

Mixed Substrates: *trans*- and *cis*-[1-¹⁴C]Monounsaturated Fatty Acids with a Fairly Uniform Distribution of Positional Isomers

Ingolf Richter, Kumar D. Mukherjee, and Nikolaus Weber *

Federal Center for Lipid Research, Münster

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Positional Isomers of [1-¹⁴C]Monounsaturated Fatty Acids, *trans*- and *cis*-[1-¹⁴C]Octadecenoic Acids, *trans*- and *cis*-[1-¹⁴C]Eicosenoic Acids, Mixed Substrates

Simple methods are described for the preparation of *trans*-[1-¹⁴C]monounsaturated fatty acids and *cis*-[1-¹⁴C]monounsaturated fatty acids, each with a fairly uniform distribution of positional isomers, which can serve as mixed substrates in biochemical studies. Methyl *trans*-[1-¹⁴C]octadecenoates and methyl *trans*-[1-¹⁴C]eicosenoates are prepared by partial catalytic hydrogenation of methyl [1-¹⁴C]linolenate and methyl [1-¹⁴C]arachidonate, respectively, followed by argentation chromatography of the partially hydrogenated products. Methyl *trans*-[1-¹⁴C]octadecenoates and methyl *trans*-[1-¹⁴C]eicosenoates, thus obtained, yield the corresponding methyl *cis*-[1-¹⁴C]octadecenoates and methyl *cis*-[1-¹⁴C]eicosenoates, respectively, after *trans*-*cis* equilibration and argentation chromatography. Hydrolysis of each of these methyl esters yields the fatty acids.

Introduction

Mixtures of a great variety of isomeric monounsaturated fatty acids are known to occur in plants [1], animals [2, 3], humans [4] and dietary fats [5] for humans. In a great number of nutritional and biochemical studies, positional and geometrical isomers of octadecenoic [6–12] and docosenoic [13] acids and their esters have been used as substrates individually -- rather than in form of isomeric mixtures. Many of the monounsaturated fatty acids differing in chain-length, position and configuration of the double bond have been synthesized individually [14, 15]. Yet, the synthetic procedures involved are rather tedious and cumbersome, especially if a great number of radioactively labelled isomers are needed. Very few species of radioactively labelled and unlabelled isomeric monounsaturated fatty acids are so far commercially available.

In metabolic studies concerned with the specificity of enzymes for certain species of monounsaturated fatty acids, it might be of advantage to use mixtures of isomeric fatty acids as mixed substrates in a single experiment rather than the pure isomers individually. We wish to report simple methods for the preparation of mixtures of *trans*-[1-¹⁴C]monounsaturated fatty acids and mixtures of *cis*-[1-¹⁴C]-

monounsaturated fatty acids, each with a nearly even distribution of positional isomers. The method involves partial catalytic hydrogenation of methyl esters of [1-¹⁴C]polyunsaturated fatty acids until the maximum formation of monounsaturated *trans*-isomers, which are then isolated by argentation chromatography. *cis*-Isomers are easily prepared from the *trans*-isomers by isomerization and subsequent argentation chromatography. Hydrolysis of the methyl esters yields the corresponding mixtures of isomeric fatty acids. Alternatively, derivatives, such as alcohols, aldehydes, acetates and alkylglycerols, which can also serve as mixed substrates or reference mixtures, are easily obtained from the methyl esters [16]. Recently we have used the mixtures of isomeric *trans*-[1-¹⁴C]octadecenoic acids and *cis*-[1-¹⁴C]octadecenoic acids, which were prepared by the methods described here, in the study of lipid metabolism in plant cell cultures [17].

Materials and Methods

[1-¹⁴C]Linolenic acid, 60 mCi/mmol, and [1-¹⁴C]-arachidonic acid, 50 mCi/mmol were obtained from Amersham Buchler GmbH, D-3300 Braunschweig, Germany. These acids, dissolved in diethyl ether-methanol, were converted to their methyl esters using diazomethane [18]. Unlabelled methyl linolenate and methyl arachidonate as well as other methyl esters used as reference compounds, were purchased from Nu-Chek-Prep, Elysian, Minn. 56028, USA. Palladium chloride/barium sulfate containing 10% palladium, thiophenol and azobis-

Requests for reprints should be sent to Dr. I. Richter, Federal Center for Lipid Research, Piusallee 68/76, D-4400 Münster.

* Present address: Deutsches Krebsforschungszentrum, D-6900 Heidelberg.



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isobutylnitrile as well as all other reagents of analytical grade were obtained from E. Merck AG, D-6100 Darmstadt, Germany.

Hydrogenations were carried out in a 20 ml screw-capped reaction tube provided with a magnetic stirrer. The tube was fitted with a Teflon-lined septum, through which two stainless steel needles were inserted to serve as inlet and outlet for hydrogen or nitrogen. Palladium chloride/barium sulfate, 10 mg, was suspended in 4 ml hexane in the reaction tube and reduced to the active catalyst by bubbling pure hydrogen through the reaction mixture under stirring at ambient temperature for 10 min. The reaction tube containing the catalyst suspension was purged with nitrogen. Mixtures of either methyl [^{14}C]linolenate, 50 $\mu\text{Ci}/0.83 \mu\text{mol}$, and methyl linolenate, 0.18 mmol, or methyl [^{14}C]arachidonate, 50 $\mu\text{Ci}/\mu\text{mol}$, and methyl arachidonate, 0.15 mmol, were dissolved in 2 ml hexane and added to the reaction tube. Hydrogenation was started by bubbling hydrogen through the reaction mixture under stirring at ambient temperature. At intervals of 2 min, the hydrogenation was interrupted by replacing hydrogen with nitrogen. Partially hydrogenated samples were withdrawn from the supernatant of the reaction mixture and analyzed by radio gas chromatography as follows.

A Hewlett-Packard Gas Chromatograph 5750 G (Hewlett Packard, San Diego, California, USA) equipped with a flame ionization detector was used in combination with a Packard Gas Proportional Counter, Model 894 (Packard Instrument Company, Inc., Downers Grove, Ill., USA). A glass column (1.8 m \times 4 mm) was packed with 10% EGSS-X on Gas-Chrom P, 100–120 mesh (Applied Science Laboratories Inc., State College, Pa. 16801, USA) and held at 175 °C. Helium (85 ml/min) served as carrier gas; the effluent carrier gas was divided by a stream splitter, 5:1, connected to the proportional flow counter and flame ionization detector, respectively. The methyl esters of ^{14}C labelled fatty acids were identified by comparing their retention times with those of reference compounds. The proportions of the various radioactively labelled components in each mixture were calculated as percentage area of the respective peak, as measured by triangulation.

Hydrogenation of polyunsaturated methyl esters was continued until a maximum proportion of monounsaturated methyl esters was formed.

Samples of the partially hydrogenated methyl esters were fractionated into *trans*- and *cis*-isomers by argentation chromatography [19] on a layer of silica gel containing 20% silver nitrate. The plates were developed twice with hexane:diethyl ether (9:1) at room temperature. The distribution of radioactivity on the thin-layer chromatograms was determined by means of a Berthold TLC Scanner LB 2760 (BF-Vertriebsgesellschaft, D-7547 Wildbach, Germany). The radioactive zones corresponding to methyl esters of *trans*- and *cis*-monounsaturated fatty acids were scraped off the plates and the methyl esters were eluted with ether, saturated with water. Both fractions of methyl esters were analyzed by radio gas chromatography, as described above.

Aliquots of all *trans*- and *cis*-methyl ester fractions were taken in scintillation vials, 10 ml of "Aquasol-2" (NEN Chemicals GmbH, D-6072 Dreieichenhain, Germany) was added and radioactivity determined in a Packard Tri-Carb C 2425 Liquid Scintillation Counter.

Fractions of methyl *trans*-[^{14}C]octadecenoates and methyl *trans*-[^{14}C]eicosenoates obtained by partial hydrogenation of methyl linolenate and methyl arachidonate, respectively, were isomerized using thiophenol and azobisisobutylnitrile [20]. The isomerized products were fractionated into *trans*- and *cis*-monounsaturated methyl esters by argentation thin-layer chromatography as described before.

Aliquots of *trans*- and *cis*-monounsaturated methyl esters, ca. 100 μg (ca. 0.2 μCi), were dissolved in pentane and ozonized at -70°C using a Supelco Micro-Ozonizer (Supelco, Inc., Bellefonte, Pa. 16823, USA) [21]. The reaction products were evaporated to dryness at room temperature, dissolved in 40 μl carbon disulfide, and the ozonides were reduced with 1–3 mg of triphenylphosphine. After standing at room temperature for 30 min, the mixture of aldehydes and aldesters was analyzed by radio gas chromatography, using a column, 1.8 m \times 4 mm, packed with a 5:1 mixture of 10% OV-17 and 3% OV-1, each on Gas-Chrom Q, 100–120 mesh (Applied Science Laboratories Inc.). The temperature was programmed from 70 °C to 270 °C, 4 °C/min. Identification of fragments was made by comparison with the retention times of aldesters, obtained by ozonolysis of monounsaturated methyl esters of different chain-lengths and known positions of double bonds. Quantitative results were obtained

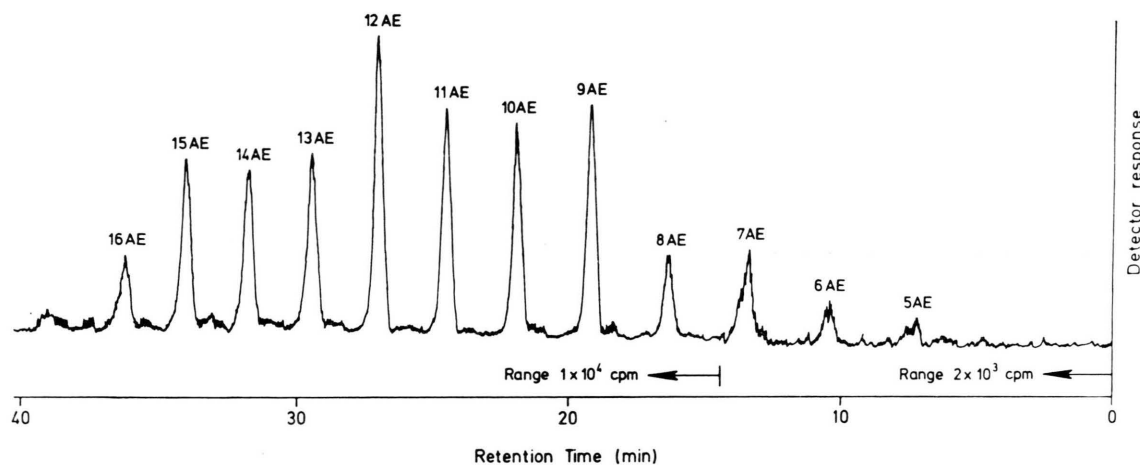


Fig. 1. Radio gas chromatogram of ozonolysis products of methyl *trans*-[1-¹⁴C]octadecenoates obtained by partial hydrogenation of methyl [1-¹⁴C]linolenate. Conditions of chromatography as described in the text. Peaks are designated by the number of carbon atoms of the aldehyde moieties of aldesters (AE).

from peak areas of [1-¹⁴C]aldesters, measured by triangulation. As an example, Fig. 1 shows a radio gas chromatogram of the ozonolysis products of methyl *trans*-[1-¹⁴C]octadecenoates, obtained by partial hydrogenation of methyl linolenate.

The *trans*- and *cis*-[1-¹⁴C]monounsaturated fatty acids were prepared by hydrolysis of the corresponding methyl esters [22].

Results and Discussion

Partial hydrogenation of polyunsaturated fatty acids and their alkyl or glyceryl esters using metal catalysts is known to yield mixtures of *trans*- and *cis*-monounsaturated fatty acids or the corresponding esters having double bonds widely distributed throughout the hydrocarbon chain [23]. Based on this fact, we have attempted to develop a simple method for the preparation of mixtures of positional isomers of *trans*- and *cis*-[1-¹⁴C]monounsaturated fatty acids with uniform distribution of isomers. The method involves partial hydrogenation of methyl esters of radioactively labelled polyunsaturated fatty acids, that are commercially available.

Methyl [1-¹⁴C]linolenate, 50 μ Ci/0.18 mmol, after hydrogenation for 6 min, yields a product containing approximately 80% methyl octadecenoates, 15% methyl octadecanoate and 5% methyl octadecadienoates. This reaction product is fractionated into methyl *trans*- and *cis*-octadecenoates by argentation chromatography. The methyl *trans*-octade-

cenoates contain ca. 45% of the radioactivity which was present in methyl linolenate. The fraction containing methyl *cis*-octadecenoates is found to contain ca. 15% of the label from methyl linolenate. The composition of positional isomers in methyl *trans*- and *cis*-[1-¹⁴C]octadecenoates, determined by reductive ozonolysis and radio gas chromatography, is given in Table I. It is evident that the methyl *trans*-octadecenoates are composed of positional isomers ranging from $\Delta 6$ to $\Delta 16$ with a fairly uniform distribution from $\Delta 9$ to $\Delta 15$. The methyl *cis*-octadecenoates contain each of the positional isomers ranging from $\Delta 8$ to $\Delta 15$, however, the distribution of these isomers is highly nonuniform; pronounced maxima are found at $\Delta 9$, $\Delta 12$ and $\Delta 15$ isomers.

Methyl [1-¹⁴C]arachidonate, 50 μ Ci/0.15 mmol, after 10 min of hydrogenation, yields a product containing approximately 60% methyl eicosenoates, 25% methyl eicosanoate and 15% methyl eicosadienoates. Argentation chromatography of this product yields methyl *trans*-eicosenoates and methyl *cis*-eicosenoates containing ca. 25% and 15%, respectively, of the radioactivity present in methyl arachidonate. The composition of mixtures of positional isomers in methyl *trans*- and *cis*-[1-¹⁴C]eicosenoates is also included in Table I. It is evident that the methyl *trans*-eicosenoates are composed of positional isomers ranging from $\Delta 4$ to $\Delta 16$ with a fairly uniform distribution from $\Delta 5$ to $\Delta 15$, whereas the positional isomers in the mixture of methyl

Table I. Percentage composition of positional isomers of methyl esters of *trans*- and *cis*-[1-¹⁴C] monounsaturated fatty acids obtained *via* partial hydrogenation and of methyl esters of *cis*-[1-¹⁴C] monounsaturated fatty acids obtained *via trans-cis* equilibration after partial hydrogenation.

Positional isomer (Δ)	Methyl [1- ¹⁴ C] octadecenoates			Methyl [1- ¹⁴ C] eicosenoates		
	<i>trans</i> ^a	<i>cis</i> ^a	<i>cis</i> ^b	<i>trans</i> ^a	<i>cis</i> ^a	<i>cis</i> ^b
4				3	2	3
5				10	19	12
6	<1		<1	7	4	6
7	2		1	6	4	7
8	5	3	4	10	14	10
9	13	21	13	7	4	6
10	12	9	12	7	4	8
11	12	9	13	13	21	12
12	19	33	19	9	4	8
13	11	7	12	10	4	9
14	10	5	10	12	17	12
15	10	13	10	5	2	5
16	5		5	1	<1	2

^a *via* partial hydrogenation.

^b *via trans-cis* equilibration after partial hydrogenation.

cis-eicosenoates show pronounced maxima at Δ5, Δ8, Δ11 and Δ14.

The results presented show that methyl esters of *trans*-[1-¹⁴C] monounsaturated fatty acids with fairly uniform distribution of positional isomers are conveniently prepared in good yields by partial hydrogenation of labelled polyunsaturated methyl esters and subsequent argentation chromatography. In order to assess the reproducibility of this method, unlabelled methyl linolenate and arachidonate were partially hydrogenated until levels ranging from 10 to 30% saturated methyl esters were attained. Methyl *trans*-octadecenoates and methyl *trans*-eicosenoates, isolated from the partially hydrogenated products, were analyzed by reductive ozonolysis and gas chromatography [24]. It was found, that although the yield of *trans*-monounsaturated methyl esters varied with the degree of hydrogenation, the distribution of positional isomers in methyl *trans*-octadecenoates and methyl *trans*-eicosenoates was independent of the degree of hydrogenation. Thus, it is evident, that the fairly uniform pattern of distribution of positional isomers in *trans*-[1-¹⁴C]-monounsaturated methyl esters, obtained by partial

hydrogenation of polyunsaturated methyl esters, can be easily reproduced.

trans-cis Isomerization of unsaturated methyl esters by free radicals is known to occur without migration of double bonds [20, 25]. We have applied this principle for the preparation of uniformly distributed mixtures of positional isomers of *cis*-[1-¹⁴C] monounsaturated fatty acids from methyl esters of *trans*-[1-¹⁴C] monounsaturated fatty acids, that are obtained by partial hydrogenation, as described above.

Methyl *trans*-[1-¹⁴C] octadecenoates, 10 μCi/0.03 mmol, prepared by partial hydrogenation and argentation chromatography, as described above, yield, on isomerization using thiophenol in the presence of azobisisobutyronitrile [20], an equilibrium mixture containing 20% methyl *cis*-octadecenoates. Separation by argentation chromatography yields methyl *cis*-[1-¹⁴C] octadecenoates representing ca. 18% of the label from methyl *trans*-[1-¹⁴C] octadecenoates. Similarly, methyl *trans*-[1-¹⁴C] eicosenoates, 5 μCi/0.015 mmol, yield, after isomerization and argentation chromatography, the methyl *cis*-[1-¹⁴C]-eicosenoates containing ca. 16% of the label from the starting material. The composition of positional isomers in methyl *cis*-[1-¹⁴C] octadecenoates and methyl *cis*-[1-¹⁴C] eicosenoates, obtained *via trans-cis* equilibration, is given in Table I. It is evident that the methyl *cis*-octadecenoates contain positional isomers ranging from Δ9 to Δ15 in a fairly uniform distribution. Similarly, the methyl *cis*-eicosenoates contain positional isomers ranging from Δ5 to Δ15 in a fairly uniform distribution. The results show that methyl esters of *cis*-[1-¹⁴C] monounsaturated fatty acids with fairly even distribution of positional isomers are conveniently prepared *via trans-cis* equilibration of the corresponding *trans*-isomers, prepared by partial hydrogenation of polyunsaturated methyl esters.

The methyl *trans*- and *cis*-octadecenoates as well as eicosenoates yield the corresponding fatty acids on hydrolysis. Reesterification with diazomethane and subsequent analysis by reductive ozonolysis and radio gas chromatography shows that the isomeric composition of the methyl esters is not altered by hydrolysis.

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