

SYNTHESIS OF FATTY-ACID ETHANOLAMIDES FROM *Linum catharticum* OILS AND *Cololabis saira* FATS

A. E. Karaulov,¹ V. G. Rybin,¹
D. V. Kuklev,² and V. N. Akulin¹

UDC 543.632.514.6

Ethanolamides of polyunsaturated fatty acids (9Z,12Z,15Z-octadecatrienoic; 5Z,8Z,11Z,14Z,17Z-eicosapentaenoic; and 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoic) and mixtures of ethanolamides of fatty acids from natural fats (oils) were synthesized. It was found that the polyunsaturated fatty acids did not isomerize under the proposed conditions for aminolysis of the ester bond. Conditions for analyzing the complicated mixtures of ethanolamides of fatty acids by HPLC-MS were determined.

Key words: fatty-acid ethanolamides, polyunsaturated fatty acids.

The biochemistry of ethanolamides of omega-3 polyunsaturated fatty acids (PUFA) is under intense study because of the presence in them of two different active factors: the omega-3 polyunsaturated hydrocarbon radical and the ethanolamide fragment [1].

Fatty-acid (FA) ethanolamides first attracted attention as bioregulators in 1957 when it was found that N-palmitoylethanolamine is an anti-inflammatory factor typical of lipids from soy beans, peanut oil, and egg yolk [2]. The interest in FA amides of primary amines is due to the discovery of the distinct modulating activity of natural N-arachidonylethanolamine (anandamide) [3] and oleoylamine (oleamide) [4]. As a result two new FA ethanolamides were isolated from porcine brain. These were di-homo- γ -linolenylethanolamine and docosatetraenylethanolamine [5, 6]. It was shown that two more PUFA ethanolamides, 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoic [7] and 5Z,8Z,11Z-eicosatrienoic [8], have distinct biological activity.

The most common method for preparing amides of carboxylic acids is the reaction of acyl chlorides or mixed anhydrides of carboxylic acids (for example, the anhydride with isobutylchloroformate) with the corresponding free amine [9]. However, the product polymerizes if isobutylchloroformate is used because of the double bonds separated by methylenes in the PUFA structures. This makes it practically impossible to use this method. The desired product formed in high (98%) yield if the commercially available chloride of 5Z,8Z,11Z,14Z-eicosatetraenoic (arachidonic) acid was used [10].

Our goal was to find a simple method for synthesizing FA ethanolamides using natural sources of FA as substrates. We used aminolysis by free monoethanolamine of FA ethyl esters to prepare the desired product [11]. The substrates for forming the FA ethanolamides were ethyl esters of 9Z,12Z,15Z-octadecatrienoic (α -linolenic), 5Z,8Z,11Z,14Z,17Z-eicosapentaenoic, and 4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoic acids in addition to ethyl esters of FA from natural fats and oils containing the FA mentioned above [flax-seed oil (*Linum catharticum* L.) and saira fat (*Cololabis saira*)].

FA ethanolamides were prepared by successive transesterification of FA triacylglycerides and aminolysis of the ester bond of the resulting FA ethyl esters by ethanolamine.

The results showed that aminolysis in sealed ampuls at 140°C for 2 h produced the desired product in yields of ~70% without formation of side products, including polymerization of the reaction mixture. Simple chromatographic purification of the reaction products from the starting materials produced pure FA ethanolamides regardless of the starting FA.

Chromatographically pure FA ethanolamides were characterized by spectral data (see Experimental).

1) Pacific Scientific-Research Fisheries Center, 690950, Vladivostok, per. Shevchenko, 4, fax (4232) 30 07 51, e-mail: karaulov2000@yandex.ru; 2) Biological Chemistry Division, University of Michigan Medical School, Ann-Arbor, USA, Katerina Roud, 1301, e-mail: dkuklev@umich.edu. Translated from *Khimiya Prirodnikh Soedinenii*, No. 3, pp. 189-192, May-June, 2004. Original article submitted May 3, 2004.

TABLE 1. Assignment of Signals (Fig. 1b) from HPLC-MS Analysis of Fatty-Acid Ethanolamides from Saira Fat

Peak No.	$[M+H]^+$, m/z	Assignment (ethanolamide)	Peak No.	$[M+H]^+$, m/z	Assignment (ethanolamide)
1	292	16:4n-3	18	312	17:1
2	292	16:4n-?	19	350	20:3n-6
3	294	16:3n-3	20	300	16:0- <i>iso</i>
4	270	14:1	20	374	22:5n-6
5	320	18:4n-3	21	400	24:6n-?
6	296	16:2n-6	22	300	16:0
7	284	15:1	23	376	22:4n-3
8	346	20:5n-3	24	326	18:1n-9
9	272	14:0	25	314	17:0- <i>iso</i>
10	322	18:3n-3	26	352	20:2n-6
11	298	16:1n-7	27	340	19:1
12	348	20:4n-3	28	328	18:0- <i>iso</i>
13	372	22:6n-3	29	328	18:0
14	348	20:4n-6	30	354	20:1n-9
15	324	18:2n-6	31	356	20:0
16	374	22:5n-3	32	382	22:1
17	312	17:1- <i>iso</i>			

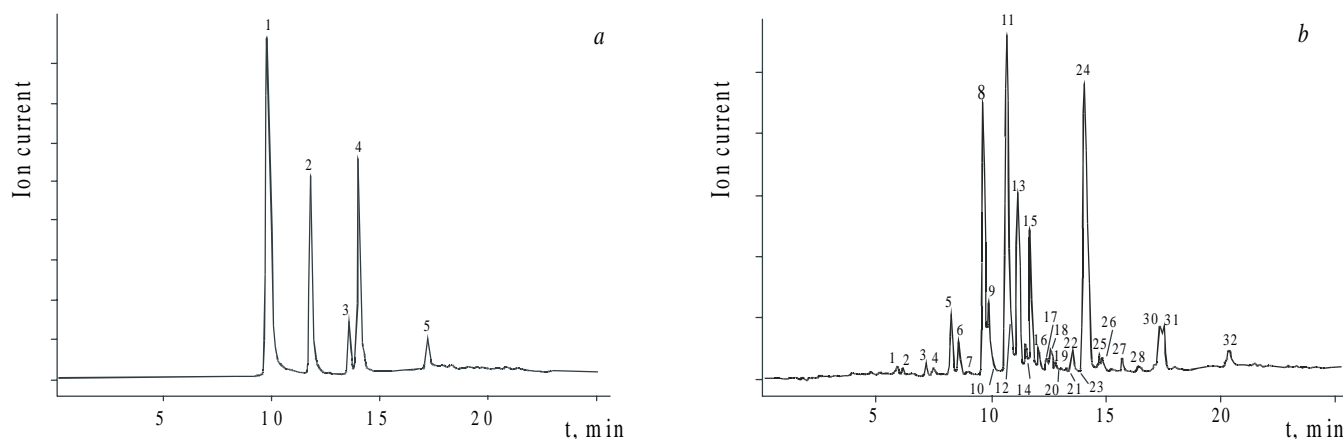


Fig. 1. HPLC-MS analysis of a mixture of fatty-acid ethanolamides from flax-seed oil (a) and saira fat (b). 9Z,12Z,15Z-Octadecatrienoic acid (2-hydroxyethyl)amide (1), 9Z,12Z-octadecadienoic acid (2-hydroxyethyl)amide (2), hexadecanoic acid (2-hydroxyethyl)amide (3), 9Z-octadecenoic acid (2-hydroxyethyl)amide (4), octadecanoic acid (2-hydroxyethyl)amide (5) (a). Assignment of signals is given in Table 1 (b).

Structural changes in the FA hydrocarbon radicals did not occur during formation of their ethanolamides according to results of spectral analysis (lack of bands near 900 cm^{-1} in the IR spectra of the aminolysis products indicating a *trans*-double bond and the lack of conjugated systems of double bonds according to UV spectrophotometry). This enabled the proposed method to be used for preparation of ethanolamides of other FA with retention of the hydrocarbon-radical native structures. This could be done both separately and in mixtures using natural sources of FA such as saira fat and flax-seed oil.

Flax-seed oil typically has five FA in its triacylglycerides. This enables this oil to be used as a standard for identification of complicated FA mixtures, for example, from fish fat. The chromatogram in Fig. 1a shows only five components in the analyzed mixture of flax-seed-oil FA ethanolamides. Therefore, the hydrocarbon radicals did not isomerize during aminolysis. In addition to the ethanolamides of flax-seed-oil FA, ethanolamides of octadecatrienoic, eicosapentaenoic, and docosahexaenoic acids were also used as standards for identification of FA ethanolamides of saira fat. We determined the conditions for HPLC analysis of complicated mixtures of FA ethanolamides (Fig. 1a and b). Mass-selective detection enabled reliable identification of each component with an even m/z value of the quasimolecular ion of composition $[M + H]^+$ (Table 1).

Assignment of structures of the hydrocarbon radicals in the saira-fat FA ethanolamides to the structures of the starting FA, the composition of which was established by GC, showed that the data obtained by the different methods were in complete agreement.

Thus, the proposed method enables the synthesis of FA ethanolamides from any natural source of FA with retention of the native hydrocarbon radical.

EXPERIMENTAL

UV spectra were recorded on a Hitachi 330 (Hitachi, Japan) UV spectrophotometer in CH₃OH; mass spectra of trimethylsilyl derivatives of FA ethanolamides, in a Finnigan MAT 4615B (Finnigan, USA) instrument using electron-impact ionization (electron energy 70 eV, direct sample introduction, positive-ion detection); IR spectra, on a Spectrum 1000 FT-IR Fourier-transform IR spectrometer (Perkin—Elmer, USA) in KBr disks.

HPLC-MS was performed on an Agilent 1100 Series LC/MSD chromatograph—mass spectrometer (Hewlett—Packard, USA) equipped with a mass-spectrometric detector (atmospheric pressure chemical ionization, APCI) and a diode matrix detector. Separation occurred over a Hypersil ODS column (4.0 × 250 mm, 5.0 μm) at 55°C with a linear elution gradient from 55% aqueous acetonitrile to 100% acetonitrile at a rate of 2.25% per minute. The elution rate was 1 mL/min. The range of detected masses was 150-1000 Da (positive-ion detection). The potential in the fragmenter was 70 V; in the ionization chamber, 4 kV. The carrier gas (N₂) flow rate was 6 L/min. The spray-gas (N₂) pressure was 50 kgs/cm². TLC was performed on plates with a fixed layer of silica gel (Silufol UV-254, Kavalier, Czech Rep.). Elution used the solvent system hexane:acetone (1:1 by vol). Compounds on the chromatograms were developed by spraying plates with phosphomolybdic acid solution (10%) in ethanol with subsequent heating to 90°C. Mass fractions of components in the reaction mixtures were determined by GLC on a Iatroscan TH-10 chromatograph (Yanaco, Japan) equipped with a flame-ionization detector using Chromarod-SIII (Yanaco, Japan). Elution used the solvent system hexane:acetone:acetic acid (50:50:0.2 by vol). GC of FA ethyl esters was carried out in a GC-14B chromatograph (Shimadzu, Japan) equipped with a capillary column (30 m × 0.35 mm, 0.25 μm) with Supelcowax 10M stationary phase (Supelco, USA) and a flame-ionization detector under the conditions: column, injector, and detector temperatures 190, 220, and 220°C, respectively; He carrier gas (pure) flow rate 40 mL/min, flow separator 1/60. FA ethyl esters were identified using Kovac retention times [12]. All reagents and solvents used in the work were chemically pure grade.

Lipid Extraction. Lipids were extracted from flax seed and saira carcasses according to the literature method [13].

Preparation of FA Ethyl Esters. KOH (0.3 g) was dissolved in aqueous ethanol (10 mL, 167 mmol, 96%) at 80°C. The resulting solution was treated with saira fat (8 g) or flax-seed oil. The mixture was refluxed for 1 h, cooled to 55°C, treated with H₂SO₄ in ethanol (50%) until the pH was 3, and washed with water (30 mL each) at 55°C. The organic fraction was separated and evaporated in vacuum at 40°C to dryness. Yield 8.3 g (8 g from flax-seed oil) of FA ethyl esters as a light yellowish-orange oil. *R_f* 0.98. Degree of conversion at least 95%. The purity of the FA ethyl esters was monitored by TLC.

Preparation of Highly Purified Ethyl Esters of α-Linolenic, Eicosapentaenoic, and Docosahexaenoic Acids. Mixtures of FA ethyl esters were separated by preparative HPLC in a LC-6A chromatograph (Shimadzu, Japan) using a Zorbax ODS column (9.4 × 250 mm, 5 μm, DuPont, USA) and a RID-6A detector (Shimadzu, Japan) at column temperature 25°C with CH₃OH:H₂O eluent (90:10) and elution flow rate 6 mL/min. Yield 100 mg of ethyl esters of α-linolenic, eicosapentaenoic, and docosahexaenoic acids of purities 98, 96.5, and 96%, respectively, as determined by GC.

Synthesis of FA Ethanolamides. A mixture of FA ethyl esters (6.56 g) was treated with freshly distilled monoethanolamine (over KOH, 6.53 g, 1:5 mole ratio) and trifluoroacetic acid (10 μL). The mixture was sealed in an ampul, vigorously shaken, held for 2 h at 140°C, cooled to 25°C, and removed from the ampul. The contents were treated with CHCl₃ (10 mL) and aqueous HCl (10%) until the pH was 3. The resulting mixture was vigorously shaken. The CHCl₃ layer was separated after layering. The extraction was repeated twice. The CHCl₃ fractions were combined, dried over anhydrous Na₂SO₄, and evaporated in vacuum at 40°C to constant mass. Yield 6.67 g (6.54 g for FA ethyl esters from flax-seed oil) as an opaque yellowish-orange oil containing 4.86 g (4.78 g for FA ethyl esters from flax-seed oil) of FA ethanolamides. The degree of conversion was about 70%. The purity of the FA ethanolamides was monitored by TLC. The FA ethanolamides were purified over a Chemapol L silica-gel column (Kavalier, Czech Rep., 40-100 μm) using a stepped gradient of acetone in hexane (0→10%) calculated as 2 g of starting ethanolamides per 60 g silica gel. Yield 900 mg of purified FA ethanolamides (*R_f* 0.47)

from saira fat and flax-seed oil as colorless oily liquids for every 2 g of starting mixture placed on the column.

An analogous procedure was performed using ethyl esters of α -linolenic, eicosapentaenoic, and docosahexaenoic acids to afford FA ethanolamides of these FA (R_f 0.47) as oily liquids in yields of 33, 34, and 35 mg, respectively, for each 100 mg of FA ethyl esters used in the reaction.

All FA ethanolamides from pure FA ethyl esters and from mixtures were analyzed by HPLC-MS (APCI).

Preparation of Trimethylsilyl Derivatives of FA Ethanolamides for MS Analysis. FA ethanolamide (3-10 mg) was dissolved in a mixture (100 μ L, 1:1 by vol) of N,O-bis-(trimethylsilyl)trifluoroacetamide and pyridine held at 60°C for 1 h, cooled, treated with hexane (1 mL) and saturated NaCl solution (1 mL), vigorously shaken, dried over a small amount of Na₂SO₄ (anhydrous), and evaporated under a stream of N₂. The dry solid was dissolved in hexane (2 mL) and analyzed by MS using EI ionization.

9Z,12Z,15Z-Octadecatrienoic acid (2-hydroxyethyl)amide (R_f 0.47, hexane:acetone, 1:1):

UV spectrum (MeOH, λ_{\max} , nm): 208 (log ϵ 3.68); IR spectrum (KBr, ν , cm⁻¹): 3311 (>NH, -OH), 2925 (-CH<), 1714 (-CO-NH-R), 1653 (>C=C<), 1550 (-CO-NH-R), 1463 (-CH₂-), 1267 (-CO-NH-R), 1070 (C-O-), 720 (>C=C<, *cis*-); mass spectrum (APCI, m/z (I_{rel} , %): 322 (91) [M + H]⁺, 304 (100); mass spectrum of trimethylsilyl derivative (EI, 70 eV, m/z , I_{rel} , %): 393 (7) [M]⁺, 378 (33), 303 (5), 175 (52), 154 (23), 134 (46), 118 (71), 73 (100), 69 (91).

5Z,8Z,11Z,14Z,17Z-Eicosapentaenoic acid (2-hydroxyethyl)amide (R_f 0.47, hexane:acetone, 1:1): UV spectrum (MeOH, λ_{\max} , nm): 211 (log ϵ 3.87); IR spectrum (KBr, ν , cm⁻¹): 3312 (>NH, -OH), 2925 (-CH<), 1712 (-CO-NH-R), 1653 (>C=C<), 1552 (-CO-NH-R), 1466 (-CH₂-), 1265 (-CO-NH-R), 1072 (C-O-), 718 (>C=C<, *cis*-); mass spectrum (APCI, m/z , I_{rel} , %): 346 (87) [M + H]⁺, 328 (100); mass spectrum of trimethylsilyl derivative (EI, 70 eV, m/z , I_{rel} , %): 417 (4) [M]⁺, 302 (25), 328 (3), 175 (50), 134 (48), 118 (100), 99 (51), 73 (100), 69 (88).

4Z,7Z,10Z,13Z,16Z,19Z-Docosahexaenoic acid (2-hydroxyethyl)amide (R_f 0.47, hexane:acetone, 1:1): UV spectrum (MeOH, λ_{\max} , nm): 215 (log ϵ 3.91); IR spectrum (KBr, ν , cm⁻¹): 3312 (>NH, -OH), 2927 (-CH<), 1714 (-CO-NH-R), 1652 (>C=C<), 1552 (-CO-NH-R), 1465 (-CH₂-), 1265 (-CO-NH-R), 1070 (C-O-), 721 (>C=C<, *cis*-); mass spectrum (APCI, m/z , I_{rel} , %): 372 (88) [M + H]⁺, 364 (100); mass spectrum of trimethylsilyl derivative (EI, 70 eV, m/z , I_{rel} , %): 443 (4) [M]⁺, 428 (20), 352 (4), 175 (48), 134 (49), 118 (98), 85 (52), 73 (100), 69 (83).

ACKNOWLEDGMENT

We thank the Scientific-Education Center Marine Biota of the Far-East State University for technical help in carrying out the structure—analytical research.

REFERENCES

1. V. V. Bezuglov, M. Y. Bobrov, and A. V. Archakov, *Biokhimiya (Moscow)*, **63**, No. 1, 27 (1998).
2. F. A. Kuehl, Jr., T. A. Jacob, O. H. Ganley, R. E. Ormond, and M. A. P. Meisinger, *J. Am. Chem. Soc.*, **79**, 5577 (1957).
3. W. A. Devane, L. Hanus, A. Breuer, R. G. Pertwee, L. A. Stevenson, G. Griffin, D. Gibson, A. Mandelbaum, A. Etinger, and R. Mechoulam, *Science*, **258**, 699 (1992).
4. B. F. Cravatt, O. Prospero-Garcia, G. Siuzdak, N. B. Gilula, J. Henriksen, D. L. Boger, and R. A. Lerner, *Science*, **268**, 1506 (1995).
5. L. Hanus, A. Gopher, S. Almog, and R. Mechoulam, *J. Med. Chem.*, **36**, No. 20, 3032 (1993).
6. R. Pertwee, G. Griffin, L. Hanus, and R. Mechoulam, *Eur. J. Pharmacol.*, **259**, No. 2, 115 (1994).
7. E. Fride, J. Barg, R. Levy, D. Saya, E. Heldman, R. Mahoulam, and Z. Vogel, *J. Pharmacol. Exp. Ther.*, **272**, No. 2, 699 (1995).
8. J. Priller, E. M. Briley, J. Mansouri, W. A. Devane, K. Mackie, and C. C. Felder, *Mol. Pharmacol.*, **48**, No. 2, 288 (1995).

9. E. Gross and J. Meienhofer, eds., *The Peptides: Analysis, Synthesis, Biology, Vol. 1: Major Methods of Peptide Bond Formation*, Academic Press, New York (1979).
10. A. Guiffridia and D. Piomelli, *FEBS Lett.*, **422**, 373 (1998).
11. D. V. Kuklev, A. E. Karaulov, V. G. Rybin, and V. I. Vysotskii, *Izv. TINRO*, **129**, 34 (2001).
12. W. W. Christie, *J. Chromatogr. A*, **447**, No. 2, 305 (1988).
13. E. G. Bligh and W. J. Dyer, *Can. J. Biochem. Physiol.*, **37**, No. 8, 911 (1959).