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Synthesis of four isomers of parinaric acid

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Abstract

A simple and reliable method for synthesizing four isomers of parinaric acid from α-linolenic acid (ALA) in high yields is described. The methylene-interrupted, *cis* triene system (1,4,7-octatriene) of ALA and common to other naturally occurring polyunsaturated fatty acids was transformed to a conjugated tetraene system (1,3,5,7-octatetraene). The synthesis involves bromination of ALA using 0.l M Br_2 in a saturated solution of NaBr in methanol, esterification of the fatty acid dibromides, double dehydrobromination by 1,8-diazabicyclo[5.4.0]undec-7-ene and saponification of the conjugated esters to a mixture of free conjugated acids. Addition of one molecule of bromine to the 12,13-double bond of ALA and subsequent dehydrobromination produces α-parinaric acid (9Z,11E,13E,15Z-octadecatetraenoic acid); addition of Br_2 to the 9,10-double bond or 15,16-double bond and then dehydrobromination and rearrangement yields 9E,11E,13E,15Z-octadecatetraenoic or 9E,11E,13E,15Z-octadecatetraenoic acids, respectively. The mixture of parinaric acid isomers is obtained in 65% yield, and the isomers can be purified by preparative HPLC; alternatively, the isomers can be converted by base catalyzed *cis–trans* isomerization (or by treatment with I_2) to exclusively β-parinaric acid (9E,11E,13E,15E-octadecatetraenoic acid). The various parinaric acid isomers were characterized by ^{1}H NMR, ^{13}C NMR, UV, GLC, HPLC and mass spectrometry.

Keywords: Parinaric acid; Fluorescent fatty acids; Conjugated fatty acids; α-Linolenic acid

1. Introduction

Parinaric acids are conjugated 9,11,13,15-octadecatetraenoic acids first isolated in 1933 (Tsujimoto and Koyanagi, 1933). The naturally occurring form, designated α-parinaric acid (**2a** (Fig. 1)), can be transformed to a higher melting form called β-parinaric acid (**2d**) via two unnamed isomers (**2b**, **2c**) by stepwise *cis–trans* isomerization of terminal double bonds (Hamberg, 1995). In 1975, Sklar, Hudson and Simoni introduced the use of parinaric acid as a fluorescent membrane probe (Sklar et al., 1975) and demonstrated (Sklar et al., 1977a,b,c, 1979) that parinaric acids could be used to detect phase transitions in bilayers as well as interactions between lipids and proteins. Since then parinaric acids have

Abbreviations: ALA, α-linolenic acid; THF, tetrahydrofuran; PUFA, polyunsaturated fatty acid; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; eq., molar equivalent; MS, mass spectrum; GC, gas chromatography; rt, room temperature (22–24 °C)

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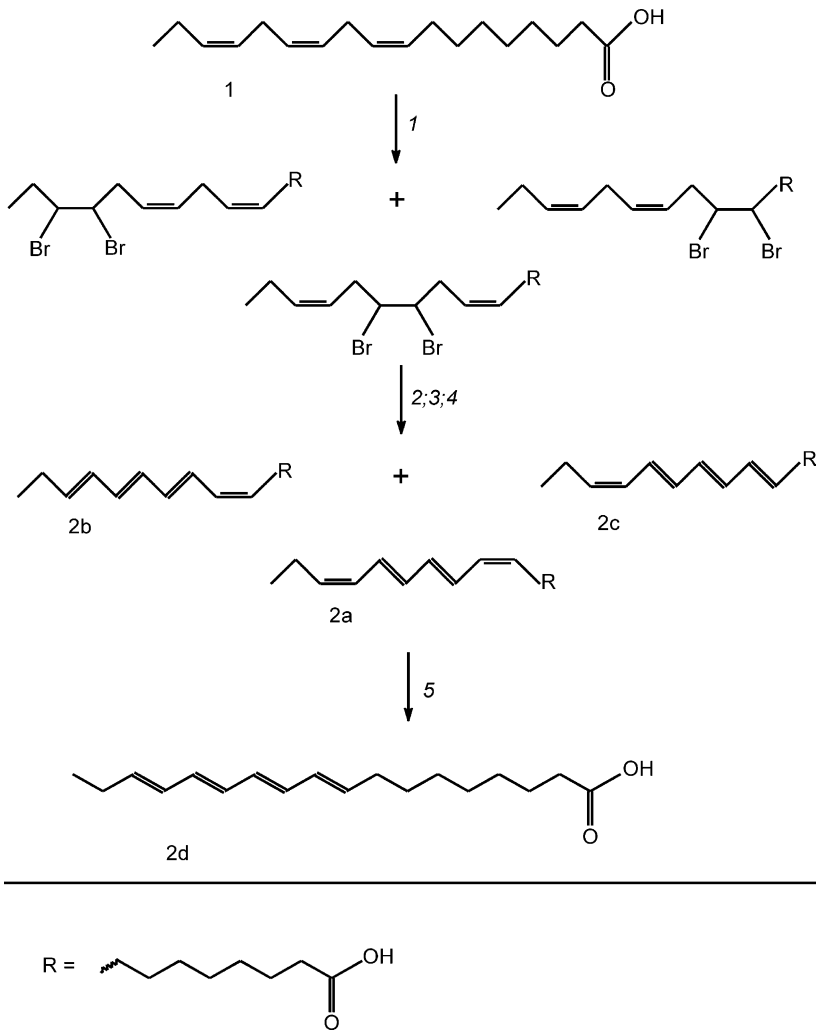

Fig. 1. Scheme for the synthesis of parinaric acids (**2a**–**d**) from α-linolenic acid (**1**). (1) – 0.1 N Br_2 in saturated NaBr in methanol, 1.1 eq., 20 min (2) HCl in methanol, 2 h, rt (3) 1.15 eq. DBU in dry benzene, 16 h (4) 10% excess of KOH in ethanol/water, 16 hr, rt (5) 50% excess of KOH, ethanol–water, boiling under reflux or I_2 in hexane, boiling under reflux 30 min.

become widely used probes for investigating membrane structure including lipid–protein interactions (Narayanaswami and McNamee, 1993), lipid clustering (Brewer and Matinyan, 1992), lipid transport processes including structural characterization of lipoproteins (Ben-Yashar and Barenholz, 1991), fatty acid-binding proteins (Hubbel and Altenbach, 1994) and phospholipid transfer proteins (Kasurinen et al., 1990). In the field of lipid peroxidation, parinaric acids are used for evaluating antioxidants (Suzuki et al., 1993), measuring peroxidation in lipoproteins (Tribble et al., 1994) and investigating relationships between peroxidation and cytotoxity (Crook et al., 1994) and apoptosis (Hockenbery et al., 1993).

Several fairly complicated methods of synthesis of β-parinaric acid have been described (Goerger and Hudson, 1988; Hayashi and Oishi, 1985; Solladie et al., 1997), that are not used widely. We are unaware of any publications on the synthesis of α-parinaric acid (**2a**) or the two other isomers (**2b**) and (**2c**).

Here, we report a simple, reliable and preparative two step technique for synthesizing four isomers

of parinaric acid from α-linolenic acid (ALA). The method is an extension of a process we developed in studying rearrangements of methylene-interrupted *cis* double bonds. The method permits transformation of 1,4,7-octatriene methylene-interrupted *cis* double bonds of naturally occurring polyunsaturated fatty acids (PUFAs) to 1,3,5,7-octatetraenes in high yield.

2. Materials and methods

2.1. Materials

1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU), acetyl chloride and urea were purchased from Sigma (St. Louis, MO). Flax Oil (57% α-linolenic acid) was purchased from Walmart. Trifluoroacetic anhydride, ethanolamine, isobutylchloroformate, pyridine, pyrrolidine and 4-methyl-1,2,4-triazoline-3,5-dione were products of Aldrich (Milwaukee, WI) with a purity of 96%. Benzene, hexane, ether and acetonitrile were distilled over phosphorus pentoxide; 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 mm was purchased from Selecto Scientific (GA, USA). Thin layer chromatography (TLC) plates were purchased from Sigma. A 5% solution of phosphomolybdic acid in methanol spray and heating of the TLC plates for 2–3 min on a hot plate (ca. 100 °C) was used to visualize products.

2.2. Equipment

All mass spectra were recorded on a Hewlett-Packard 5890 gas chromatograph connected to a Hewlett-Packard 5970 series mass selective detector operated with a Hewlett-Packard 7946 computer. Gas chromatography conditions for GC–MS were as follows: He was used as the carrier gas at a flow rate of 35 cm/s, the oven temperature was maintained at 210 °C, the injector temperature was 250 °C and the interface temperature was 250 °C. GC analysis was performed with the use of a capillary column DB-5 ms (30 m × 0.32 mm, 1 μm) (J&W, USA); the injector split ratio was kept constant at 1:60. Mass detector conditions were as follow: electron energy 70 eV, emission current 0.8 mA, accelerating voltage 8 kV, scale from 50 to 1000. GLC of fatty acid methyl esters was performed on a Shimadzu GC-17A3 gas chromatograph, equipped with Restek Stabilwax (30 m × 0.32 mm × 0.25 μm) or with Restek Rtx-5 (15 m × 0.25 mm × 0.25 μm) columns, a flame ionization detector and He as the carrier gas. HPLC analysis and preparative separations were performed on a Shimadzu LC-10 HPLC system equipped with a Shimadzu SPD-M10AVP photodiode array detector. Analytical RP-HPLC was performed on a Nucleosil-C18 analytical column (4.6 mm × 250 mm, 5 mm) (Xpertek, USA). Preparative separations were performed on a Kromasil C18 column (10 mm × 250 mm, 5 μm) (Xpertek, USA). ^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian INOVA-300 and INOVA 500 operated at 300 and 500 MHz, respectively. Samples were dissolved in $CDCl_3$,and tetramethylsilane was used as an internal standard. All the signal assignments were performed based on selective decoupling experiments. All UV–vis spectra were recorded on a Hewlett-Packard 8453 UV–vis spectrophotometer operated with ChemStation data processing software.

2.3. Preparation of Br_2 solution

The bromine solution was prepared by saturating methanol with NaBr (about 65 g per 500 ml), filtering and adding then 10.1 g of liquid bromine with stirring to achieve a 0.1 M solution of the reagent.

2.4. Preparation of flax oil free fatty acids

Flax oil (500 g) was dissolved in a boiling ethanolic solution of potassium hydroxide (120 g of KOH in 1000 ml of ethanol) and boiled under reflux for 30 min. Water (500 ml) was added to the boiling reaction mixture, and it was boiled for an additional 30 min; after that, one more part of cool water (300 ml) was added and the reaction mixture was acidified with 5N HCl to pH 5 and 500 ml of hexane was added to the warm solution. The organic layer was separated and washed sequentially with water (2 × 500 ml) and saturated aqueous NaCl (200 ml), dried over anhydrous Na_2SO_4 and evaporated under vacuum to yield 410 g of flax oil free fatty acids with ALA concentration of 55% as estimated by GC analysis.

2.5. Preparation of an ALA concentrate from flax oil fatty acids

The mixture of free fatty acids from flax oil was subjected to crystallization with urea to produce a mixture of fatty acids enriched in ALA. To a clear hot solution of 640 g of analytical grade urea in 1600 ml methanol, a portion of the 400 g of the fatty acids of flax oil was dissolved with stirring. The mixture was allowed to cool slowly to room temperature for about four hr and then placed overnight at 0 °C. The precipitate of urea complexes was filtered with suction, the filtrate evaporated to dryness, and warm water (200 ml) was added to the residue to dissolve the excess urea. The filtrate acids were isolated by separation with a separatory funnel, and the lower layer was extracted with hexane (200 ml). The combined layers were washed with warm water (40 °C, 3 × 200 ml) and saturated aqueous NaCl (200 ml), and then dried over anhydrous Na_2SO_4 and evaporated under vacuum to yield 191 g of an ALA concentrate as a dark yellow oil (82% ALA, 18% linoleic acid as determined by GC analysis); the recovery of ALA was 68%.

2.6. Bromination of the ALA concentrate

To a solution of 10.0 g of the ALA concentrate in 500 ml of methanol, was added dropwise with rigorous stirring over 30 min a solution of 0.1N of bromine reagent (310 ml, 1.1 eq.). Then 100 ml of a 1% solution of HCl in methanol (prepared immediately before use by dissolving acetyl chloride in methanol) was added to the reaction mixture, which was kept for 2 h at room temperature. The reaction mixture was evaporated under vacuum, and the dry residue was dissolved in 100 ml of water and extracted with ethyl ether (3 × 100 ml), washed with saturated aqueous NaCl (100 ml), dried over anhydrous Na_2SO_4 and evaporated under vacuum. The resulting mixture of dibromides and unreacted ALA (14% as determined by GC) was used without additional purification.

2.7. Dehydrobromination of ALA bromides

DBU (12.2 ml; 1.15 eq.) was added to the solution of the bromides obtained in the previous step in 100 ml of dry benzene with stirring. The reaction mixture was kept under nitrogen with stirring overnight. The yield of the target conjugated tetraenoic fatty acids was 65% as estimated spectrophotometrically using an extinction coefficient for conjugated tetraenes of $\varepsilon_{302} = 70{,}000$ l/mol cm^{-1}. The reaction mixture was filtered, the filtrate evaporated in vacuo, and the dry residue was stirred with 200 ml of hexane; the dissolved materials were separated from a dark oily deposit, and the hexane solution was washed with 2N HCl (2 × 200 ml), water (2 × 200 ml), saturated aqueous NaCl (200 ml) and dried over anhydrous Na_2SO_4. An HPLC chromatogram of the processed reaction mixture is shown in Fig. 2 (upper trace).

2.8. Isolation of α-parinaric acid and two related isomers

The dry extract obtained in the previous step was filtered, evaporated, dissolved in 200 ml of ethanol and combined with 20 ml of a 10% solution of KOH (1.1 eq.) with stirring. After 16 h at room temperature, the reaction mixture was acidified with 2N HCl to pH 5 and extracted with ethyl ether (2 × 200 ml). The combined ether layers were washed with water (500 ml) and saturated aqueous NaCl (200 ml), dried over anhydrous Na_2SO_4 and evaporated under vacuum. The dry oily residue was crystallized at −60 °C three times from 100 ml of hexane. The light crystalline precipitate of the mixture of three parinaric acid isomers was separated by preparative HPLC using a Kromasil® column (250 mm × 10 mm, C18, 5 mm) with methanol:water:acetic acid (85:15:0.3) at a flow rate of 5 ml/min.

α-Parinaric acid (**2a**; Fig. 1); peak #1 (methyl ester) (Fig. 2). GLC (41%, ECL = 19.23, Rtx 5). HPLC: $k' = 6.1$ (methanol:H_2O:acetic acid; 85:15:0.3). UV: l_{max} (methanol) = 291, 304 ($\varepsilon = 70{,}000$ l/mol cm^{-1}) and 319; MS, EI, methyl ester of **2a**, *m*/*z* (I%): 290 (14%, M^+), 261 (4%, M^+ -Et), 259 (4%, M^+ -OMe), 161 (11%), 147 (20%), 133 (31%), 119 (38%), 105 (70%), 91 (100%), 79 (75%), 55 (42%). ^{1}H NMR (*d*, ppm, 300 MHz, $CDCl_3$): 1.03 (t, 3H, $J_{18,17}$ 7.5, H-18), 1.28 (m, 8H, H-4,5,6,7), 1.59 (m, 2H, H-3), 2.23 (dq, 2H, $J_{17,16}$ 7.2, $J_{17,18}$ 7.5, H-17), 2.2 (m, 2H, H-8), 2.28 (t, 2H, $J_{2,3}$ 7.5), 5.41 (m, 2H, $J_{9,8}$ 7, $J_{16,17}$ 7.2, $J_{9,10} = J_{16,15}$ 10.0, H-9,H-16,), 6.01 (dd, 1H, $J_{15,16}$ 10.0, $J_{15,14}$ 11, H-15), 6.17 (m, 4H, H-10,11,12,13), 6.48 (m, 2H, $J_{14,15} = J_{11,10}$ 11, $J_{14,13} = J_{11,12}$ 14.5, H-11,14). ^{13}C NMR (125 MHz, $CDCl_3$): 14.45 (C-18),

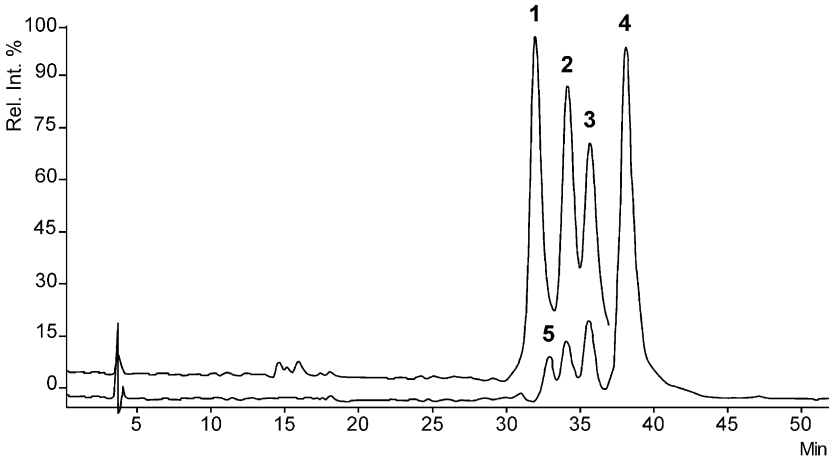

Fig. 2. HPLC analysis of reaction mixtures containing methyl esters of α-parinaric acid and isomers **2b** and **2c** (upper trace) and β-parinaric acid (lower trace). HPLC was performed on a Nucleosil C18 column, 250 mm × 4.6 mm, 5 μm, methanol:H_2O:acetic acid (80:20:0.3), flow rate 1.5 ml/min, detection—photo diode array detector, trace—maximum spectrum plot (200–400 nm). Peak legends, methyl esters of: #1: α-parinaric acid (**2a**), #2: 9Z,11E,13E,15E-octadecatetraenoic acid (**2b**), #3L: 9E,11E,13E,15Z-octadecatetraenoic acid (**2c**), #4: β-parinaric acid (**2d**); #5: full trans isomer of β-parinaric acid with unknown position of conjugated double bonds.

21.42 (C-17), 25.12 (C-3), 28.03 (C-8), 29.29, 29.33, 29.45 (C-5,6,7), 29.56 (C-4), 34.05 (C-2), 128.23, 128.29, 128.34, 128.99, 132.94, 133.04, 133.99, 134.66 (C-8,9,10,11,12,13,14,15,16), 176.30 (C-1).

9Z,11E,13E,15E-Octadecatetraenoic acid (**2b**; Fig. 1), peak #2 (methyl ester) (Fig. 2). GLC (33%, ECL = 19.34 Rtx 5). HPLC: $k' = 6.6$ (methanol:H_2O:acetic acid; AcOH; 85:15:0.3). UV: l_{max} (methanol) = 289, 302 ($\varepsilon = 71{,}000$ l/mol cm^{-1}) and 316; MS, EI, methyl ester of **2b**, *m/z* (I%): 290 (17%, M^+), 261 (5%, M^+ -Et), 259 (5%, M^+ -OMe), 161 (13%), 147 (22%), 133 (33%), 119 (41%), 105 (73%), 91 (100%). ^{1}H NMR (*d*, ppm, 300 MHz, $CDCl_3$): 1.07 (t, 3H, $J_{18,17}$ 7.5, H-18), 1.28 (m, 8H, H-4,5,6,7), 1.59 (m, 2H, H-3), 2.11 (dq, 2H, $J_{17,16}$ 7.2, $J_{17,18}$ 7.5, H-17), 2.2 (m, 2H, H-8), 2.28 (t, 2H, $J_{2,3}$ 7.5), 5.36 (dt, 1H $J_{9,8}$ 7.2, $J_{9,10}$ 10.0, H-9), 5.78 (dt, 1H, $J_{9,8}$ 7, $J_{9,10}$ 10, H-16), 6.04 (dd, 1H, $J_{10,9}$ 10.0, $J_{10,11}$ 11, H-10), 6.2 (m, 4H, H-12,13,14,15), 6.43 (m, 1H, $J_{11,10}$ 11, $J_{11,12}$ 14.4, H-11). ^{13}C NMR (125 MHz, $CDCl_3$): 13.92 (C-18), 25.32 (C-3), 26.28 (C-17), 28.26 (C-8), 29.44, 29.47, 29.49 (C-5,6,7), 29.97 (C-4), 34.48 (C-2), 129.21, 130.00, 131.29, 132.82, 133.20, 133.24, 133.48, 137.38 (C-8,9,10,11,12,13,14,15,16), 174.71 (C-1).

9E,11E,13E,15Z-Octadecatetraenoic acid (**2c**; Fig. 1), peak #3 (methyl ester) (Fig. 2). GLC (27%, ECL = 19.53 Rtx 5). HPLC: $k' = 6.9$ (methanol:H_2O:acetic acid; 85:15:0.3). UV: l_{max} (methanol) = 289, 302 ($\varepsilon = 71{,}000$ l/mol cm^{-1}) and 316; MS, EI, methyl ester of **2c**: the same as for 2b. ^{1}H NMR (*d*, ppm, 300 MHz, $CDCl_3$): 1.04 (t, 3H, $J_{18,17}$ 7.5, H-18), 1.28 (m, 8H, H-4,5,6,7), 1.59 (m, 2H, H-3), 2.1 (m, 2H, H-8), 2.22 (dq, 2H, $J_{17,16}$ 7.2, $J_{17,18}$ 7.5, H-17), 2.28 (t, 2H, $J_{2,3}$ 7.5), 5.44 (dt, 1H $J_{16,17}$ 7.2 $J_{16,15}$ 10.0, H-16), 5.73 (dt, 1H, $J_{9,8}$ 7, $J_{9,10}$ 10, H-9), 6.04 (dd, 1H, $J_{15,16}$ 10.0, $J_{15,14}$ 11, H-15), 6.2 (m, 4H, H-10,11,12,13), 6.42 (m, 1H, $J_{14,15}$ 11, $J_{14,13}$ 14.4, H-14). ^{13}C NMR (125 MHz, $CDCl_3$): 14.65 (C-18), 21.64 (C-17), 25.36 (C-3), 29.31, 29.37, 29.47 (C-5,6,7), 29.96 (C-4), 33.51 (C-8), 34.44 (C-2), 127.94, 128.55, 129.18, 129.22, 130.67, 132.38, 133.24, 134.88 (C-8,9,10,11,12,13,14,15,16), 174.71 (C-1).

2.9. Isolation of crude β-parinaric acid

The dry extract of the reaction product from the dehydrobromination reaction was filtered, evaporated, dissolved in 200 ml of ethanol and saponified using the technique described above for saponification of flax oil. The resulting fatty acids were crystallized from 100 ml hexane three times at −60 °C and β-parinaric acid was by HPLC purified further.

β-Parinaric acid (**2d**; Fig. 1); peak #4 (methyl ester) (Fig. 2). GLC (ECL = 19.80, Rtx 5). HPLC: k' = 7.3 (methanol:H_2O:acetic acid; 85:15:0.3). UV: l_{max} (methanol) = 286, 299 (ε = 73,000 l/mol cm^{-1}) and 313 nm; MS, EI, methyl ester of **2d**, *m/z* (I%): 290 (22%, M^+), 261 (7%, M^+ -Et), 259 (6%, M^+ -OMe), 161 (14%), 147 (23%), 133 (40%), 119 (44%), 105 (70%), 91 (100%). ^{1}H NMR (*d*, ppm, 300 MHz, $CDCl_3$): 1.08 (t, 3H, $J_{18,17}$ 7.5, H-18), 1.29 (m, 8H, H-4,5,6,7), 1.60 (m, 2H, H-3), 2.11 (dq, 2H, $J_{17,16}$ 7.2, $J_{17,18}$ 7.5, H-17), 2.1 (m, 2H, H-8), 2.32 (t, 2H, $J_{2,3}$ 7.5, H-2), 5.75 (m, 2H, H-9, H-16), 6.08 (m, 6H, H-10,11,12,13,14,15). ^{13}C NMR (125 MHz, $CDCl_3$): 13.94 (C-18), 25.05 (C-3), 26.27 (C-17), 29.35, 29.42, 29.47 (C-5,6,7), 29.63 (C-4), 33.21 (C-8), 34.16 (C-2), 130.04, 131.05, 131.26, 131.29, 132.81, 132.90, 135.39, 136.98 (C-8,9,10,11,12,13,14,15,16), 178.96 (C-1).

3. Results and discussion

The synthesis of parinaric acid isomers (**2a–d**) involved brominating naturally occurring ALA with 1.1 eq. of $NaBr_3$ and subsequent double dehydrobromination with DBU. The resulting reaction mixture was comprised of three major components—α-parinaric acid **2a**: a well-known fluorescent reagent whose synthesis has never been reported previously, and two rarely described isomers **2b** and **2c**. These latter compounds originated from a "double E2 elimination with shift" rearrangement that we discovered and described recently (Kuklev and Smith, 2004).

The specificity of molecular bromine addition to double bonds of PUFAs depends on several parameters and has not been investigated in detail. We established that the bromination of a PUFA by 0.1 M bromine in methanol saturated with sodium bromide at room temperature proceeds smoothly with up to 45% regioselectivity at the 12,13-double bond of ALA. Under these conditions, addition of 1.1 eq. of bromine leads to conversion of 83–88% of the parent ALA to bromides with 12–17% of the starting ALA left intact (according to GC analysis). Both PUFAs and their esters can be brominated in high yields. Brominating PUFAs with molecular bromine in organic solvents (ether, methylene chloride, chloroform, hexane or acetic acid) proceeds with the formation of by-products together with the target dibromides, but the dibromides are always the main products of the reaction (data not shown).

Dehydrobromination of the dibromides occurs in high yields using DBU in dry benzene. However, it is essential before dehydrobromination to protect the fatty acid bromides by converting them to esters. Dehydrobromination of free fatty acid dibromides with DBU leads to a complex mixture of by-products (80%), possibly due to various polymerization or condensation reactions arising from the DBU salt of the fatty acid dibromides. Esterified bromides undergo dehydrobromination within 16 h with yields of more than 80%; the yields of tetraenoic compounds were 62–65% for the three stage synthesis (bromination, esterification, and dehydrobromination). The reaction mixture contains the parinaric fatty acid isomers **2a–c** in a ratio of about 0.5:0.3:0.2 as determined by GC. Only traces of β-parinaric acid (**2d**) were detected.

We found that the compounds **2a–c** are not stable to alkaline treatment at elevated temperatures and were isomerized to the all *trans* β-parinaric acid in high yield. Thus, saponification of the methyl esters of compounds **2a–c** with a 10% molar excess of KOH at room temperature for 16 h leads to decreasing amounts of the α-parinaric acids (from 45 to 35–40%) and a corresponding increase in β-parinaric acid. Use of a 50% excess of KOH and boiling the reaction mixture under reflux for 2 h leads to practically complete transformation of isomers **2a–c** to β-parinaric acid; there are also minor components with an absorption maximum at 299 nm, but these have chromatographic mobilities different than that of β-parinaric acid (peak #5, Fig. 2). We rationalize this as occurring by migration of the system of four conjugated *trans*-only-double bonds along the carbon chain with the formation of regioisomers of β-parinaric acid. We did not observe migration of the conjugated system of double bonds along the chain if there is at least one *cis* double bond; a *cis* double bond in the system of conjugated double bonds seems to serve as an anchor preventing movement along the carbon chain. We do not have an explanation for why having a *cis* double bond in a tetraene system prevents formation of regioisomers with parinaric acid or other conjugated fatty acids (20).

All spectral properties (^{1}H NMR, mass spectra, UV) of compounds **2a–d** are in excellent agreement with those reported for the naturally occurring

compounds (Hamberg, 1993). From ^{1}H NMR and ^{13}C NMR experiments it was clear that all the isomers **2a**–**d** (Fig. 1) contain eight aliphatic methylenes, eight olefinic methines, one methyl group and one carbonyl carbon. All NMR spectra of isomers **2a**–**d** were similar but obviously not identical. Thus, all isomers had a system of four conjugated double bonds starting at the *w*3 position but the structures are different. A ^{1}H–^{1}H chemical shift correlation spectroscopy (COSY) experiment with **2a** demonstrated coupling from the C18-methyl at *d*1.03 through the aliphatic protons at C17 (*d*2.23) to the C16 olefinic proton at *d*5.In contrast, in the spectrum of **2d** there was clearly coupling of C18-methyl protons at *d*1.08 through the aliphatic protons at C17 (*d*2.11) to the C16 olefinic proton at *d*5. Similarly, a COSY experiment with isomer **2b** showed coupling of the C18-methyl protons at *d*1.07 through aliphatic protons at C17 (*d*2.11) to the C16 olefinic proton at *d*5.These shifts are very close to those for isomer **2d**, that has a full *trans* structure, and this means that isomer **2b** has a *trans* double bond at C15. In the case of isomer **2c**, a COSY experiment revealed coupling of the C18-methyl protons at *d*1.04 through the aliphatic protons at C17(*d*2.22) to the C16 olefinic proton at *d*5.These shifts are very close to those for isomer **2a**, that has a *cis* double bond at C15. On the basis of COSY experiments and experiments with selective proton-proton decoupling, the structures of isomers **2b** and **2c** were assigned as 9Z,11E,13E,15E- and 9E,11E,13E,15Z-octadecatetraenoic acids, respectively.

An additional confirmation of the structure assignments was made from ^{13}C NMR spectroscopy. Thus, the signal for C17 of isomer **2b** is located at *d*26.28 which is very close to that of isomer **2d**: *d*26.27 (a diagnostic shift for a carbon adjacent to a *trans*-double bond taking into account the effect of a terminal methyl group); in contrast the signal for C17 of isomer **2c** is located at *d*21.64, and this is very close to that of isomer **2a**: *d*21.42 (a diagnostic shift for a carbon adjacent to a *cis*-double bond).

In summary, the parinaric acid isomers **2a**–**d** can be synthesized using our new method with common laboratory equipment in hundred milligram yields with high (>96%) purity. The approach developed for the synthesis of conjugated PUFAs seems to be generally applicable for the synthesis of sets of conjugated fatty acids. Accordingly, additional experiments are in progress to synthesize ω-6 isomers of parinaric acid from γ-linolenic acid.

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