Chemical Synthesis and Gas Chromatographic Behaviour of γ-Stearidonic (18:4n-6) Acid*

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Abstract γ-Stearidonic acid, 18:4n-6, a potential product of β-oxidation of arachidonic acid (20:4n-6), was only recently reported for a living organism - a thermophilic cyanobacterium *Tolypothrix* sp., albeit at low levels, whilst some indirect evidence suggests its wider presence, e.g. in a unicellular marine alga. We have prepared 18:4n-6 using an iodolactonisation chain-shortening approach from 22:5n-6 and obtained its ^{1}H-, ^{13}C-, COSY- and HSQC NMR spectra, with 18:5n-3 spectra also recorded for a comparison. The GC and GC-MS behaviour of its methyl ester was also studied. Like another Δ3 polyunsaturated acid, octadecapentaenoic (18:5n-3), 18:4n-6 rapidly yields 2-*trans* isomer upon formation of dimethyloxazoline derivative. On a polar ionic liquid phase (SLB-IL100, 200 °C) the methyl ester could be mistaken for 18:3n-3, while on methylsilicone phase (BP1, 210 °C) it eluted ahead of 18:3n-6 and 18:4n-3, suggesting that when present it may be easily misidentified during GC analysis of fatty acids.

Keywords Stearidonic · Δ3 PUFA · Octadecatetraenoic · Octadecapentaenoic · GC · GC-MS · NMR

Introduction

Until recently, the only naturally occurring methylene-interrupted Δ3 PUFA known was octadecapentaenoic acid (18:5n-3, ODPA), a product of β-oxidation of eicosapentaenoic (20:5n-3) acid. It is rapidly metabolized by animal cells into 18:4n-3 and then into 20:5n-3 and 16:3n-3 [1]. Due to this rapid conversion 18:5n-3 is rarely found in animals, but may be found in certain groups of marine phytoplankton, dinoflagellates, haptophytes and prasinophytes, sometimes at levels of up to 15% of total fatty acids, ([1] and the references therein). ODPA was reported as toxic for fish [2], and glyceroglycolipids, namely digalactosyldiacylglycerol (DGDG) containing ODPA, are cytotoxic [3] and may induce apoptosis [4].

A potential product of β-oxidation of another important Δ5 PUFA, arachidonic acid (20:4n-6), would also be a Δ3 fatty acid, 18:4n-6. A full chemical synthesis of this fatty acid was described [5]. When used in the early studies of prostaglandin biosynthesis, this fatty acid proved to be about 6.5 times poorer substrate than 20:4n-6, as judged by the yield of prostaglandins (determined by absorbance at 278 nm after alkali treatment) after 30 min incubation of fatty acids with the particulate enzyme fraction of sheep vesicular glands [6]. Whilst the publication describing the synthesis of 18:4n-6 is occasionally cited, it is usually because of another synthesis described in the same paper, namely, that of 20:4n-6.

Only recently 18:4n-6 acid was reported for a living organism - a thermophilic cyanobacterium *Tolypothrix* sp., albeit at low levels. The newly discovered acid was named "γ-stearidonic" [7]. Apart from *Tolypothrix* sp., 18:4n-6 acid has been mentioned only once in relation to structure elucidation of a natural product in which the ODPA-containing total lipids of the unicellular marine alga *Prorocentrum minimum* were converted to methyl esters and subjected to a partial hydrazine reduction [8]. In this report, one of the less prominent

peaks was tentatively identified as that of 18:4n-6, despite the difference between the calculated and observed equivalent chain length (ECL) being quite significant (0.10-0.15).

As a laboratory involved in lipid analysis of a range of marine and terrestrial organisms, we pay special attention to the identification of unusual fatty acids and determination of their GC behavior as well as assess other features useful in their analysis (e.g., MS, NMR). Therefore, it was of interest to prepare a reference sample of 18:4n-6 and to study its characteristic features that could help in further identification of this compound, if present, in any natural sources.

Experimental Procedures:

Materials

Frozen pig testes were a gift from Taranaki Abattoir Company, North Island, New Zealand. The Vegetarian DHA (Source Natural® Inc., USA) was purchased via online store. The fatty acid composition of the batch used was found to be as follows (in weight % of total fatty acids): 22:6n-3, 38.6; 18:1n-9, 18.6; 22:5n-6,15.8; 16:0, 12.9; 14:0, 4.9; 18:2n-6, 1.5; 20:5n-3, 1.0; 18:0, 1.0; the rest of the fatty acids were present at levels of below 1 % each. Concerning weight, the Vegetarian DHA contained 35.3 g 22:6n-3 and 14.4 g 22:5n-6 per 100 g of oil. All solvents were of HPLC grade. Chemicals were analytical grade.

GC analysis

Fatty acid methyl esters were prepared according to Carreau and Dubacq [9], of which technique only the acidic methylation step was employed to esterify free fatty acids (FFAs). GC analysis of fatty acid methyl esters was performed on a Trace GC Ultra instrument (Thermo Finnigan, Waltham, MA, USA), equipped with flame ionization detector (FID). Helium was used as the carrier gas; the split ratio was 1:60. The FID response factors were determined with the use of the reference standards and were employed in analysis of the starting materials. Alternatively, a GC-14A gas chromatograph (Shimadzu, Japan) was used with the split ratio 1:50. The capillary columns employed and the relevant temperature regimes used are listed in Table 3.

GC-MS

GC-MS analysis of fatty acid methyl esters was performed on a GCMS-QP2010 Ultra (Shimadzu, Japan) gas chromatograph equipped with FID and MS detector. A TG-WAXMS (30 m x 0.25 mm i.d., 0.25 μm) capillary column (Thermo Finnigan, USA) and detector splitting system, which allows splitting a column flow and record signal data for both the MS

detector and FID, were used. Helium was used as the carrier gas; the split ratio was 1:80. Injector and detector temperatures were 280°C and 290°C, respectively. Separation temperature was 195°C. Dimethyloxazoline (DMOX) derivatives were prepared according to the method [10].

NI ESI QToF MS

Experiments were performed with the use of Waters Q-ToF Premier mass spectrometer working under MassLynx version 4.1 software. Calibration was performed with sodium formate solution. Capillary voltage was 2,500V, cone voltage 50V, source temperature 80 °C, desolvation temperature 180 °C, mass range 100-2,000 Da. Typical run duration was between 0.5 and 1 min, with scan time 1 s, and interscan time 0.1 s. Cone gas flow was off; desolvation gas flow was 600 L/h. Collision energy in tandem MS experiments was within 10-50 eV range.

NMR Spectroscopy

The compounds were dissolved in $CDCl_3$ and their ^{1}H and ^{13}C NMR spectra of compounds and referenced to internal TMS at 0 ppm were obtained on a Bruker Avance-III 500 Spectrometer equipped with a Bruker 5-mm broadband probe at 303 K. The ^{1}H and ^{13}C frequencies were 499.8 and 125.7 MHz, respectively, and 90° pulses 13.0 and 14.0 μs, respectively. Standard Bruker supplied NMR pulse programs were used for recording all spectra. The pulse program COSYGPMFQF was used to record the gradient selected absolute value proton homonuclear double quantum filtered COSY spectrum. The phase-sensitive gradient selected pulse programme HSQCETGP was used for the two-dimensional (2D) inverse detected ^{1}H, ^{13}C heteronuclear (HSQC) spectra with isotropic proton mixing. Parameters for the ^{1}H spectrum were: sweep width 10,302 Hz, 65,536 data points, 30° excitation pulse, 32 transients taken, each with a 1-s delay time and f.i.d. acquisition time of

3.18 s. Spectra were processed with a standard exponential weighting function with a 0.3-Hz line broadening before Fourier transformation. Parameters for the WALTZ ^{1}H decoupled ^{13}C spectrum were: 30° excitation pulse, sweep width 31,250 Hz, 65,536 data points and 4,192 transients each taken with a 0.5-s delay time and f.i.d. acquisition time of 1.05 s for a total acquisition time of 2 h. Spectra were processed with a standard exponential weighting function with 5-Hz line broadening before Fourier transformation.

Parameters for the proton homonuclear double quantum filtered COSY spectrum were as follows: two transients each taken with a delay time 1.69 s and f.i.d. acquisition time 0.46 s, sweep width F2 2,222 Hz in 2,048 data points and sweep width F1 2,222 Hz in 320 slices for a total acquisition time of 25 min. The absolute value spectrum was processed with a sine bell window function in both directions. Parameters for the HSQC spectrum were: 16 scans, each taken for 320 slices in the indirect dimension with a delay time 0.96 s and acquisition time 0.46 s, sweep width F2 2,212 Hz in 1,024 real points, sweep width F1 3,142 Hz in 512 slices and MLEV17 mixing at 8.3 kHz for 60 ms. ^{13}C GARP decoupling (globally optimised alternating phase rectangular pulse) was at 3.8 kHz during acquisition for a total acquisition time of 2 h. Data was processed with a squared cosine bell window function in both directions.

Preparation of 18:4n-6 from a Natural Source of 22:5n-6

The process included the following stages:

1. Extraction of total lipids from the feed (not required for the Vegetarian DHA oil).
2. Preparation of FFA concentrate.
3. Conversion of Δ4 PUFA into γ-iodolactones and isolation of γ-iodolactones.
4. Conversion of γ-iodolactones into dihydroxy fatty acids.
5. Periodate oxidation of dihydroxy fatty acids into shorter chain aldehydes.
6. Oxidation of aldehydes into Δ3-fatty acids.

7. Isolation of individual Δ3-fatty acids by preparative HPLC.

1. Extraction of Total Lipids From the Feed

Pig testes were cut into pieces and freeze-dried. In a typical extraction 8.800 kg of frozen testes were freeze-dried, producing 1.349 kg of dry material (15.3 wt %). Freeze-dried testes were extracted with near-critical dimethyl ether (DME) [11], yielding 199.8 g lipids extract (14.8 % dry weight, or 2.3 % wet testes weight). The residue was re-extracted according to [12] with a cumulative 3.5 % lipid yield. DME extract contained 4.3 % 22:5n-6 and 4.7 % 22:6n-3, whereas Folch extract of the residue contained 2.5 and 4.1 % 22:5n-6 and 22:6n-3, respectively. Only DME extract was used for further transformations. This preparation was employed only in the preliminary experiments until an improved source of 22:5n-6 was used. No extraction step was required when the Vegetarian DHA (Source Natural® Inc., USA) was used.

2. Preparation of the FFA Concentrate

The Vegetarian DHA oil, 12.15g, was dissolved in 200 mL 1M NaOH in 95 % EtOH and refluxed for 1 h. Distilled water, 100 mL, was added, followed by repeated extraction of unsaponifiable material with 30 mL of petroleum ether (b.p. 60-80 °C). The unsaponifiable phase was washed with 3 x 30 mL water; water washings were added to the aqueous layer, which was acidified with hydrochloric acid to pH 2. FFAs were extracted with 3 x 100 mL petroleum ether (b.p. 60-80°C). FFA extract was evaporated under reduced pressure on a rotor evaporator yielding 11.226g of FFA. FFA contained 15.2 % 22:5n-6 and 36.4 % 22:6n-3 as revealed by GC.

3. Conversion of Δ4 PUFA into γ-Iodolactones, and Isolation of γ-Iodolactones

The mixture of FFA prepared from the Vegetarian DHA was used for iodolactonization that was performed as described by [13]: to 2.5 g FFA dissolved in 7.5 mL of ethanol, 20 ml of

20 % aq. potassium carbonate was added. The mixture was stirred for 10 min until it produced a clear solution. A solution of 1.02 g iodine in 10 mL ethanol was added drop wise during 1 h and stirred for 2 h. Reaction products were extracted with 3 x 30 mL hexane-diethyl ether (4:1, v/v), and evaporated under reduced pressure on a rotor evaporator yielding 1.17g crude iodolactones.

Alternatively, 10.05g of the FFA mixture was dissolved in 33 mL ethanol; 88 mL of 20 % aq. potassium carbonate was added. The mixture was stirred for 10 min until it produced a clear solution. A solution of 4.05 g iodine in 40 mL ethanol was added dropwise during 2.5 h and stirred for 1 h. Reaction products were extracted with 3 x 100 mL hexane-diethyl ether (4:1, v/v), washed by 30 mL 10 % aq. potassium carbonate followed by 30 mL water and evaporated under reduced pressure on a rotary evaporator yielding 3.995 g of crude iodolactones.

4. Conversion of γ-Iodolactones into Dihydroxy Fatty Acids

In a typical experiment 1.17g of the iodolactones mixture from the Vegetarian DHA was dissolved in 80 mL of 0.2 M NaOH in 1,4-dioxane/water (1:1, v/v) and left in the dark for 68.5 h at room temperature. The solution was acidified with HCl to pH 2, followed by extraction with 3 x 50 mL hexane-diethyl ether (1:1, v/v), and washed with 30 mL water; the hexane-diethyl ether was removed using a rotary evaporator. The yield of the dihydroxy fatty acid mixture was 1.018 g.

In a scale-up preparation, the 3.995 g iodolactone mixture derived from the Vegetarian DHA oil was dissolved in 300 mL of 0.2 M NaOH in 1,4-dioxane/water (1:1, v/v) and left in the dark for 68 h at room temperature. The solution was acidified with HCl to pH 2, followed by extraction with 3 x 100 mL hexane-diethyl ether (1:1, v/v), and washed with 50 mL water; the hexane-diethyl ether was removed using a rotary evaporator. The yield of the dihydroxy fatty acid mixture was 3.2g.

5. Periodate Oxidation of Dihydroxy Fatty Acids into Shorter Chain Aldehydes.

The dihydroxy fatty acid mixture (from the Vegetarian DHA oil), 1.018g, was dissolved in 180 mL methanol and combined with 43 mL 0.2 M aqueous sodium periodate. The reaction mixture was stirred for 1.5 h at room temperature in the dark. Water, 150 mL, was added and aldehydes extracted with 3 x 50 mL of chloroform. The yield was 851 mg.

Alternatively, the dihydroxy fatty acid mixture, 3.2g, was dissolved in 300 mL methanol and combined with 132.2 mL of 0.2M aqueous sodium periodate. The reaction mixture was stirred for 1.5 h at room temperature in the dark. Water, 200 mL, was added and aldehydes extracted with 3 x 100 mL chloroform. The yield was 2.825g.

Any attempts to purify fatty aldehydes resulted in a great reduction of yields.

6. Oxidation of Aldehydes into Δ3-Fatty Acids

The mixture of fatty aldehydes derived from the Vegetarian DHA oil, 851mg, was dissolved in 150ml acetone and cooled to -70 °C on dry ice. Jones reagent [14], 0.85 mL, was added, and the reaction mixture was allowed to warm up to +10 °C. Water, 150 mL, was then added, and FFAs were extracted with 3 x 50 mL hexane. The extract was washed with 2 x 50 mL water followed by evaporation under reduced pressure on a rotary evaporator. The yield was 607 mg of crude FFA mixture.

Alternatively, the mixture of aldehydes, 2.825 g, was dissolved in 400 mL acetone and cooled to -70 °C on dry ice. Jones reagent, 2.825 mL, was added, and the reaction mixture was allowed to warm up to +10 °C. Water, 300 mL, was then added, and FFAs were extracted with 3 x 100 mL hexane. The extract was washed with 2 x 50 mL water followed by evaporation under reduced pressure on a rotor evaporator. The yield was 2.113 mg of crude FFA mixture.

7. Isolation of Individual Δ3-Fatty Acids by Preparative HPLC

A crude mixture of Δ3-FFA was purified using SPE before HPLC separation. For purification, 607 mg was dissolved in 10 mL of hexane and applied to a Strata SI-1 Silica SPE (1 g/6 mL) pre-washed with hexane. FFAs were eluted with 50 mL hexane-diethyl ether (9:1, v/v). The yield was 481 mg.

In a scale-up experiment, a crude mixture of Δ3 FFA was purified using lager SPE cartridges as follows: 2.113 g was dissolved in 20 mL of hexane and split in half. Each half was applied on a Strata SI-1 Silica SPE (2 g/12 mL) prewashed with hexane. FFAs were eluted with 90 mL hexane-diethyl ether (9:1, v/v). The combined yield of FFA from two SPEs was 1.455 g. FFA contained 31.3 % 18:4n-6 and 65.0% 18:5n-3 as revealed by GC.

Preparative HPLC separation of 18:4n-6 and 18:5n-3 acids was performed as follows: 100 mg FFA was dissolved in 0.5 mL ethanol and filtered through a 2.2-μm nylon filter before injection. Preparative HPLC separation of the 18:4n-6 and 18:5n-3FFA was carried out using a Gilson 321 preparative HPLC pump, an Agilent 1100 photodiode array detector (at 205 nm) and a C12 column (Synergy 4 μ Max-RP 80A, 250 x 30 mm i.d.) with a guard column (Phenomenex Security Guard cartridge C12, 15 x 30 mm i.d.). Data were collected and analysed using Gilson Unipoint 3.20 software. Solvents used were: solvent A: 85 % aqueous methanol with 0.05 % acetic acid; solvent B: methanol with 0.05 % acetic acid. The eluent flow rate was 10 mL/min. The concentration of injected sample was 200 mg/mL in chloroform; 0.3 mL was injected. The eluent gradient employed was: 0 to 2 min—100% solvent A; 35 min— 65 % Solvent A, 35 % solvent B; 69 min—100% solvent B. Yields: 18:4 – 23 mg (95 % purity by GC); 18:5 – 49 mg (96 % purity by GC).

Results and Discussion:

While both 18:4n-6 and 18:5n-3 may be isolated from natural sources, the expected yields are either low (e.g. in the case of 18:4n-6 the highest content reported was 0.7 ± 0.19 % total fatty acids in *Tolypothrix* sp. collected in the Tatry Mountains, Slovakia, while the specimen collected in Prague possessed only 0.2 ± 0.08 % of this acid, according to [7]) or may not always be guaranteed because of the limited availability of the source material (e.g. collection from microalgae during red tides).

Our approach to the preparation of 18:4n-6 was based on a more viable option - the chain-shortening technique (Fig. 1), originally developed by us for identification of very-long chain fatty acids of the Lake Baikal sponge [15] and successfully used later in our laboratory[1] for the synthesis of 18:5n-3 from 22:6n-3 [13]. Since the iodolactonisation step in our technique required a Δ4 or Δ5 fatty acid as a longer-chain precursor, we started with 22:5n-6 fatty acid. There are relatively few sources with elevated content of this acid available, with the notable exception of testes (and recently commercially available single cell DHA oil, e.g. from *Schizochytrium* sp.). While the highest level of 22:5n-6 in multicellular organisms was reported [16] for hamster, dog and rat testes (14.9, 14.0 and 13.5 % of total fatty acids, respectively), we selected pig testes as a more convenient source, with 22:5n-6 content of 12.1%, according to [17]. The fatty acid profile of locally obtained pig testes was, however, quite different, with 22:5n-6 present in the lipid extracts at only 4.1-4.3 % of total fatty acids; a level closer to that of 22:5n-6 in testes of pigs, 5.9%, was reported in [18]. The content of DHA in the lipid extracts of testes used in our experiments was 4.1-4.7% of total fatty acids ([17]: 4.4%; [18]: 2.2%).

For preparation of 18:4n-6, a commercially available single-cell oil DHA product was found to be a better alternative to pig testes lipids, in terms of 22:5n-6 content (average in pig testes lipids 4.4 % of total fatty acids vs. 14.9 % of total fatty acids in the Vegetarian DHA)

and in total lipid content (pig testes – 3.5 % extractable lipids of wet weight, while Vegetarian DHA oil as virtually pure lipid did not require extraction), and was therefore employed in all subsequent Δ3-fatty acids preparations. Starting compounds, intermediates and final products are presented at Fig. 2.

A mixture of FFAs containing on average 47 % of 18:4n-6 and 36 % of 18:5n-3 was obtained with yields 24-33 % of theoretical (starting from crude iodolactones). Attempts to purify intermediate products, especially aldehydes, resulted in greatly reduced overall yields, down to as low as 8 %. The target compound, 18:4n-6, was isolated from this mixture by HPLC with 96.7 % purity (by GC), with the major contaminants being 18:5n-3 (0.5 %), 14:0 (0.6 %), 16:3n-4 (0.3 %), 20:5n-3 (0.3 %) and the long chain linear aliphatic hydrocarbon "grease" [19]. In NI QToF-MS the molecular ion of 18:4n-6 was observed at *m/z* 275.2008 ($C_{18}H_{27}O_2^-$, calculated 275.2011). The other Δ3 acid, 18:5n-3, was obtained simultaneously at 96 % purity by GC (observed *m/z* 273.1854, $C_{18}H_{25}O_2^-$, calculated 273.1855).

The structures of 18:4n-6 and 18:5n-3 were confirmed by ^{1}H-, ^{13}C-, COSY- and HSQC NMR spectra (see Table 1, Table 2; Fig. 3 for assignment of the signals). The signals of olefinic protons of the Δ3 double bond are noticeably shifted downfield from those of common PUFA. As for the most characteristic feature in the ^{13}C-NMR spectrum, the chemical shift of the C3 carbon, about 121.3 ppm, falls well outside of that of the range of the chemical shifts of olefinic carbons in more familiar PUFA (e.g. see http://lipidlibrary.aocs.org/nmr/nmrpufa/index.htm).

On a highly polar ionic liquid phase SLB-IL100 methyl ester of 18:4n-6 could be mistaken for 18:3n-3, while on methylsilicone phase BP1 it eluted ahead of 18:3n-6 and 18:4n-3, suggesting that when present it may be easily misidentified during GC analysis of fatty acids (see Table 3).

Whist the molecular weights of the Δ3 esters may not be deduced from the spectra obtained by GCMS of methyl esters (Fig. 4), some structural information may still be gathered: according to [20] in GC-MS of fatty acids methyl esters of three ions, *m/z* 108, 150, and 192, the peak at *m/z* 108 is dominating for the n-3 family, *m/z* 150 for the n-6 family, and *m/z* 192 for the n-9 family. Indeed, the spectrum of 18:4n-6 methyl ester (Fig. 4, the lower spectrum) shows the ion with *m/z* 150 that is absent from the spectrum of 18:5n-3 methyl ester (Fig. 4, the upper spectrum), which, in turn displays the ion with *m/z* 108.

Attempts to produce DMOX derivatives of 18:4n-6 and 18:5n-3 for GC-MS analysis using the method [10] resulted in the formation of 2-*trans* isomers, as confirmed by the ^{1}H-NMR-spectrum showing a disappearance of signals of olefinic protons of the Δ3 double bond (5.58 ppm), accompanied by a presence of two novel olefinic protons, at C2 and C3, a doublet at 5.81 ppm and a multiplet at 6.86 ppm, respectively, as well as a novel group of allylic protons at C4 and C5 (a multiplet at 2.25 ppm). Due to a rapid conversion of the 3-*cis*- to 2-*trans*-double bond GC-MS studies of DMOX derivatives were not performed. Such a conversion has been reported earlier this year by Svetashev and Imbs who noted that "all methods of preparation of N-acyl derivatives for GC-MS that [they] tested led to fast isomerization of 18:5n-3" [21]. They also observed that 18:5n-3 acid quickly isomerises if an attempt to produce its methyl ester is made with the use of base-catalysed derivatisaion. Since in our study 18:4n-6 was produced in a form of FFA, there was no need to use the alkakine methylation reagent to produce its methyl ester. The absence of isomerisation under conditions used was confirmed by both GC and NMR data.

The search performed with the use of the SciFinder service resulted in finding 33 more references (apart from references [7] and [8]) that mentioned 18:4n-6 in the tables or figures. Only one of these publications [22] has a caveat that this fatty acid was only tentatively identified, and none of these papers or patents discusses the fact of finding such a peculiar

fatty acid. Identification in the rest of these publications either referred to the use of commercial reference mixtures of FAME (but 18:4n-6 was not listed as a component in any manufacturers' specifications) or by the use of GCMS of methyl esters (which, as can be seen from Fig. 4, is not very informative). Tables in some publications owe the presence of impressive levels of 18:4n-6 to typographic errors (e.g. the components actually discussed in the text were 18:3n-6 or 18:4n-3, or even 20:4n-6). In the work where the authors tentatively identified 18:4n-6 as a minor component of a menhaden fish oil-derived Ag^{+}-SPE fraction, it possessed an ECL value of 19.449, which matches the ECL value of 19.45 determined in our work using a similar column [22].

Finally, it should be noted that to the best of the authors' knowledge, the name "γ-stearidonic acid" is the only common name ever used for 18:4n-6 [7]. The use of a distinctive name, e.g. "2,3-dinor arachidonic acid", might be better, albeit more cumbersome, to avoid confusion with its more familiar, stearidonic acid, 18:4n-3.

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Conclusion

The Vegetarian DHA oil that contains high levels of 22:5n-6 acid was found to be a convenient starting material in the preparation of γ-stearidonic acid, 18:4n-6, a potential product of β-oxidation of arachidonic acid, 20:4n-6, which was only recently positively identified in a living organism. The structure of γ-stearidonic acid was confirmed by QToF MS, ^{1}H-, ^{13}C-, COSY- and HSQC NMR, with all of these spectra recorded for the first time. NMR spectra display a characteristic pattern of Δ3 methylene-interrupted PUFA, allowing monitoring isomerisation into Δ2 non-methylene interrupted arrangement of double bonds upon derivatisation. The GC and GC-MS behaviour of γ-stearidonic acid methyl ester suggests that this acid may be easily misidentified during GC analysis of fatty acids.

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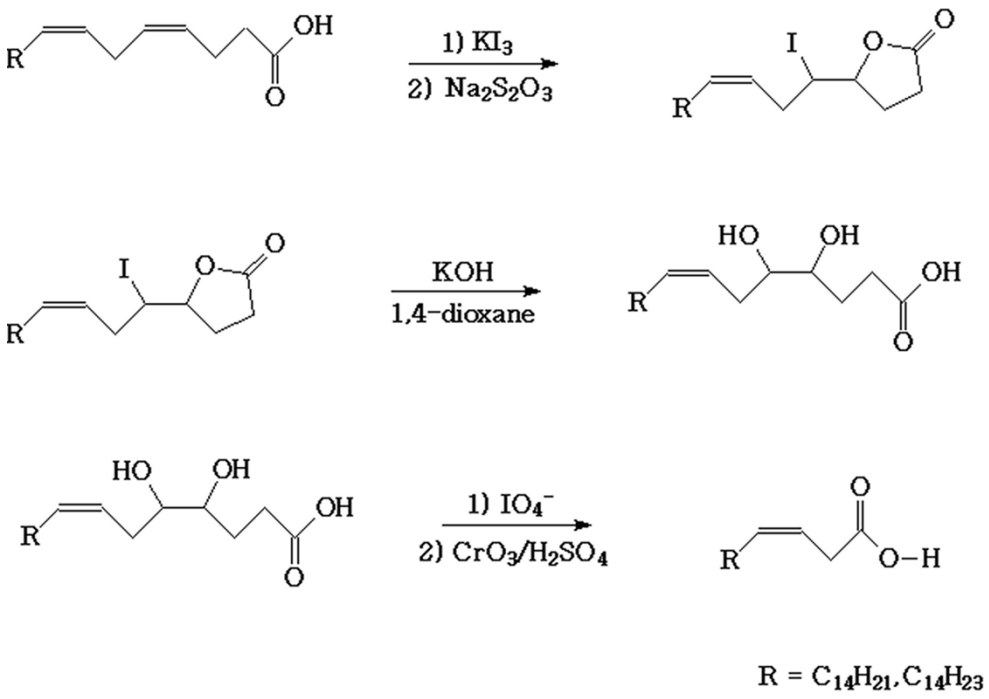

Fig. 1 Synthetic approach employed in the preparation of Δ3 fatty acids (after [15]).

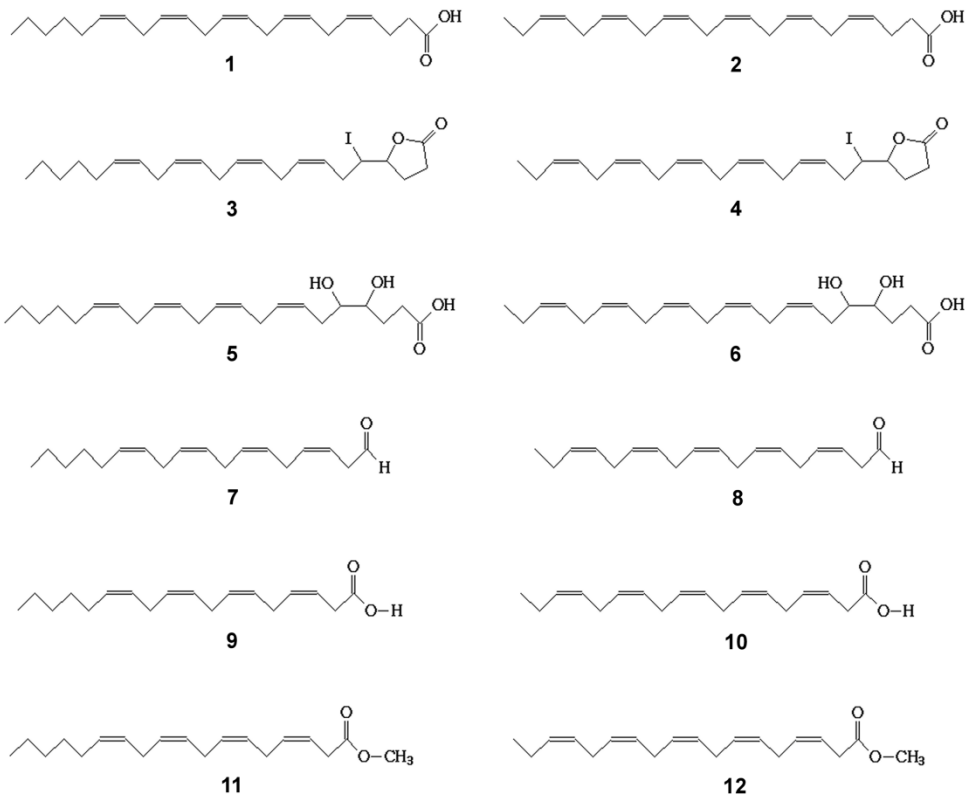

Fig. 2 Starting compounds, intermediates and final products. *1* Osbond acid, 22:5n-6; *2* docosahexaenoic acid, 22:6n-3, DHA; *9* γ-stearidonic acid, 18:4n-6; *10* octadecapentaenoic acid, 18:5n-3, ODPA

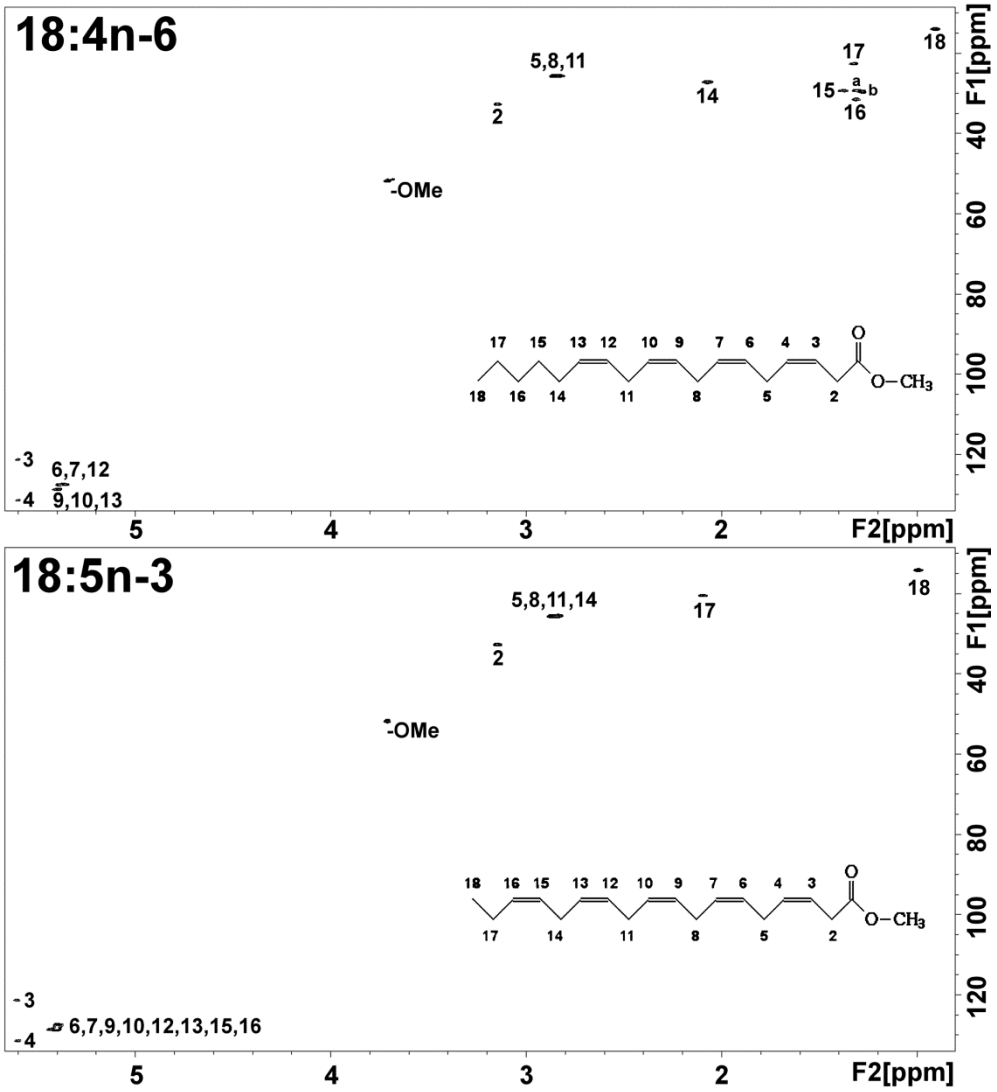

Fig. 3 HSQC spectra of methyl esters of 18:4n-6 (*upper*) and 18:5n-3 (*lower*). Axes: F1–^{13}C; F2–^{1}H. See also Tables 1 ad 2 for the signals' assignments. **a** – CH_2 signal from myristic acid impurity; **b** – CH_2 signal from "grease" [19]

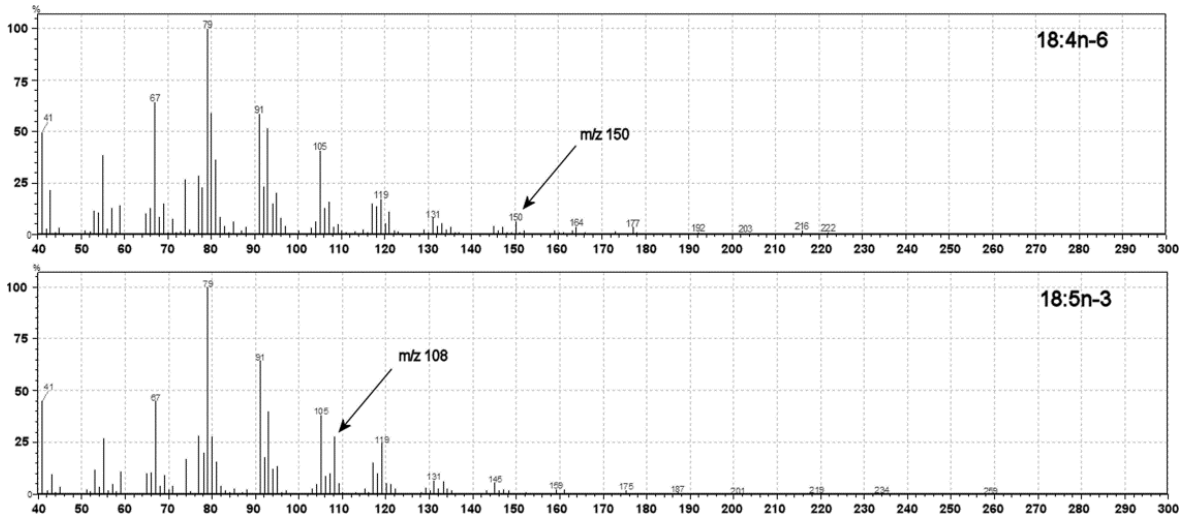

Fig. 4 Mass-spectra of methyl esters of 18:4n-6 and 18:5n-3 (positive ion EI GC-MS)

Table 1 ^{1}H-NMR spectra of 18:4n-6 and 18:5n-3 in $CDCl_3$, in ppm relative to TMS.

	18:4n-6 methyl ester (**11** in Fig. 2)	18:5n-3 methyl ester (**12** in Fig. 2)
H2	3.13 d	3.13 d
H3	5.58 m	5.58 m
H4	5.58 m	5.58 m
H5	2.83 m	2.83 m
H6	5.38 m	5.36 m
H7	5.38 m	5.36 m
H8	2.83 m	2.83 m
H9	5.38 m	5.36 m
H10	5.38 m	5.36 m
H11	2.83 m	2.83 m
H12	5.38 m	5.36 m
H13	5.38 m	5.36 m
H14	2.05 m	2.83 m
H15	1.36 m	5.36 m
H16	1.29 m	5.36 m
H17	1.29 m	2.08 m
H18	0.89 t	0.97 t
$-OCH_3$	3.69 s	3.69 s

Atoms numbered as in Fig. 3

Table 2 ^{13}C-NMR spectra of 18:4n-6 and 18:5n-3 in $CDCl_3$, in ppm relative to TMS.

18:4n-6 Methyl ester (**11** in Fig. 2)	
C1	172.19
C2	32.77
C3	121.26
C4	131.36
C5, C8, C11	25.63, 25.63, 25.76
C6, C7, C9, C10	127.49, 127.70, 128.70, 128.76
C12	127.29
C13	130.53
C14	27.22
C15	29.31
C16	31.51
C17	22.55
C18	14.03
-OCH_3	51.82

18:5n-3 Methyl ester (**12** in Fig. 2)	
C1	172.18
C2	32.77
C3	121.28
C4	131.34
C5, C8, C11, C14	25.54, 25.63, 25.63, 25.76
C6, C7, C9, C10, C12, C13	127.36, 127.83, 127.94, 128.38, 128.62, 128.68
C15	127.01
C16	132.05
C17	20.55
C18	14.24
-OCH_3	51.83

Atoms numbered as in Fig. 3.

Table 3 Equivalent chain lengths (ECL) of methyl esters of 18:4n-6 and relevant fatty acids on different phases.

Column	BP1	BP5	SolGelWax	TG-WAXMSA	Omegawax 320	Omegawax 320	Silar 5CP	SLB-IL100	SLB-IL100
Oven temperature	210 °C	210 °C	195 °C	195 °C	180 °C	200 °C	200 °C	190 °C	200 °C
Dimensions	60 m * 0.32 mm i.d.	50 m * 0.32 mm i.d.	30 m * 0.55 mm i.d.	30m * 0.25 mm i.d.,	30 m * 0.32 mm i.d.	30 m * 0.32 mm i.d.	60 m * 0.25 mm i.d.	100 m * 0.25 mm i.d.	100 m * 0.25 mm i.d.
18:3n-6	17.48	n.d.	18.99	19.03	n.d.	n.d.	n.d.	n.d.	n.d.
18:3n-3	17.69	17.79	19.29	19.37	n.d.	19.34	n.d.	n.d.	21.13
18:4n-6	17.43	17.54	19.46	19.55	19.45	19.52	19.58	20.86	21.18
18:4n-3	17.51	n.d.	n.d.	19.69	19.59	n.d.	19.82	21.65	n.d.
18:5n-3	17.46	17.60	20.08	20.21	20.09	20.15	20.20	21.95	22.27

n.d. – not determined