-CH_3), 1.26–1.39 (6 H, m, 17–19 CH_2), 1.70 (2H, tt, J = 7.0, 7.6, 3-CH_2), 2.07 (2H, dq, J = 7.6, 7.6, 16-CH_2), 2.14 (2H, dt, J = 7.0, 7.4, 4-CH_2), 2.39 (2H, t, J = 7.0, 2-CH_2), 2.81–2.87 (6H, m, 7- 10- and 13-CH_2), 3.29 (2H, bt, J = 5.0, 2′-CH_2), 4.37 (2H, bt, J = 5.0, 1′-CH_2), 5.33–5.45 (8H, m, 5- 6- 8- 9- 11- 12- 14- and 15-CH), the NH_3^+ signal was too broad to be detected. Anal. Calcd for $C_{24}H_{38}F_3NO_4$ (461.56): C, 62.45; H, 8.30; N, 3.03. Found: C, 62.51; H, 8.45; N, 3.02.

2.4.5. O-palmitoylethanolamine-N-trifluoroacetate salt (**2b**)

The title compound **2b** was obtained from palmitic acid as white solid; isolated overall yield 55.8 mg (90%). *Rf* 0.22 (CH_2Cl_2/MeOH 95:5). Mp 104–105 °C. ^{1}H NMR δ: 0.90 (3H, t, J = 7.6, 16-CH_3), 1.23–1.33 (24 H, m, 4–15 CH_2), 1.58–1.65 (2H, m, 3-CH_2), 2.36 (2H, t, J = 7.0, 2-CH_2), 3.26 (2H, bt, J = 5.0, 2′-CH_2), 4.35 (2H, bt, J = 5.0, 1′-CH_2), the NH_3^+ signal was too broad to be detected. Anal. Calcd for $C_{20}H_{38}F_3NO_4$ (413.52): C, 58.09; H, 9.26; N, 3.39. Found: C, 58.21; H, 9.12; N, 3.24.

2.4.6. O-stearoylethanolamine-N-trifluoroacetate salt (**2c**)

The title compound **2c** was obtained from stearic acid as colourless white solid; isolated overall yield 60.9 mg (92%). *Rf* 0.26 (CH_2Cl_2/MeOH 95:5). Mp 114–115 °C. ^{1}H NMR δ: 0.91 (3H, t, J = 7.0, 18-CH_3), 1.25–1.36 (28 H, m, 4–17 CH_2), 1.61–1.77 (2H, m, 3-CH_2), 2.35 (2H, t, J = 7.4, 2-CH_2), 3.27 (2H, bt, J = 5.5 2′-CH_2), 4.36 (2H, bt, J = 5.5, 1′-CH_2), the NH_3^+ signal was too broad to be detected. Anal. Calcd for $C_{22}H_{42}F_3NO_4$ (441.57): C, 59.84; H, 9.59; N, 3.17. Found: C, 59.98; H, 9.45; N, 3.21.

2.4.7. O-oleoylethanolamine-N-trifluoroacetate salt (**2d**)

The title compound **2d** was obtained from oleic acid as amorphous solid; isolated overall yield 58.7 mg (89%). *Rf* 0.20 (CH_2Cl_2/MeOH 95:5). ^{1}H NMR δ: 0.90 (3H, J = t, 6.8, 18-CH_3), 1.25–1.37 (20 H, m, 4–7 and 12–17 CH_2), 1.59–1.66 (2H, m, 3-CH_2), 2.04–2.08 (4H, m, 8- and 11-CH_2), 2.38 (2H, t, J = 7.4, 2-CH_2), 3.26 (2H, bt, J = 5.5 2′-CH_2), 4.37 (2H, bt, J = 5.5, 1′-CH_2), 5.35–5.41 (2H, m, 9- and 10-CH), the NH_3^+ signal was too broad to be detected. Anal. Calcd for $C_{22}H_{40}F_3NO_4$ (439.55): C, 60.11; H, 9.17; N, 3.19. Found: C, 60.21; H, 9.25; N, 3.16.

2.4.8. O-linoleylethanolamine-N-trifluoroacetate salt (**2e**)

The title compound **2e** was obtained from linoleic acid as colourless oil; isolated overall yield 53.2 mg (81%). *Rf* 0.18 (CH_2Cl_2/MeOH 95:5). ^{1}H NMR δ: 0.91 (3H, t, J = 7.0, 18-CH_3), 1.28–1.39 (14 H, m, 4–7 and 15–17 CH_2), 1.59–1.67 (2H, m, 3-CH_2), 2.07 (4H, dt, J = 6.4, 7.6, 8-CH_2 and 14-CH_2), 2.36 (2H, t, J = 7.4, 2-CH_2), 2.79 (2H, t, J = 6.7, 11-CH_2), 3.27 (2H, bt, J = 5.5, 2′-CH_2), 4.36 (2H, bt, J = 5.5, 1′-CH_2), 5.32–5.43 (4H, m, 9- 10- 12- and 13-CH), the NH_3^+ signal was too broad to be detected. Anal. Calcd for $C_{22}H_{38}F_3NO_4$ (437.54): C, 60.39; H, 8.75; N, 3.20. Found: C, 60.51; H, 8.85; N, 3.22.

2.4.9. O-α-linolenylethanolamine-N-trifluoroacetate salt (**2f**)

The title compound **2f** was obtained from α-linolenic acid as colourless oil; isolated overall yield 52.3 mg (80%). *Rf* 0.16 (CH_2Cl_2/MeOH 95:5). ^{1}H NMR δ: 0.99 (3H, t, J = 7.0, 18-CH_3), 1.30–1.38 (8H, m, 4–7 CH_2), 1.59–1.65 (2H, m, 3-CH_2), 2.05–2.13 (4H, m, 8-CH_2 and 17-CH_2), 2.37 (2H, t, J = 7.6, 2-CH_2), 2.82–2.84 (4H, m, 11- and 14-CH_2), 3.27 (2H, bt, J = 4.9, 2′-CH_2), 4.37 (2H, bt, J = 4.9, 1′-CH_2), 5.31–5.44 (6H, m, 9- 10- 12- 13- 15- and 16-CH), the NH_3^+ signal was too broad to be detected. Anal. Calcd for $C_{22}H_{36}F_3NO_4$ (435.52): C, 60.67; H, 8.33; N, 3.22. Found: C, 60.79; H, 8.25; N, 3.14.

2.4.10. O-eicosapentaenoylethanolamine-N-trifluoroacetate salt (**2g**)

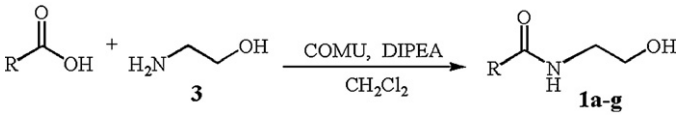
The title compound **2g** was obtained from eicosapentaenoic acid as colourless oil; isolated overall yield 53.8 mg (78%). *Rf* 0.19 (CH_2Cl_2/MeOH 95:5). ^{1}H NMR δ: 1.00 (3H, t, J = 7.0, 20-CH_3), 1.73 (2H, tt, J = 6.9, 7.6, 3-CH_2), 2.10 (2H, dq, J = 7.6, 7.6, 19-CH_2), 2.14 (2H, dt, J = 6.9, 7.6, 4-CH_2), 2.39 (2H, t, J = 7.6, 2-CH_2), 2.81–2.87 (8H, m, 7- 10- 13- and 16-CH_2), 3.29 (2H, bt, J = 5.0, 2′-CH_2), 4.37 (2H,bt, J = 5.0, 1′-CH_2), 5.32–5.45 (10H, m, 5- 6- 8- 9- 11- 12- 14- 15- 17- and 18-CH), the NH_3^+ signal was too broad to be detected. Anal. Calcd for $C_{24}H_{36}F_3NO_4$ (459.54): C, 62.73; H, 7.90; N, 3.05. Found: C, 62.85; H, 7.75; N, 3.15.

3. Results and discussion

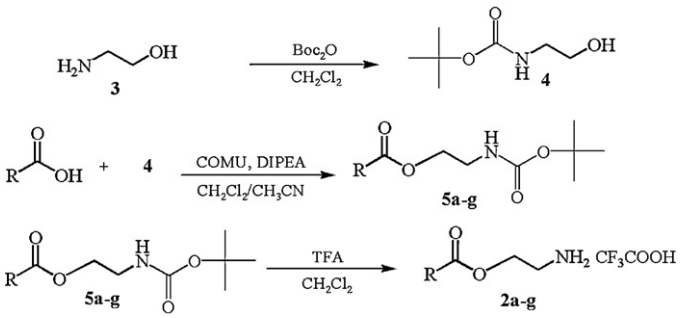
3.1. Chemical synthesis of NAEs (**1a–1g**) and OAEs (**2a–2g**)

Structures and numeration of NAEs and OAEs (**1a–1g** and **2a–2g**) are shown in Fig. 1. NAEs (**1a–1g**) of matched acyl chain lengths (n = 16–20) have been synthesized in fair yields by the reaction of the corresponding acid with 2-ethanolamine (**3**), as shown in Scheme 2.

The starting compound **3** was linked to palmitic acid (C16), stearic acid (C18), oleic acid (C18:1), linoleic acid (C18:2), α-linolenic acid (C18:3), arachidonic acid (C20:4), or 5,8,11,14,17-eicosapentaenoic acid (C20:5) by coupling in the presence of COMU and the base N,N-diisopropylethylamine (DIPEA). After purification via flash chromatography, substituted products (**1a**, **1e**, **1f**, **1g**) were obtained in high yield (85–90%). Column purification



Scheme 2. Synthetic route to the target compounds NAEs (**1a–1g**).



Scheme 3. Synthetic route to the target compounds OAEs (**2a–2g**).

was not necessary for **1b–1d** as the final compounds readily precipitated out of CH_2Cl_2. COMU is a third generation uronium-type coupling reagent used in peptide synthesis. For the first screening we tested HATU (O-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate), the most potent of currently commercially available coupling reagents. Nevertheless, for our reactions, COMU was better than HATU because only 1 equiv. of coupling reagent performed extremely well in the presence of 2 equiv. of base (DIPEA) giving higher yields. COMU reduces even reaction time indeed reactions are complete in 3–6 h instead of 10–24 h. Moreover the byproducts of COMU are water soluble and simply removed by extraction allowing easier purification by flash chromatography. All these considerations make COMU an excellent choice as coupling reagent. Additionally COMU shows a less hazardous safety profile than benzotriazole-based reagents, such as HATU, which in addition exhibit unpredictable autocatalytic decompositions and therefore a higher risk of explosion.

The synthesis of OAEs (Scheme 3) required a straightforward three-step synthetic route.

To obtain fatty acid derivatives with ester linkage the protection of 2-ethanolamine (**3**) amino group is necessary. For this purpose di-*tert*-butyl dicarbonate (Boc_2O) is widely applied to introduce the *tert*-butoxycarbonyl (Boc) protecting group. Removal of Boc is easily performed under various reaction conditions (Greene et al., 1999; Tarbell et al., 1972).

A variety of reagents and methodologies, developed over the years, for the preparation of N-*tert*-butyl carbamate using Boc_2O have been carried out either in the presence of a base (aq NaOH, K_2CO_3, DMAP, NaHMDS, Et_3N) or, more recently, acid catalyst (Jahani et al., 2011). In our experiments it was found that when the ethanolamine (**3**) was reacted with Boc_2O in anhydrous CH_2Cl_2 in the absence of catalysts (Becht et al., 2003) the Boc protected key intermediate *tert*-butyl 2-hydroxyethylcarbamate (**4**) was obtained in quantitative yield.

Compounds **5a–5g** were easily achieved by coupling *tert*-butyl 2-hydroxyethylcarbamate (**4**) and the opportune fatty acid in the presence of COMU and DIPEA (Twibanire et al., 2011). For these syntheses we assessed even EDAC (*N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride), usually used in lipids chemistry (Vadivel et al., 2011), as coupling reagent but also in this case COMU gave better results. Column purification was not necessary and crude derivatives **5a–5g** were dissolved in 5% trifluoroacetic acid (TFA) in CH_2Cl_2 and stirred overnight at room temperature. For the usual work-up the pH is adjusted to 7 but, contrary to expectations, the isolated material was the corresponding amide instead of the desired ester derivative. This is the consequence of intramolecular nucleophilic attack promoted by basic conditions during the washing of the organic phase with an aqueous solution of $NaHCO_3$. A pH-dependent "O-N intramolecular acyl migration", a well known reaction seen in Ser/Thr-containing peptides (Tamamura et al., 2003; Mouls et al., 2004) have been using for over a decade. Moreover this migration was documented long ago in the chemical literature for N-ethanolamines. In aqueous solution above pH 7.5 the aminoethyl esters were found to rearrange to N-ethanolamines almost instantaneously (Phillips et al., 1947).

This acyl group migration can be avoided by the choice of work-up conditions. We decided to remove the solvent skipping the neutralization step because even careful addition of a basic resin (DOWEX) till pH 7 gave a partial migration to NAEs. Acidic condition allows to obtain more stable TFA salts of OAEs. The presence of NH_3^+ instead of NH_2 moiety is fundamental for the stability of

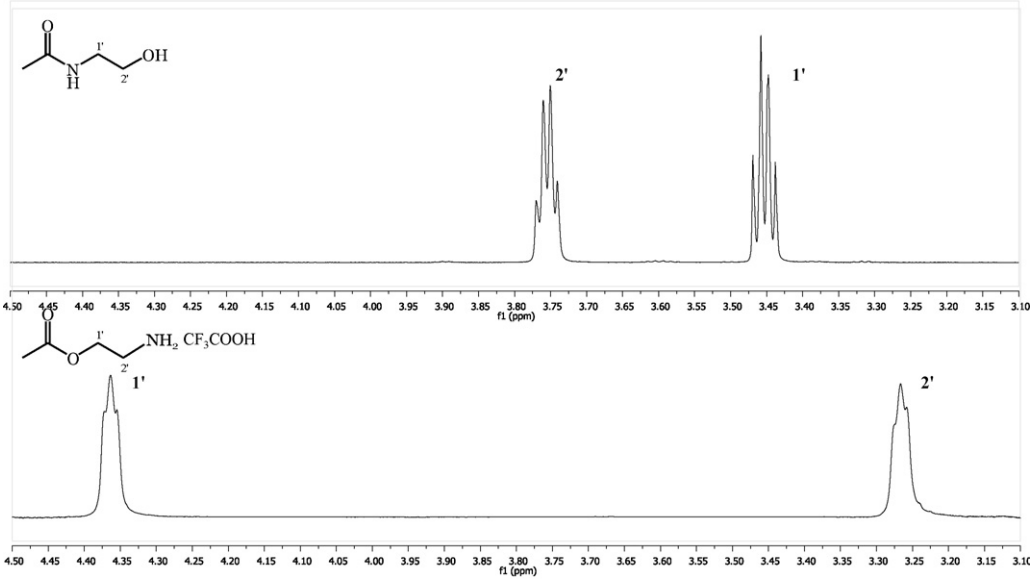


Fig. 2. Comparison of ^{1}H NMR significant signals of amide (compounds **1a–1g**) and ester (compounds **2a–2g**) dissolved in $CDCl_3$.

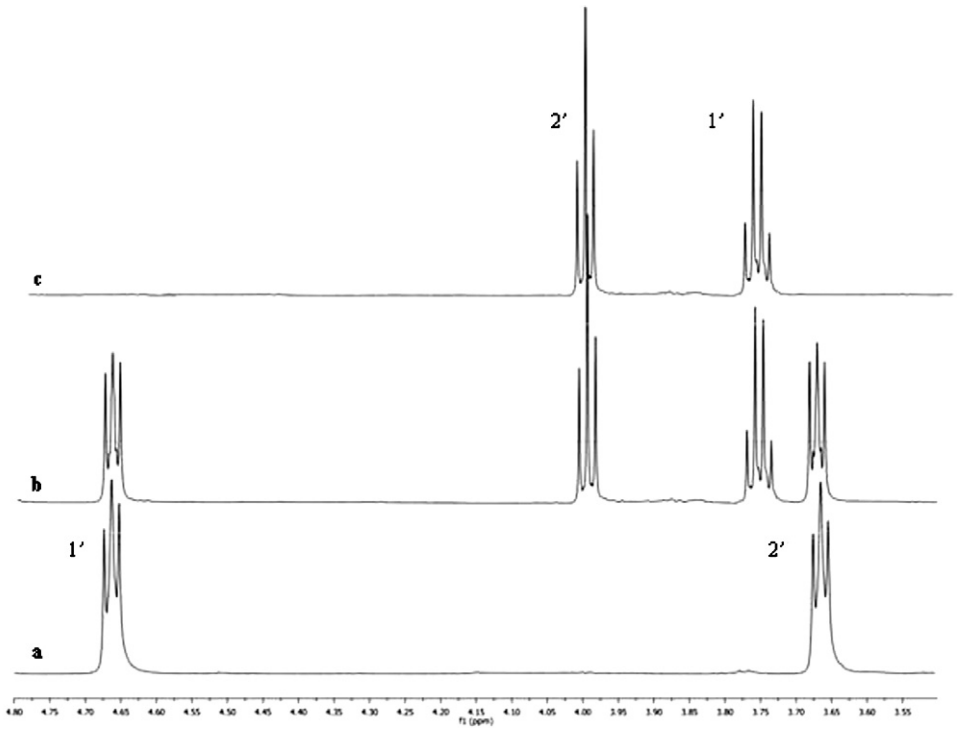


Fig. 3. (a) ^{1}H spectrum of compound **2c** dissolved in pyridine-d_5 (isotopic enrichment 99.98%) solution at 300 K, t = 0. The higher field signal of pyridine-d_5 (7.19 ppm) was used as internal reference standard, (b) ^{1}H spectrum of compound **2c** at t = 12 h and (c) ^{1}H spectrum of compound **2c** at t = 24 h.

OAEs indeed flash chromatography performed with CH_2Cl_2/MeOH 95:5 gave a partial migration to NAEs while the addiction of 0.5% TFA in the eluent preserve the desired esters. This device let us to obtain pure OAEs. The yields of the final products (**2a–2g**) for the two synthetic steps were quite quantitative (Scheme 3).

3.2. Structure confirmation of NAEs (*1a–1g*) and OAEs (*2a–2g*)

Synthesized NAEs (**1a–1g**) and OAEs (**2a–2g**) were subjected to detailed NMR analyses and the complete ^{1}H signals were unambiguously assigned using one and two dimensional experiments. The assignment of ^{1}H frequencies, made by examining the observed chemical shifts and multiplicities caused by homonuclear couplings, confirmed the structural backbone.

The chemical shift values of protons of ethanolamine portion were most informative because significant differences allow to distinguish the two isomers (Fig. 2) while the fatty acid portion exhibited essentially identical ^{1}H chemical shifts and spectral patterns. In particular a distinctly different ^{1}H-signal pattern was observed for 1′-CH_2 which resonates at 3.44–3.45 ppm as a double triplet (J 5.1 and 5.4 Hz) in NAEs (**1a–1g**) whereas in OAEs (**2a–2g**) was downshielded at 4.35–4.37 ppm as a broad triplet (J 5.0 Hz). The difference is less significative for 2′-CH_2 which resonates at 3.72–3.75 ppm as a broad triplet (J 5.0 Hz) in NAEs (**1a–1g**) and at 3.26–3.29 ppm as a double triplet (J 5.1 and 5.4 Hz) in OAEs (**2a–2g**).

NOE analysis allowed the determination of the correct position of 1′-CH_2 and 2′-CH_2 signals in NAEs (**1a–1g**). The NOE contacts between NH and less shielded 1′-CH_2 were observed in NAEs (**1a–1g**), whereas in the OAEs (**2a–2g**) the NOE response was not observed owing to broadening of the NH_3^+.

3.3. Rate of acyl chain migration under basic and acidic conditions

The O → N acyl group migration was also followed by 500 MHz ^{1}H NMR spectroscopy. ^{1}H-chemical shifts of protons of ethanolamine portion are excellent structural parameters to monitor the acyl migration. To prove the real conversion of OAEs into NAEs, spectra of OAEs were recorded at different time under basic conditions. For these experiments a 40 mM solution (20 μmol of product in 0.5 ml of solvent) of compound **2d**, taken as model, was prepared in pyridine-*d5*. We selected pyridine-*d5* because only weakly basic conditions give slow migration rate allowing to follow the reaction. After 24 h the O-ethanolamine **2d** totally rearranged to *N*-ethanolamine **1d** (Fig. 3).To monitor conversion of NAEs into OAEs the experiments were performed in acidic conditions ($CDCl_3$/TFA) adding 10 μl of 2.0 M TFA in $CDCl_3$ (data not shown). The N → O acyl group migration was slower than N → O in fact only after 72 h the *N*-ethanolamine **1d** rearranged totally to O-ethanolamine **2d**.

4. Conclusions

In conclusion, we have developed an effective protocol for the synthesis of NAEs (**1a–1g**) and OAEs (**2a–2g**) characterized by an easy work-up and quite quantitative yields. It is noteworthy that during the synthesis of esters (**2a–2g**) on removal of the N-protecting group and following work-up a pH-induced O → N acyl group migration occurs. O-Acyl migration is even possible under physiological pH. Our protocol for the synthesis of OAEs schedules to keep slightly acidic conditions during the entire work-up to obtain more stable TFA salts of OAEs in order to prevent O → N acyl migration. The synthetic procedures optimized in this article allow to obtain pure NAEs and/or OAEs. This optimization is important for the availability of OAEs that could be utilized to evaluate their biological properties for comparison to NAEs. Moreover OAEs could be used as analytical standards to properly quantify endogenous OAEs that could have escaped detection in the past for their conversion to NAEs under working conditions (extraction, purification or detection). In fact we have proved that in aqueous solution above pH 7.5 the O-ethanolamine esters rearrange to N-ethanolamines almost instantaneously so any work-up procedure from tissue and/or

biological sample has to be conducted at lower pH. Since N → O acyl migration promoted by acid is slow (12 h about 10%) acidic condition could be applied for extraction and work-up without affecting NAEs or OAEs amount. A detailed NMR spectroscopic characterization allowed the assignment of all proton signals of NAEs and OAEs useful for the identification of newly isolated or synthesized derivatives. Moreover NMR studies performed in acidic and basic environments let to better define O → N and N → O acyl migrations conditions.

Acknowledgement

This study was supported by a fellowship cofinanced by "Dote ricerca" FSE, Regione Lombardia.

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