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Chemistry and Physics of Lipids 130 (2004) 145–158
CPL
CHEMISTRY AND PHYSICS OF LIPIDS
www.elsevier.com/locate/chemphyslip

Synthesis of long chain n-3 and n-6 fatty acids having a photoactive conjugated tetraene group

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Received 11 December 2003; received in revised form 25 February 2004; accepted 25 February 2004

Available online 26 April 2004

Abstract

Fatty acids of the n-3 and n-6 families containing a photoactive conjugated tetraene group near the carboxylate were prepared from several naturally occurring fatty acids by sequential iodolactonization and treatment with excess 1,8-diazabicyclo[5.4.0] undec-7-ene. The new conjugated fatty acids include 5*E*,7*E*,9*E*,11*Z*,14*Z*- and 5*E*,7*E*,9*E*,11*E*,14*Z*-eicosapentaenoic acids derived from arachidonic acid; 5*E*,7*E*,9*E*,11*Z*,14*Z*,17*Z*- and 5*E*,7*E*,9*E*,11*E*,14*Z*,17*Z*-eicosahexaenoic acids from eicosapentaenoic acid; and 4*E*,6*E*,8*E*,10*Z*,13*Z*,16*Z*,19*Z*- and 4*E*,6*E*,8*E*,10*E*,13*Z*,16*Z*,19*Z*-docosaheptaenoic acids from docosahexaenoic acid. All of the newly synthesized fatty acids were characterized by UV, ^{1}H NMR and mass spectroscopy. These new products represent the first examples of directed conjugation of methylene interrupted double bond systems. The products can be synthesized in gram quantities and in high yields (>50%). Interestingly, it did not prove possible to synthesize fatty acids having a triene group near the carboxyl group even using mild conditions and different synthetic approaches. Once initiated, the isomerization always continued until a tetraene group was formed. Because of the sensitivity of the tetraene group to light, these fatty acids have the potential for being used in tracking fatty acid movements in cells employing fluorescence techniques and in UV light-induced cross linking to membrane proteins.

Subj. Classification: Parinaric acid; Arachidonic acid; Docosahexaenoic acid; Eicosapentaenoic acid; Conjugated fatty acids

Keywords: Photoactive; Tetraene group; Fatty acid

Abbreviations: AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; Q^a, fluorescent yield; MTAD, 4-methyl-1,2,4-triazoline-3,5-dione; 4-HDHE, 4-hydroxy,5*E*,7*Z*,10*Z*,13*Z*,16*Z*,19*Z*-docosahexaenoic acid; TMS, trimethylsilyl; 5-HEPE, 5-hydroxy,6*E*,8*Z*,11*Z*,14*Z*,17*Z*-eicosapentaenoic acid; 5-HETE, 5-hydroxy,6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid; THR, tetrahydrofuran; PUFA, polyunsaturated fatty acid; HUFA, highly unsaturated fatty acid; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; eq, molar equivalent; BHT, butylated hydroxytoluene; MS, mass spectrum

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doi:10.1016/j.chemphyslip.2004.02.006

1. Introduction

Fatty acids with conjugated double bonds have been known for many years to be naturally occurring compounds (Solodovnik, 1967), and more recently there has been an interest in exploring the biological activities of these fatty acids (Banni and Martin, 1998; Pariza et al., 2000; Scimeca et al., 1994; Solodovnik, 1967; Yurawecz et al., 1999). Conjugated fatty acids are claimed to exhibit anti-atherosclerotic effects, to potentiate immune responses and to modulate energy metabolism (e.g. by promoting protein as opposed to fat deposition). Another aspect of the interest in conjugated fatty acids has been in their use as fluorescent probes for studying membrane structure (Sklar et al., 1975). Sklar et al. (1975, 1976, 1977) demonstrated that parinaric acid (9*Z*,11*E*,13*E*,15*Z*-octadecatetraenoic acid) can be used to detect phase transitions in bilayers as well as interactions among lipids and proteins (Comfort and Howell, 2002). *cis*-Parinaric acid can be incorporated into phospholipids by lipid biosynthetic pathways and spectroscopic investigations can be performed. *cis*-Parinaric acid has become widely used as a membrane probe and is now available commercially. Additional information about the dynamics of membrane behavior as well as interactions between proteins and lipids can be obtained by using other conjugated double bond systems (e.g. with the chromophore in the middle of the fatty acid or near the carboxyl group (Goerger and Hudson, 1988).

During the past fifteen years several polyconjugated and polyunsaturated fatty acids have been isolated from marine plants mostly by Gerwick and co-workers (Michailova et al., 1995; Wise et al., 1994). In developing biochemical tools that might be used to study fatty acids containing n-3 and n-6 double bonds, we synthesized fatty acids bearing a photoactive conjugated tetraene group near the carboxyl group and a natural methylene interrupted n-3 or n-6 grouping. We believe this to be the first report of a directed synthetic method for creating this type of polyconjugated system of double bonds close to carboxyl group from a methylene interrupted system of double bonds in natural polyunsaturated fatty acids. Another aspect of our studies involved investigating the stability of the methylene interrupted system of *cis*-double bonds in naturally occurring essential fatty acids in the context of devising synthetic techniques for constructing other novel fatty acids.

2. Materials and methods

2.1. Reagents

Arachidonic acid (AA; 90%) and 1,8-diazabicyclo [5.4.0]undec-7-ene (DBU) were purchased from Sigma Chemical Co. (St. Louis, MO). Fish oil (28% eicosapentaenoic acid (EPA) and 23% docosahexaenoic acid (DHA)) was purchased from Wal-Mart. Trifluoroacetic anhydride, ethanolamine, isobutylchloroformate, pyridine, pyrrolidine and 4-methyl-1,2,4-triazoline-3,5-dione were products of Aldrich Chemical Co. (Milwaukee, WI) with purities $\geq$ 96%. Benzene, hexane, ether and acetonitrile were distilled over phosphorus pentoxide and triethylamine, tetrahydrofuran (THF) and methanol were distilled over metallic sodium before use. DBU was distilled over CaH_2 in vacuo. Silica gel "Selecto" 32–63 μm was purchased from Selecto Scientific (Suwanee, GA). Thin layer chromatography (TLC) plates were purchased from Sigma. Compounds on TLC plates were visualized by spraying the plates with a 5% solution of phosphomolybdic acid in methanol and then heating the plates for 2–3 min at about 100 °C.

2.2. Mass spectrometry and HPLC

Mass spectra were obtained using a Hewlett-Packard 5890 gas chromatograph coupled to a Hewlett-Packard 5970 series mass selective detector operated with a Hewlett-Packard 7946 computer. Gas chromatography conditions were as follows: He was used as the carrier gas at a flow rate of 35 cm/s; the oven temperature was kept at 210 °C; the injector temperature was 250 °C; the interface temperature was 250 °C; separations were on a capillary column DB-5ms (30 m × 0.32 mm, 1 μm; J&W, USA); the injector split ratio was kept constant at 1:60. The mass detector conditions were as follows: the electron energy was 70 eV; the emission current was 0.8 mA; the accelerating voltage was 8 kV; the scale was from 50 to 1000. HPLC analysis and preparative separations were performed on an Alliance HPLC system (Waters, USA) equipped with a Waters 2695 separation

module and a Waters 2996 photodiode array detector. Analytical RP-HPLC was performed on a Nucleosil-C18 analytical column (4.6 mm × 250 mm, 5 μm; Xpertek, USA). Solid phase extraction was performed on Luna-2 C18 guard column (10 mm × 50 mm, 10 μm; Phenomenex, Torrance, CA). Preparative separations were performed on a Kromasil C18 column (10 × 250, 5 μm; Xpertek, Cobert Associates, St. Louis, MO). ^{1}H NMR spectra were recorded on a Varian INOVA-300 spectrometer operated at 300 MHz; for samples dissolved in $CDCl_3$, tetramethylsilane was used as the internal standard. All of the signal assignments were performed on the basis of selective decoupling experiments. All of the UV-Vis spectra were recorded on a Hewlett-Packard 8453 instrument; the UV-Vis spectrophotometer was operated with ChemStation data processing software.

2.3. Preparation of iodolactones of DHA, EPA and AA

A mixture of fish oil fatty acids (43.5 g, 28% EPA and 23% DHA) was dissolved in 150 ml of ethanol and 230 ml of a 7.5% solution of $KHCO_3$ (17.3 g of $KHCO_3$; 1.2 eq. per total fatty acid) was added to give a clear solution of fatty acid salts. A solution of 27 g of I_2 in 300 ml of ethanol (1.5 eq. per EPA plus DHA) and 500 ml of hexane were added to the clear solution with vigorous stirring. The reaction mixture was kept at room temperature (24 °C) for 16 h, the hexane layer was removed, and the reaction mixture was extracted with hexane (3 × 500 ml). The combined hexane layers were washed sequentially with aqueous 5% $Na_2S_2O_3$ (250 ml), water (500 ml) and saturated aqueous NaCl (200 ml) and then dried over anhydrous Na_2SO_4 and evaporated under vacuum. The crude oily product (24.9 g, indicating ≥75% yield) was dissolved in dry benzene (30 ml) and after addition of 2 g of BHT was stored at −80 °C. Aliquots of this crude mixture of the γ-iodolactone of DHA (Fig. 1, Ic) and the δ-iodolactone of EPA (Fig. 1, Ib) were purified by column chromatography on silica gel as required to isolate the individual iodolactones. The purification needs to be performed quickly using a 25–33-fold weight excess of silica gel as the adsorbent and elution with a gradient of ether in benzene (0–5%). To prevent on column degradation of the iodolactones, the column is prewashed with 1% BHT in dry benzene.

In the case of arachidonic acid its δ-iodolactone (Fig. 1, Ia) was synthesized from a commercially available concentrate with an AA content of about 90%. The yield of chromatographically pure product was 62%.

2.4. Reaction of iodolactones with DBU to form polyconjugated fatty acids (Fig. 1, reaction (1))

The δ-iodolactone of eicosapentaenoic acid (Fig. 1, Ib) (1.30 g, 3.0 mmol) was dissolved in 15 ml of dry benzene and a solution of 1.2 g (7.9 mmol) of dry DBU in 15 ml of dry benzene was added. The reaction mixture was left at room temperature under nitrogen for 72 h in the dark. The yield of the expected conjugated fatty acids (Fig. 1, IIIb) determined spectrophotometrically was 93% (ε (303 nm) = 74,000 l mol^{-1} cm^{-1}). Spectral information is presented in Section 3.

Similarly from 1.4 g (3.1 mmol) of the γ-iodolactone of docosahexaenoic acid (Fig. 1, Ic) through the action of 1.2 g (7.9 mmol) of dry DBU, the conjugated fatty acids (IIIc) were obtained with a yield of 95%. The composition of the mixture of tetraenoic fatty acids was ∼90% 4*E*,6*E*,8*E*,10*Z*,13*Z*,16*Z*,19*Z*-docosaheptaenoic acid and ∼10% 4*E*,6*E*,8*E*,10*E*,13*Z*,16*Z*,19*Z*-docosaheptaenoic acid as determined by HPLC.

4*E*,6*E*,8*E*,10*Z*,13*Z*,16*Z*,19*Z*-Docosaheptaenoic acid exhibited the following spectral properties: UV (methanol): λ_{max} = 290, 303, 317 nm; ε (303 nm) = 74,000 l mol^{-1} cm^{-1}. Fluorescence (methanol): Em λ_{max} = 428 nm; Q^a = 0.017. MS of oxazoline (*m*/*z*, (I%)): $[M]^+$ 351 (17), $[M-1]^+$ 350 (10), $[M–CH_3]^+$ 336 (5), $[M–C_2H_5]^+$ 322 (7), 202 (12), 242 (12), 282 (18), 322 (5), 85 (100), 98 (55), 111(30) (Δ4 double bond) and 141 (33). RP-HPLC (C_{18} column eluted with 85:15:0.1, methanol–H_2O–acetic acid): k' = 8.8. ^{1}H NMR: δ 0.95 (3H, t, J = 7.5 Hz, H-22), 2.06 (2H, m, H_2-21), 2.35 (2H, m, H_2-3), 2.4 (2H, m, H_2-2), 2.80 (4H, m, H_2-15,18), 2.88 (2H, m, H_2-12), 5.36 (7H, m, H-11,13,14,16,17,19,20), 5.66 (1H, dt, $J_{5,6}$ = 14.4 Hz, J_{54} = 6.9 Hz, H-4), 6.08 (1H, m, H-10), 6.18 (4H, m, H-5,6,7,8), 6.46 (1H, dd, $J_{9,8}$ = 13.5, $J_{9,10}$ = 11.1, H-9).

4*E*,6*E*,8*E*,10*E*,13*Z*,16*Z*,19*Z*-Docosaheptaenoic acid yielded the following spectral data: UV (methanol): λ_{max} = 289, 300, 315 nm; ε 300 nm) ∼ 80,000 l mol^{-1} cm^{-1}. Fluorescence: Em λ_{max} = 422 nm; Q^a =

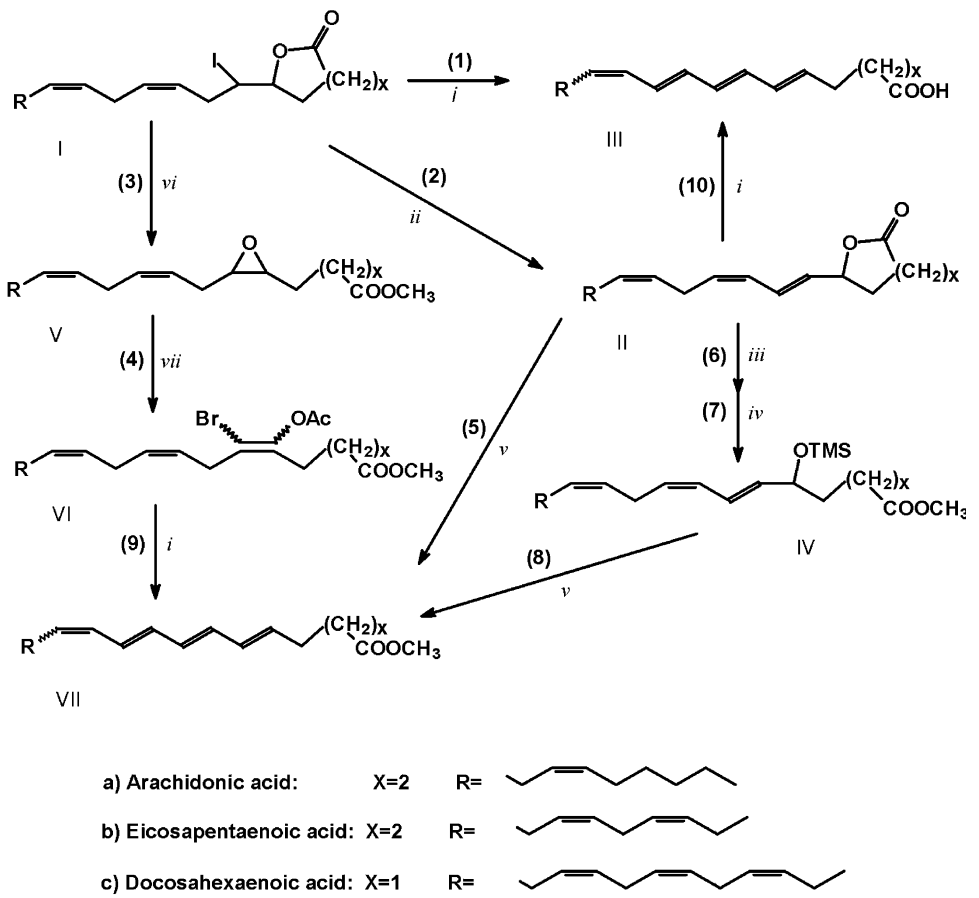

Fig. 1. Formation of polyconjugated fatty acids from naturally occurring highly unsaturated fatty acids. Reagents and conditions: (i) 2.2 mol DBU, 72 h, 24 °C; (ii) 1.1 mol DBU, 12 h, 24 °C; (iii) 0.2 N KOH, 40% ethanol, 16 h, 24 °C; (iv) CH_2N_2/ether; chlorotrimethylsilane/pyridine, 1 h, 70 °C; (v) 0.5% H_2SO_4 in methanol in a sealed ampule, 80 °C, 1 h; (vi) 3 mol triethylamine in methanol, boiling under reflux, 5 h; (vii) 1.15 mol acetylbromide in ether, 1 h, 24 °C.

0.013. RP-HPLC (C_{18} column eluted with 85:15:0.1, methanol–H_2O–acetic acid) $k' = 9.5$. MS of oxazoline the same as for its isomer noted above; MS of the MTAD adduct (*m/z* (I%): $[M]^+$453 (3), $[M–C_4H_7O_2]^+$ 366(11), $[M–C_4H_7O_2–C_2H_3NO]^+$ 309 (6), $[M–C_{11}H_{17}]^+$ 304(8). ^{1}H NMR: δ 0.95 (3H, t, $J = 7.5$ Hz, H-22), 2.06 (2H, m, H_2-21), 2.35 (2H, m, H_2-3), 2.4 (2H, m, H_2-2), 2.80 (4H, m, H_2-15,18), 2.88 (2H, m, H_2-12), 5.36 (6H, m, H-13,14,16,17,19,20), 5.66 (2H, m, $J_{4,5} \approx J_{10,11} = 14.5$, $J_{4,3} \approx J_{11,12} = 7$, H-4,11), 6.13 (6H, m, H-5,6,7,8,9,10).

Similarly from 1.58 g (3.7 mmol) of the δ-iodolactone of arachidonic acid (Fig. 1, Ia) and the action of 1.4 g (9.2 mmol) of dry DBU, the conjugated fatty acids (Fig. 1, IIIa) were obtained in a yield of 95% (determined spectrophotometrically). The composition of the mixture of tetraenoic fatty acids determined by HPLC was 85% 5*E*,7*E*,9*E*,11*Z*,14*Z*-eicosapentaenoic acid and 15% 5*E*,7*E*,9*E*,11*E*,14*Z*-eicosapentaenoic acid.

5*E*,7*E*,9*E*,11*Z*,14*Z*-Eicosapentaenoic acid exhibited the following spectral properties: UV (methanol): $\lambda_{max} = 290, 303, 317$ nm; ε (303 nm) = 74,000 l mol^{-1} cm^{-1}. RP-HPLC (C_{18} column eluted with 85:15:0.1, MeOH–H_2O–acetic acid) $k' = 9.7$. Fluorescence (methanol): Em $\lambda_{max} = 428$ nm; $Q^a = 0.015$. MS of oxazoline (*m/z*, (I%)): $[M]^+$ 327 (41), $[M - 1] + 326$ (35), $[M–1Me]^+$ 312 (15), $[M–C_5H_{11}]^+$ 256 (11), $[M–C_7H_{13}]^+$ 230 (7),

$[M–C_8H_{11}]^+$ 216 (10), 98 (35), 85 (100). ^{1}H NMR (300 MHz, $CDCl_3$, δ, ppm): 0.89 (3H, t, $J = 7$ Hz, H_3-20), 1.33 (6H, m, H_2-17,18,19), 1.73 (2H, tt, $J_{32} = 7.5$; $J_{34} = 7.4$, H-3), 2.03 (2H, m, H_2-16), 2.23 (2H, bdt, $J_{43} = 7.4$, $J_{45} = 6.9$, H_2-4), 2.33 (2H, t, $J_{23} = 7.5$, H_2-2), 2.95 (2H, m, H-13), 5.36 (3H, m, H-12,14,15), 5.64 (1H, dt, $J_{5,6} = 14.4$ Hz, $J_{54} = 6.9$ Hz, H-5), 6.09 (1H, m, H-6), 6.18 (4H, m, H-7,8,9,11), 6.47 (1H, dd, $J_{10,9} = 13.7$, $J_{10,11} = 11$, H-10).

5*E*,7*E*,9*E*,11*E*,14*Z*-Eicosapentaenoic acid exhibited the following spectral properties: UV (methanol): $\lambda_{max} = 289, 300, 315$ nm; ε (300 nm) $\sim$80,000 l mol^{-1} cm^{-1}. RP-HPLC (C_{18} column eluted with 85:15:0.1, MeOH–H_2O–acetic acid) $k' = 10.6$. Fluorescence (methanol): Em $\lambda_{max} = 428$ nm; $Q^a = 0.013$. MS of oxazoline is the same as that noted above for its isomer. Mass spectrometry of methyl ester of MTAD adduct (*m/z* (I%)): $[M]^+$ 429 (8), $[M–C_8H_{15}]^+$ 318 (50). ^{1}H NMR: 0.89 (3H, t, $J = 7$ Hz, H_3-20), 1.33 (6H, m, H_2-17,18,19), 1.73 (2H, tt, $J_{32} = 7.5$; $J_{34} = 7.4$, H-3), 2.03 (2H, m, H_2-16), 2.23 (2H, bdt, $J_{43} = 7.4$, $J_{45} = 6.9$, H_2-4), 2.33 (2H, t, $J_{23} = 7.5$, H_2-2), 2.95 (2H, m, H_2-13), 5.39 (2H, m, H-14,15), 5.68 (2H, m, $J_{5,6} \approx J_{11,12} = 14.5$, $J_{5,4} \approx J_{12,13} = 7$, H-5,12), 6.13 (6H, m, H-6,7,8,9,10,11).

Conjugated fatty acids were purified as follows. The reaction mixture was transferred to a centrifuge tube and centrifuged at 1000 × *g* for 5 min. The clear solution was separated from the precipitate which was then stirred vigorously with dry benzene (25 ml) and centrifuged again, and the clear benzene solutions were combined. The solution was transferred to an evaporating flask, 5 ml of dry diglyme (2-methoxyethyl ether) was added, and the mixture was evaporated under vacuum keeping the bath temperature at less than 30 °C. To the resulting solution of the reaction mixture in diglyme were added 5 ml of methanol, 5 ml of water and 600 μl of acetic acid. The mixture was stirred for 30 s and put on a solid phase extraction cartridge (pre-washed with 10 ml of methanol and then 10 ml of water). The cartridge was eluted with 15 ml of water, 30 ml of a methanol–water mixture (50:50, v/v) and 2 ml of methanol. All the fatty acid was found in the methanol fraction. To the solution of the conjugated fatty acid was added 6 ml of water, and the resulting solution was pumped through a guard column (50 × 10 mm, C18, 10 μm, equipped with 0.5 in. × 0.031 in., 2 μm PEEK frits (Alltech)). After concentrating the fatty acid, the guard column was attached to a preparative HPLC column (C18, 250 × 10 mm, 5 μm, equipped with PEEK frits), and the system was washed with methanol–water–acetic acid (85:15:0.1). The fractions containing the purified fatty acid product were combined and placed −80 °C. After an overnight crystallization, a white precipitate of the conjugated fatty acid was collected.

2.5. Reaction of iodolactones with DBU to form allylic lactones (Fig. 1, reaction (2))

The γ-iodolactone of docosahexaenoic acid (Fig. 1, Ic) (2.10 g; 4.63 mmol;) was dissolved in 15 ml of dry benzene and a solution of dry DBU (770 μl; 5.1 mmol) in 15 ml of dry benzene was added. The reaction mixture was stirred at room temperature under nitrogen for 12 h in the dark, then transferred to a centrifuge tube and centrifuged at 1000 × *g* for 5 min. The clear solution was separated from the precipitate which was then vigorously stirred with dry benzene (25 ml) and centrifuged again, and the clear benzene solutions were combined. The resulting solution was evaporated under vacuum, the dry residue dissolved in a small amount of benzene and after addition of 0.25 g of BHT, the sample was purified by column chromatography on 60 g of silica gel using a gradient of dry ether in dry benzene (0–7%). The yield was 1.03 g (68%) of the γ-lactone of 4-HDHE (4-hydroxy,5*E*,7*Z*,10*Z*,13*Z*,16*Z*,19*Z*-docosahexaenoic acid (IIc, Fig. 1)) as a pale yellow oil.

Similarly, from δ-iodolactones of EPA (Fig. 1, Ib) and AA (Fig. 1, Ia) the corresponding δ-lactones of 5-HEPE (5-hydroxy,6*E*,8*Z*,11*Z*,14*Z*,17*Z*-eicosapentaenoic acid (Fig. 1, IIb) and 5-HETE (5-hydroxy,6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid (Fig. 1, IIa) were synthesized in yields of 72 and 71%, respectively. The spectral properties of these newly synthesized compounds are in agreement with those reported previously (Corey et al., 1983, 1980; Kuklev and Bezuglov, 1998; Wright et al., 1987).

2.6. Reaction of allylic lactones with excess DBU to form polyconjugated fatty acids (Fig. 1, reaction (10))

The conditions for the reactions of allylic lactones with excess DBU to form polyconjugated fatty

acids are as described above for the reaction between iodolactones and DBU. Thus, from the γ-lactone of 4-HDHE (Fig. 1, IIc) the corresponding polyconjugated fatty acids were obtained in a yield of 93% (determined spectrophotometrically). Similarly, from δ-lactones of EPA (Fig. 1, IIb) and AA (Fig. 1, IIa) the corresponding polyconjugated fatty acids were obtained in yields of 86%.

2.7. Base cleavage of allylic lactone ring—hydroxy acid formation (Fig. 1, reaction (6))

To a solution of 236 mg (0.72 mmol) of the γ-lactone of 4-HDHE in 5 ml of ethanol was added 1 ml of 1.5N KOH, 3 ml of ethanol and 3 ml of water, and the reaction mixture was stirred for 16 h at room temperature. Water (10 ml) was added, and the solution was acidified to pH 4 with 1.5N HCl and extracted with hexane–ether (1:1) (3 × 10 ml). The resulting organic extract was washed with water (2 × 20 ml) and then a saturated solution of NaCl and dried over Na_2SO_4. The dry extract was filtered and evaporated under vacuum to yield 214 mg (86%) of 4-HDHE. Similarly, 5-HETE and 5-HEPE were synthesized from their corresponding γ-lactones in yields of more than 85%. The spectral properties of these hydroxy fatty acids were identical to those reported previously (Corey et al., 1980; Kuklev and Bezuglov, 1998; Solodovnik, 1967).

2.8. Synthesis of methyl esters of TMS-ethers of hydroxy acids (Fig. 1, reaction (7))

4-HDHE (60 mg) was converted to its methyl ester using freshly distilled diazomethane in ether. The solvent was evaporated and the sample dissolved in 1 ml of dry pyridine (freshly distilled over CaH_2). Chlorotrimethylsilane (50 μl) was added, and the reaction mixture kept at 70 °C for 2 h. After cooling, the reaction mixture was evaporated under a stream of nitrogen and the dry residue was dissolved in 2–5 ml of hexane and filtered through 500 mg of silica gel. The trimethylsilylether of the methyl ester of 4-HDHE had an $R_f \sim 0.9$ (hexane–ether, 1:1) and was used without further purification. Similar procedures were used to obtain the methyl esters of 5-HETE and 5-HEPE and their corresponding trimethylsilylethers.

2.9. Cleavage of TMS-ethers of allylic alcohols under acidic conditions: formation of tetraenoic polyconjugated and polyunsaturated fatty acid methyl esters (Fig. 1, reaction (8))

The dry oil of the trimethylsilylether of the methyl ester of 5-HEPE (∼50 mg) (Fig. 1, IVb) was dissolved in 1 ml of a 0.5% solution of H_2SO_4 in methanol (1 ml of diglyme and 100 μl of 1N HCl or 50 μl trifluoroacetic acid may be used as well), the reaction mixture was put in a sealed ampule and kept at 80 °C for 2 h. After cooling, the ampule was opened, and the contents were dissolved in 5 ml of methylene chloride and washed in turn with water (3 × 5 ml) and saturated aqueous NaCl (50 ml), and then dried over anhydrous Na_2SO_4, filtered and stored at −80 °C. The trimethylsilylethers of methyl esters of 5-HETE and 5-HEPE were converted to their corresponding esters using similar protocols.

The yields of the conjugated tetraenoic acid methyl esters measured spectrophotometrically were 70–74% based on the parent trimethylsilylether of the methyl ester of the hydroxy acid. The isomer compositions determined by HPLC analysis after a 2-h reaction time were the same as for the synthesis involving the use of excess DBU. There was a tendency for the amount of the all *trans*-isomers to increase with the reaction time (data not shown).

2.10. Acid catalyzed cleavage of the allylic lactone ring (Fig. 1, reaction (5))

To a solution of 350 mg of the γ-lactone of 4-HDHE in 3 ml of absolute methanol was added 2 ml of 0.5% concentrated sulfuric acid in absolute methanol. The mixture was put in an ampule, sealed and kept at 80 °C for 1 h. The reaction mixture changed from colorless to yellow. After cooling, the ampule was opened and the reaction contents diluted with 50 ml of water and 50 ml of methylene chloride. The organic layer was separated and the aqueous layer extracted with methylene chloride (2 × 50 ml). The combined organic layers were washed sequentially with water (50 ml) and saturated aqueous NaCl (50 ml), dried over anhydrous Na_2SO_4, filtered and stored at −80 °C. The yield determined spectrophotometrically was 85%. The resulting crude mixture of methyl esters of 4*E*,6*E*,8*E*,10*Z*,13*Z*,16*Z*,19*Z*-docosaheptaenoic

acid (∼80%) and 4*E*,6*E*,8*E*,10*E*,13*Z*,16*Z*,19*Z*-docosaheptaenoic acid (∼20%) was purified by HPLC and analyzed further (Fig. 1, VIIc). Similarly, from the δ-lactone of 5-HEPE, a mixture of 5*E*,7*E*,9*E*,11*Z*,14*Z*,17*Z*-eicosahexaenoic (∼80%) and 5*E*,7*E*,9*E*,11*E*,14*Z*,17*Z*-eicosahexaenoic (∼20%) acids (Fig. 1, VIIb) were synthesized with a total yield determined spectrophotometrically of 81%; from the δ-lactone of 5-HETE a mixture of 5*E*,7*E*,9*E*,11*Z*,14*Z*-eicosapentaenoic (∼80%) and 5*E*,7*E*,9*E*,11*E*,14*Z*-eicosapentaenoic (∼20%) acids (Fig. 1, VIIa) were synthesized with a total yield determined spectrophotometrically of 83%. Spectral data for these newly synthesized compounds were the same as those for the conjugated polyunsaturated fatty acids synthesized with the use of excess of DBU (as described above).

2.11. Synthesis of epoxides from iodolactones and epoxide ring opening to corresponding isomeric bromoacetates (Fig. 1, reactions (3) and (4))

The methyl ester of 4,5-epoxy,7*Z*,10*Z*,13*Z*,16*Z*,19*Z*-docosapentaenoic acid was synthesized as follows. To a solution of 9.7 g (21 mmol) of the γ-iodolactone of DHA (Fig. 1, Ic) in 50 ml of methanol was added 16 ml of triethylamine (11.6 g, 5.5 eq.). The reaction mixture refluxed for 3 h and then evaporated under vacuum. The residue was stirred with hexane (3 × 70 ml), filtered, evaporated under vacuum and purified by column chromatography on 30 g of silica gel using a gradient of ether in hexane (0 → 10%). The yield was 5.40 g (71%) of Vc (Fig. 1) as a colorless, mobile oil with an R_f ∼ 0.58 (hexane–ether, 1:1). MS: *m*/*z* (I%): $[M]^+$ 358 (3), $[M–MeO]^+$ 327 (8). ^{1}H NMR ($CDCl_3$, *δ*, ppm): 0.99t (H-22; 3H), 1.86m—(H-3; 2H), 2.40m (H-6; 2H), 2,50m (H-2; 2H), 2.84m (H-9,12,15,18; 8H), 2.98m—(H-4,5; 2H), 3.69s—($COOCH_3$, 3H), H-7,8,10,11,13,14,16,17,19,20—5.37m). Spectral data for this compound were in full agreement with those reported previously (Kuklev et al., 1991, 1992).

The mixture of isomeric bromoacetates (VI) was synthesized as follows. To a solution of 305 mg of the methyl ester of 4,5-epoxy,7*Z*,10*Z*,13*Z*,16*Z*,19*Z*-docosapentaenoic acid in 10 ml of dry ether was added a solution of 120 mg (1.15 eq.) of acetylbromide in 10 ml of dry ether. The reaction mixture was kept at room temperature for one hour and then washed with water (3 × 20 ml) and saturated NaCl (25 ml) and dried over Na_2SO_4. The dry extract was filtered, evaporated under vacuum and purified by column chromatography on 10 g of silica gel using a gradient of ether in hexane (0 → 30%). The yield was 355 mg (87%) as a colorless oil with R_f ∼ 0.65–0.8 (hexane–ether, 1:1). The properties of the compounds in good agreement with those reported earlier (Kuklev et al., 1996, 1997).

2.12. Reaction of bromoacetates with DBU to form tetraenoic conjugated fatty acids (Fig. 1, reaction (9))

To a solution of isomeric bromoacetates (Fig. 1, V) (200 mg) in 5 ml of dry benzene was added 200 μl (ca. 1.5 eq.) of dry DBU. The reaction mixture was kept at room temperature for 96 h. The yield of conjugated tetraene determined spectrophotometrically was 55%. The final reaction mixture was handled as for the synthesis of conjugated fatty acids from iodolactones (as described above) and stored in methanol at −60 °C.

2.13. Mass spectrometry

4-Methyl-1,2,4-triazoline-3,5-dione (MTAD) adducts of conjugated fatty acids were prepared as described by Dobson (Dobson, 1998). An aliquot of the reaction mixture was analyzed by GC-MS immediately after derivatization. Oxazolines of conjugated fatty acids were prepared and analyzed by GC-MS as reported recently (Kuklev and Smith, 2003).

3. Results and discussion

3.1. Overview

The overall goal of the work described in this report was to prepare derviatives of n-3 and n-6 highly unsaturated fatty acids (HUFAs) that could be employed as photoactive probes for defining further the biological functions of essential fatty acids. Using naturally occurring HUFAs as starting materials (Kuklev and Bezuglov, 1998), we were able to develop photosensitive conjugated tetraene systems neighboring the carboxyl groups while leaving the ω-termini of the acids intact. This involved sequential iodolactonization of the carboxyl group of the starting HUFAs and then treatment with at 2–2.5 molar excess of 1,8-diazobicyclo[5.4.0]undec-7-ene (DBU) (Fig. 1). The

iodolactonization protocol is mild and provides for regioselective modification of the double bond closest to the carboxyl group of HUFAs in high yield while leaving other double bonds unaltered (Kuklev and Bezuglov, 1994; Solodovnik, 1967). Treatment of the iodolactone with an equimolar amount of DBU yielded the corresponding lactone of an allylic alcohol that, in turn, could be saponified to form the corresponding hydroxyacids—widely distributed products of the oxidative metabolism of HUFAs (Corey et al., 1980; Wright et al., 1987).

Importantly, we discovered that using a 2–2.5-fold molar excess of DBU to HUFA iodolactones led to the formation of fatty acids having four conjugated double bonds instead of the expected triene conjugation. The success of this procedure required the use of dry benzene (distilled over P_2O_5) as the solvent and dry reagent—DBU (distilled over CaH_2 at reduced pressure); using benzene or DBU that had not been subjected to these treatments led only to the formation of various by-products (data not shown). We also found that the formation of the conjugated tetraene cassette does not depend on the size of the iodolactone ring. This was seen in comparing of the reactions of the γ-iodolactone of DHA having a ring containing five atoms and the δ-iodolactones of EPA and AA that have lactone rings with six atoms (Fig. 1). In all the cases the reactions involved an initial formation of the lactone of an allylic alcohol followed by opening of the lactone ring.

Interestingly, when alcoholysis of the lactone ring of allylic lactones was performed under acidic conditions (i.e. H^+/methanol) methyl esters of the corresponding tetraene conjugated acids are the main products. The double bond positions and structures of the methyl esters correspond to the methyl esters synthesized through the action of DBU on the iodolactone and then esterified with diazomethane; again the size of the lactone ring was unimportant.

An additional confirmation of the transformation of the natural system of methylene interrupted double bonds was obtained while investigating the reaction of isomeric mixtures of vicinal bromoacetates with DBU (Fig. 1). The bromoacetates were synthesized from the corresponding epoxides that were obtained from iodolactones as described in Section 2. The reaction between these bromoacetates and DBU leads to formation of methyl esters with the same conjugated tetraene group as those present in methyl esters obtained by acidic alcoholysis of allylic lactones and those obtained directly from iodolactones. We also stress that treatment of trimethylsilyl ethers of allylic alcohols (5-HETE, 5-HEPE, 4-HDHE) with aqueous hydrochloric or trifluoroacetic acid leads to formation of the same conjugated tetraene group as those obtained directly from natural PUFAs as starting materials. Thus, an intermediate lactone is not required for the formation of conjugated tetraenes.

Analysis of the reaction mixtures by HPLC demonstrated: (a) the absence of any remarkable amounts of conjugated triene-containing species (i.e. materials with absorption maxima at 270–280 nm), (b) the identity of the products obtained from a given fatty acid using different synthetic techniques as determined by spectral and chromatographic properties, and (c) systems consisting only of carbon–carbon double bonds possessing well-defined UV spectral features and insensitivity to reduction by $LiAlH_4$.

Purification of the conjugated polyunsaturated fatty acids did present problems because these acids readily undergo isomerization, polymerization and oxidation (Frankel, 1979; Johnson, 1979; Johnson and Pryde, 1979; Lopez and Gerwick, 1987). It is essential to use the procedures that are detailed in Section 2 paying particular attention to the use of dry solvents, mild temperatures and minimizing exposure to metals. Using the appropriate techniques it is possible to transfer these fatty acids not only to methanol but to methylene chloride or chloroform (or $CDCl_3$) (e.g. for analysis by ^1H NMR).

Because the highest yields were observed in reactions of iodolactones with an excess of DBU, we discuss further here formation of the conjugated tetraenoic fatty acids involving these reaction conditions. Because the procedures and outcomes were very similar to one another for AA, EPA and DHA, we describe in the following sections only the compounds derived from EPA. Experimental data on compounds derived from AA and DHA are detailed in Section 2.

3.2. Preparation of iodolactone of EPA

A mixture of the γ-iodolactone of DHA (Fig. 1, Ic) and the δ-iodolactone of EPA (Fig. 1, Ib) was prepared in about 75% yield by treating a mixture of the potassium salts of these two fatty acids with I_2. The

reaction was optimized to eliminate other I_2 addition products. The individual iodolactones were isolated by column chromatography on silica gel which removed any unreacted fatty acids (i.e. fatty acids lacking a Δ3, Δ4 or Δ5 double bond). The spectral properties of compounds Ia–c (Fig. 1) were in excellent agreement with those reported earlier (Corey et al., 1983; Kuklev et al., 1991; Wright et al., 1987).

3.3. Reaction of the iodolactone of EPA with DBU to form polyconjugated fatty acids

Two major peaks were always present upon RP-HPLC of reaction mixtures involving iodolactones and excess DBU. Thus, HPLC analysis of the product mixture from treatment of the δ-iodolactone of EPA with 2.2 eq. of DBU for 72 h provided a Compound 1 with $k'_1 = 5.75$ (10.87 min) and a Compound 2 with $k'_2 = 6.16$ (11.53 min) (Fig. 2). The difference in the retention times between the compounds was sufficient to permit them to be separated on a preparative scale in high purity (>95%).

UV spectra of the compounds separated by HPLC showed characteristic UV absorption characteristics of a *trans*, *trans*, *trans*, *cis* tetraene functionality (λ = 290.1, 303.2, 317.5 nm) for Compound 1 that was replaced in the absorption spectrum of Compound 2 with a pattern characteristic of an all *trans* tetraene (λ = 289, 300.8, 315 nm) (Hamberg, 1995) (Fig. 3). The main difference between the spectra is in the hypochromic shift of the peak maxima for Compound 2 by about 3 nm. Preliminary observations of the spectroscopic properties of the two molecules, their mobilities on HPLC and the spontaneous conversion of Compound 1 to Compound 2 at room temperature (as monitored by HPLC) suggested that these two compounds were geometrical isomers.

The fluorescence emission spectrum of Compound 1 derived from EPA was determined in methanol at 23 °C. The emission origin is at about 350 nm and there is a broad maximum at about 422 nm for the all *trans* isomer and at about 428 for the *trans*, *trans*, *trans*, *cis* isomer. The emission spectrum is essentially independent of the solvent (data not shown). The flu-

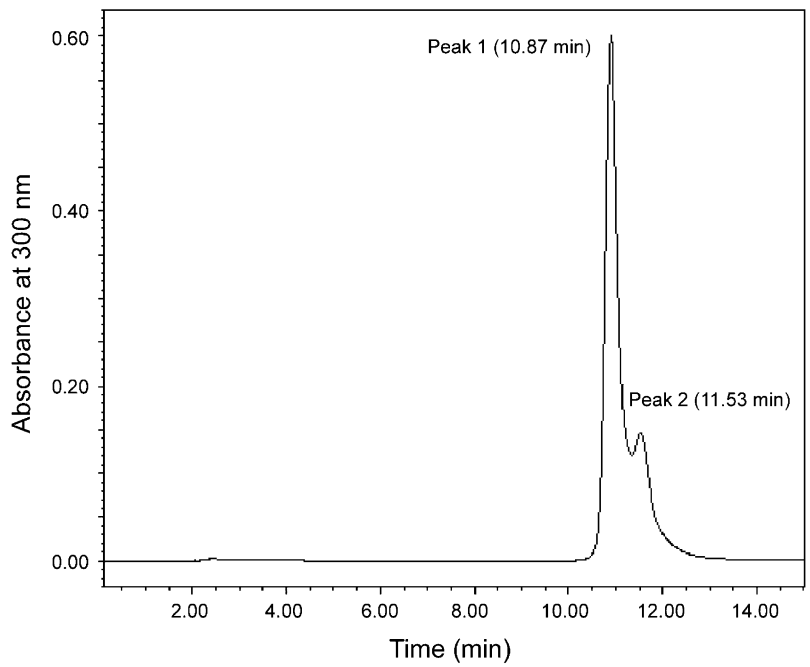

Fig. 2. HPLC analysis of the reaction mixture (following solid phase extraction to remove DBU) of the conjugated fatty acids derived from eicosapentaenoic acid. Column: Nucleosil-C18 (4.6 × 250, 5 μm); mobile phase: methanol–water–acetic acid (85:15:0.1); flow rate: 1.5 ml/min; detection: 300 nm.

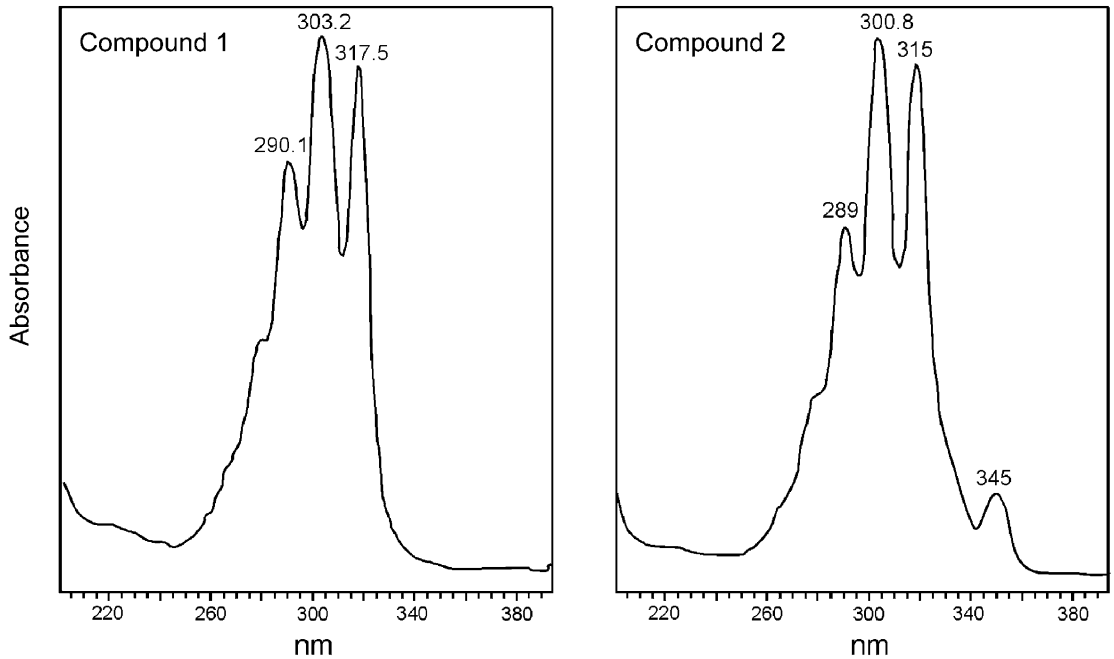

Fig. 3. UV spectra (in methanol) of Compounds 1 and 2 derived from eicosapentaenoic acid.

orescence quantum yield was determined as described earlier (Sklar et al., 1977; Solodovnik, 1967) and was similar (i.e. $Q^a = 0.015$) to that of *cis* parinaric acid (Sklar et al., 1977). The fluorescence data on poly-conjugated fatty acids derived from DHA and AA are provided in Section 2.

Compounds 1 and 2 derived from EPA were analyzed in as oxazolines (Kuklev and Smith, 2003). In the mass spectrum of the oxazoline of Compound 2 (the mass spectrum of Compound 1 was almost the same) a prominent molecular ion is present at *m*/*z* 325 (18%), and this is accompanied by a peak at *m*/*z* 324 (14%). This suggests that the molecular mass of Compound 2 is two a.m.u. less than that of the starting EPA and correlates with the structure of Compound 2 being an eicosahexaenoic acid. The spectrum can be separated into two parts (Kuklev and Smith, 2003). The first part, located in the higher mass region, contained prominent ions at *m*/*z* 216 (17%), *m*/*z* 256 (20%), *m*/*z* 296 (8%) and *m*/*z* 310 (45%) that represent two methylene interrupted double bonds in the ω3 position (i.e. fragments $[M–CH_3]^+$ at *m*/*z* 310, and $[M–CH_2CH_3]^+$ at *m*/*z* 296). The second part of the spectrum, in the lower mass region with the *m*/*z* below 216, represented a very complicated fragmentation of the molecule at sites of conjugation. Nonetheless, prominent peaks were clearly seen for ions at *m*/*z* 85 (100%), 98 (65%), 138 (94%) (Δ5 double bond). Therefore, the normal fragmentation of oxazolines peculiar to fatty acids having methylene interrupted double bond systems terminates at C-12, but starts, as anticipated, for non-conjugated fatty acids with C-5. This indicates that the position of the conjugated system of double bonds is between C-5 and C-12 in Compound 2.

To confirm the position of the conjugated system of double bonds the 4-methyl-1,2,4-triazoline-3,5-dione (MTAD) adduct of Compound 2 derived from EPA was analysed by GC-MS (Dobson, 1998) (Fig. 4). MTAD reacted with conjugated tetraene to form at least three major 1:1 adducts together with some amounts of products having the same spectra but different mobilities on gas chromatography—a problem of the method originally described by Dobson (Dobson, 1998). That is, the reaction between Compound 2 and MTAD proceeded as if Compound 2 had three independent diene systems. We detected all three regioisomers of the 1:1 adducts between MTAD

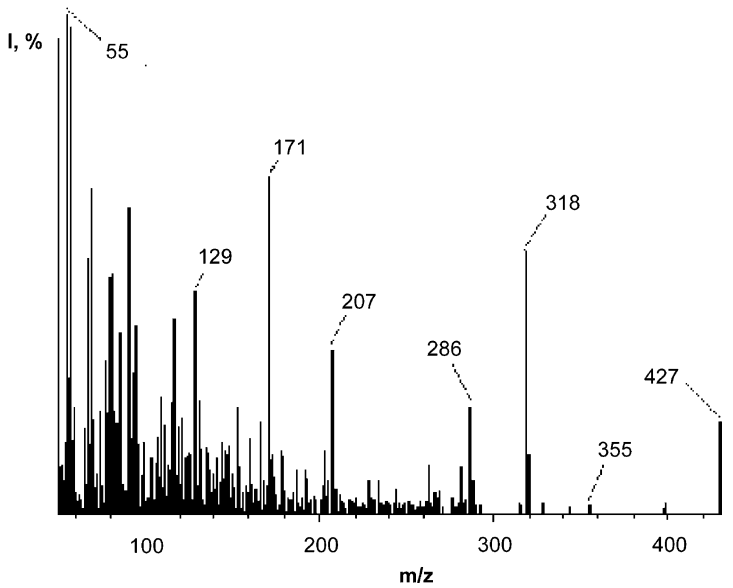

Fig. 4. Mass spectrum of C9–C12 isomer of the MTAD adduct of Compound 2 derived from eicosapentaenoic acid. Molecular *m/z* 427 and diagnostic *m/z* 318 ions are clearly visible.

and Compound 2 and all of them had a prominent molecular ion $[M]^+$: *m/z* 427, ≈15%; and diagnostic ions of $[M–C_8H_{13}]^+$: *m/z* 318, 22%—identifying the location of the ring adduct between C-9 and C-12 (and therefore a 9,11-diene system in Compound 2) (see Fig. 4); $[M–C_{10}H_{15}]^+$: *m/z* 292, 17% as expected for an adduct ring located between C-7 and C-10 (and therefore a 7,9-diene system in Compound 2; $[M–C_{12}H_{17}]^+$: *m/z* 266, 20% and $[M–C_5H_9O_2]^+$: *m/z* 326, 8%—identifying an adduct ring between C-5 and C-8 (and therefore a 5,7-diene system in Compound 2). Hence, in Compound 2 the conjugated tetraene group is located between C-5 and C-12, and thus, the double bonds are at positions C-5, C-7, C-9 and C-11. The data from mass spectroscopy of oxazolines and MTAD adducts of the polyconjugated fatty acids synthesized from DHA and AA are presented in Section 2.

Complete data for the ^{1}H NMR spectra shown in Fig. 5 are: (a) for Compound 1 synthesized from EPA: δ 0.96 (3H, t, $J = 7.5$ Hz, H-20), 1.73 (2H, m, H-3), 2.06 (2H, m, H-19), 2.14 (2H, m, H-4), 2.35 (2H, t, 7.4, H-2), 2.80 (2H, m, H-16), 2.96 (2H, m, H-13), 5.37 (5H, m, H-12,14,15,17,18), 5.65 (1H, dt, $J_{5,6} = 14.4$ Hz, $J_{54} = 6.9$ Hz, H-5), 6.08 (1H, m, H-6), 6.18 (4H, m, H-7,8,9,11), 6.47 (1H, dd, $J_{10,9} = 13.7$, $J_{10,11} = 11.1$, H-10); and (b) for Compound 2 obtained from EPA: δ 0.95 (3H, t, $J = 7.6$ Hz, H-20), 1.73 (2H, m, H-3), 2.05 (2H, m, H_2-19), 2.16 (2H, m, H-4), 2.35 (2H, t, 7.2, H-2), 2.76 (2H, m, H-16), 2.86 (2H, m, H-13), 5.39 (4H, m, H-14,15,17,18), 5.68 (2H, m, $J_{5,6} \approx J_{11,12} = 14.5$, $J_{5,4} \approx J_{12,13} = 7.1$, H-5,12), 6.13 (6H, m, H-6,7,8,9,10,11).

It is clear from these ^{1}H NMR data (Fig. 5) that there are 12 olefinic methines, 6 aliphatic methylenes, 1 methyl group and 1 carbonyl carbon in both compounds. Two of the methylenes were bis allylic groups (as indicated by their chemical shifts, one at δ 2.80 and another at δ 2.96). Selective decoupling experiments demonstrated coupling from the C-2-methylene at δ 2.35 through the aliphatic protons at δ 1.73 (C-3) to the δ 2.14 (C-4) and further to the olefinic proton at approximately δ 5.6 at C-5. Similarly, coupling from the terminal methyl at δ 0.96 through the methylene protons at δ 2.06 (C-19) to the cluster of olefinic protons at δ 5.3–5.4 established the structure at the ω terminus of the molecule. The relative positions in the conjugated tetraene functionality of the eight low-field proton signals in Compound 1 at δ 5.65 (1H), δ 6.08 (2H), δ 6.18 (4H), and δ 6.47 (1H) were assigned on the basis of selective decoupling experiments and supported by comparison with ^{1}H NMR spectra of Com-

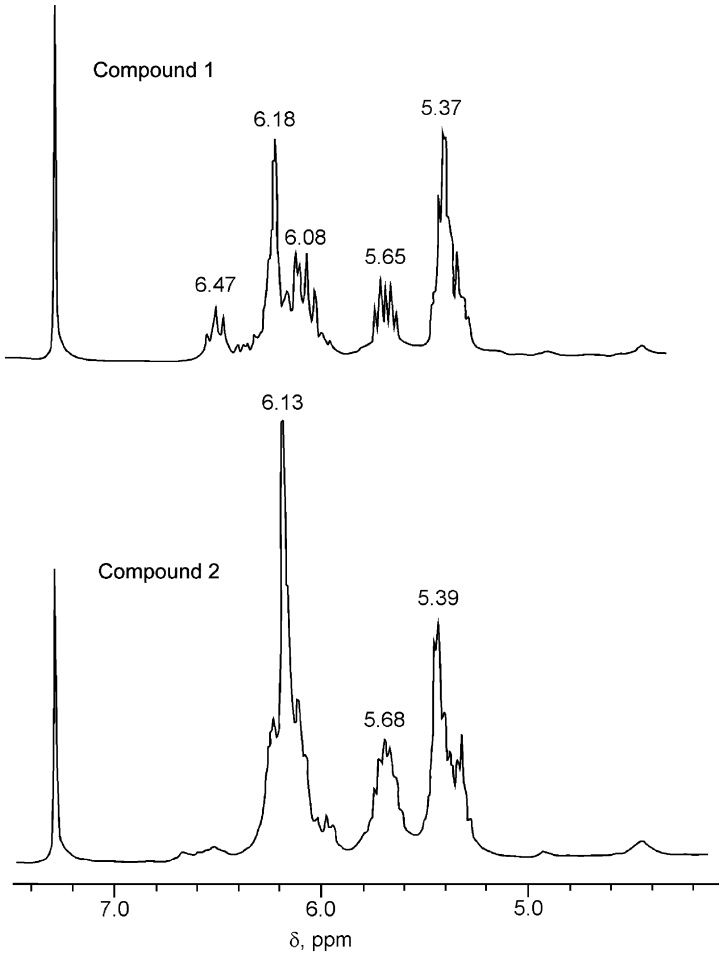

Fig. 5. Low field fragments of ^{1}H NMR (δ, ppm, $CDCl_3$, 300 MHz) spectra of Compounds 1 and 2 derived from eicosapentaenoic acid.

pound 2 where eight low-field proton signals formed two clusters at δ 5.7 (2H, H-5,H-12) and at δ 6.13 (6H, H-6 to H-11). Additional support for the assignments was obtained by comparison of our chemical shifts and coupling constants with those from experiments with naturally occurring conjugated fatty acids (Lopez and Gerwick, 1987; Michailova et al., 1995; Wise et al., 1994).

The configuration of the conjugated double bonds in Compound 1 can be seen from the data presented in Fig. 5. Thus, the doublet of doublets at δ 6.47 (H-10) with coupling constants of 13.7 and 11.1 Hz is a very common signal for a methyne proton at the third carbon in from the end of a conjugated system of double bonds if the terminal double bond (C-11 to C-12) has a *cis*-configuration and the second double bond has a *trans* configuration (C-9 to C-10) (Michailova et al., 1995; Wise et al., 1994). One more diagnostic signal is the nicely resolved doublet of triplets at δ 5.65 that is well known for a terminal methyne proton at a *trans* double bond in a conjugated system (Wise et al., 1994). Isomerization of Compound 1 to Compound 2 results in the disappearance of the well shaped signals at δ 5.65 and δ 6.47 and formation of only two signal clusters at 6.1 (6H) and 5.7 (2H). The coupling constants for the external methyne protons were almost the same with values at 14.5 Hz (*trans* double bond) and 7.1 Hz (aliphatic methylene). These data are in excellent agreement with the structures of a 5*E*,7*E*,9*E*,11*Z*-tetraene system for Compound 1 and a

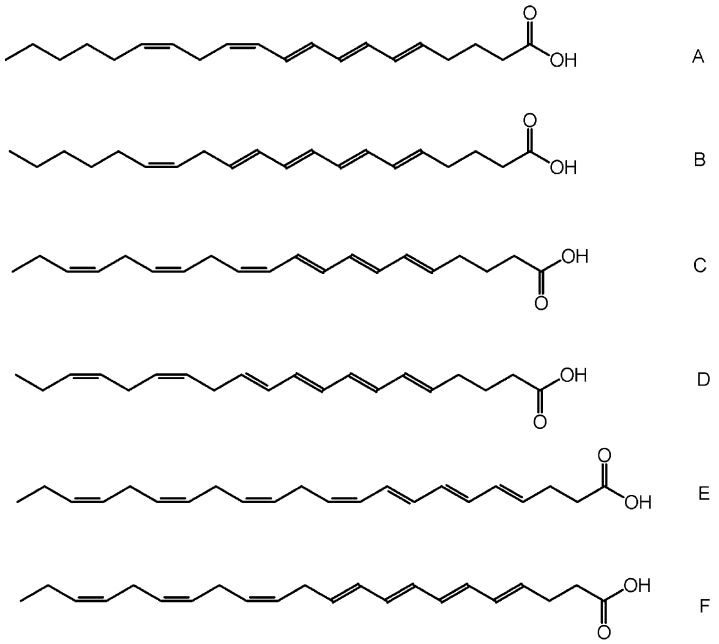

Fig. 6. Structures of newly synthesized polyconjugated and polyunsaturated fatty acids (A) 5*E*,7*E*,9*E*,11*Z*,14*Z*-eicosapentaenoic acid; (B) 5*E*,7*E*,9*E*,11*E*,14*Z*-eicosapentaenoic acid; (C) 5*E*,7*E*,9*E*,11*Z*,14*Z*,17*Z*-eicosahexaenoic acid; (D) 5*E*,7*E*,9*E*,11*E*,14*Z*,17*Z*-eicosahexaenoic acid; (E) 4*E*,6*E*,8*E*,10*Z*,13*Z*,16*Z*,19*Z*-docosaheptaenoic acid; (F) 4*E*,6*E*,8*E*,10*E*,13*Z*,16*Z*,19*Z*-docosaheptaenoic acid.

5*E*,7*E*,9*E*,11*E*-conjugated system in Compound 2 derived from EPA.

Based on results from HPLC, UV spectroscopy, fluorescence spectroscopy, ^{1}H NMR and GC-MS of derivatives we assigned structures for the compounds derived from AA: Compound 1 as 5*E*,7*E*,9*E*,11*Z*,14*Z*-eicosapentaenoic acid (Fig. 6, structure A) and Compound 2 as 5*E*,7*E*,9*E*,11*E*,14*Z*-eicosapentaenoic acid (Fig. 6, structure B); for compounds derived from EPA: Compound 1: 5*E*,7*E*,9*E*,11*Z*,14*Z*,17*Z*-eicosahexaenoic acid (Fig. 6, structure C) and Compound 2: 5*E*,7*E*,9*E*,11*E*,14*Z*,17*Z*-eicosahexaenoic acid. (Fig. 6, structure D); and for compounds derived from DHA: Compound 1: 4*E*,6*E*,8*E*,10*Z*,13*Z*,16*Z*,19*Z*-docosaheptaenoic acid (Fig. 6, structure E) and Compound 2: 4*E*,6*E*,8*E*,10*E*,13*Z*,16*Z*,19*Z*-docosaheptaenoic acid (Fig. 6, structure F).

3.4. Formation of tetraene versus triene group

The results of our synthetic studies suggest that it is not possible to introduce a triene structure neighboring a methylene interrupted system of double bonds; instead the apparently more stable conjugated tetraene system forms spontaneously. This occurs independent of the position of the most carboxyl proximal double bond or the length of the methylene interrupted double bond system. Formation of the tetraene is also independent of the nature of the reagents (e.g. acidic versus basic). The location of the conjugated double bond system remains the same as the position of the first double bond in the parent fatty acid (e.g. the 5,8,11 methylene interrupted system of AA (C-5 to C-12) converts to the conjugated 5,7,9,11 system (C-5 to C-12). In surveying the literature, we were unable to find any naturally occurring fatty acids with a conjugated triene system linked to a system of methylene interrupted double bonds. This along with the results of our synthetic studies led us to conclude that this type of structure is unstable and would not exist in nature. We synthesized and characterized the conjugated tetraene system, which in contrast to the conjugated triene system, is quite stable in the form of *E*,*E*,*E*,*Z*-system before undergoing a slow spontaneous isomerization to a *E*,*E*,*E*,*E*-system. This rearrangement has not been described previously. All of the fatty acids

that we have synthesized have spectral properties and chemical properties that may prove useful in investigating the biochemistry of essential fatty acids.

Acknowledgements

This work was supported in part by Grant No. DK22042 from the National Institutes of Health.

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