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Preparation of methyl *cis*-9, *trans*-11- and *trans*-9, *trans*-11-octadecadienoate-17,17,18,18-d_4, two of the isomers of conjugated linoleic acid[1]

R. Adlof *

USDA, Agricultural Research Service, National Center for Agricultural Utilization Research, Food Quality and Safety Research, 1815 N. University Street, Peoria, IL 61604, USA

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Abstract

A multi-step synthesis was used to prepare the *cis*-9, *trans*-11- and *trans*-9, *trans*-11-isomers (in a ratio of 46/54) of conjugated linoleic acid (*cis*-9, *trans*-11-octadecadienoic acid) labeled with deuterium atoms on the 17- and 18-carbon atoms (17,17,18,18-d_4). The methyl *cis*/*trans*-9, *trans*-11-octadecadienoate-17,17,18,18-d_4 isomer pair were obtained from the Wittig coupling of *trans*-2-nonenyltriphenyl-phosphonium bromide (8,8,9,9-d_4) and methyl 9-oxononanoate. To prepare the phosphonium bromide, 5-hexyn-1-ol was reduced with deuterium gas/Wilkinson's catalyst to yield 1-hexanol-5,5,6,6-d_4. The alcohol was converted to the iodide with phosphorous pentoxide/phosphoric acid/potassium iodide. Coupling of the iodide with 2-propyn-1-ol via lithium amide in liquid ammonia gave 2-nonyn-1-ol-d_4. The acetylenic alcohol was reduced with lithium metal in liquid ammonia to yield the *trans*-2-nonen-1-ol-d_4. The alcohol was converted to the bromide (using triphenylphosphine dibromide) and then converted to the phosphonium salt. The aldehyde ester was prepared by the reductive ozonization of methyl 9-*cis*-octadecenoate. The two conjugated linoleic acid isomers, formed during the final Wittig coupling reaction, were readily separated by a combination of reversed-phase and silver resin chromatography. Isotopic and chemical purities were $>95\%$ for each geometric isomer. Overall yield (both isomers) from the 8-step synthesis was 12%. © 1997 Elsevier Science Ireland Ltd.

Keywords: Fats; Deuterium; Conjugated; *Cis*; *Trans*; Isomers

* Tel.: +1 309 6816278; Fax: +1 309 6816679; E-mail: adlofro@mail.ncaur.usda.gov

[1] Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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1. Introduction

To study the metabolism and interaction of fatty acid (FA) *cis* and *trans* isomers in humans (Emken, 1979; Emken et al., 1980), we have synthesized various deuterium-labelled monoenoic (Adlof and Emken, 1978), dienoic (Adlof and Emken, 1981, 1985, 1987), and trienoic (Adlof and Emken, 1985, 1987) FAs (Rakoff, 1982). Conjugated linoleic acid (CLA; *cis*-9, *trans*-11-octadecadienoic acid) has been associated with the reduction of chemically-induced cancers in mice and rats and the suppression of atherosclerosis in rats (Steinhart, 1996; Chin et al., 1992; Belury et al., 1996; Ip et al., 1996). The *cis*-9, *trans*-11-isomer is considered to be the active constituent (Chin et al., 1992), but contributions by other isomers (*trans*-9, *trans*-11-) have not been ruled out. Deuterium-labeled CLA isomers can be safely used in humans to trace their metabolism and interaction(s) with other fats. A synthesis was therefore developed to generate both CLA isomers, deuterium atom-labeled, in sufficient yield and of high enough isotopic and chemical purity for use in humans.

2. Experimental

2.1. Reagents

Lindlar catalyst, *n*-butyl lithium (2.6 M in hexane), triphenylphosphine, lithium metal, magnesium turnings, ethyl bromide, and 2-propynol were obtained from Aldrich (Milwaukee, WI). 5-Hexynol was purchased from Farchan Laboratories (Gainesville, FL) and tris-(triphenylphosphine) chlororhodium from Strem Chemicals (Newburyport, MA). Silica gel (60-200 mesh) was obtained from J.T. Baker (Jackson, TN) and deuterium gas, 98%, from Matheson Gas Products (Secaucus, NJ).

2.2. Analyses of HPLC fractions

Fatty acid methyl esters (FAMEs) were analyzed with a Varian 3400 GC (Varian Instruments, Palo Alto, CA) equipped with a 30 m × 0.32 mm SP2380 (Supelco, Bellefonte, PA) capillary column and flame ionization detector (FID). Helium was utilized as carrier gas. Methyl ester peaks were identified by comparison with standard FAME mixtures of known composition.

Gas chromatography/mass spectrometry (GC/MS) was used to determine the quantity of deuterated and non-deuterated FAME. Analyses were made on a Hewlett-Packard Model 5889 GC/MS (quadrapole; positive chemical ionization mode; isobutane as ionizing gas) equipped with a 30 m × 0.25 mm Supelcowax 10 fused silica capillary column (Supelco, Bellefonte, PA). Data collection and manipulation have been described previously (Rohwedder et al., 1985).

2.3. Liquid chromatography:

Silver resin chromatography for preparative separation of CLA isomers was done on a 2.5 × 45 cm glass column packed with 200/270 mesh, silver ion-saturated Rohm and Hass XN1010 macroreticular sulfonic acid resin (Emken et al., 1978). For silver ion incorporation and packing of the glass columns see Adlof (1994). Samples were applied by needle-tipped syringe through a septum to the top of the column. The C18 RP system consisted of a 50 × 250 mm stainless steel column (5 μm particle size; Serva Feinbiochemica, Heidelberg, Germany) and a Rheodyne 7125 injector (Rheodyne, Cotati, CA) with a 2 ml injection loop. Both systems utilized Waters 510 HPLC pumps and R403 refractive index detectors (both Waters Associates, Milford, MA).

2.4. Ag-HPLC:

A Spectra-Physics P2000 solvent delivery system (Spectra-Physics Analytical, Fremont, CA), a Rheodyne 7125 injector (Rheodyne, Cotati, CA) with a 20 μl injection loop, and an ISCO V4 Absorbance Detector (Isco, Lincoln, NE) were used. The ChromSpher Lipids columns (Cat. No. 28313; 4.6 mm i.d. × 250 mm stainless steel; 5 micron particle size; silver ion impregnated) were purchased from Chrompack International, Middelburg, The Netherlands, and used as received. Two Lipids columns connected in series resulted

in improved peak-to-peak resolutions. Solvent flow was standardized at 1.0 ml/min; isocratic conditions [0.35% acetonitrile (ACN) in hexane; 23°C] were used to minimize variations in FAME retention(s) and resolution(s). The void volume of this pair of columns was 4.2 ml.

3. Synthetic procedures

Both compounds were synthesized from a single synthetic sequence (Fig. 1) involving a combination of acetylenic and Wittig coupling reactions. Experimental details for most of these steps (or for the preparation of analogues) have been published elsewhere (Adlof and Emken, 1978, 1981, 1985, 1987).

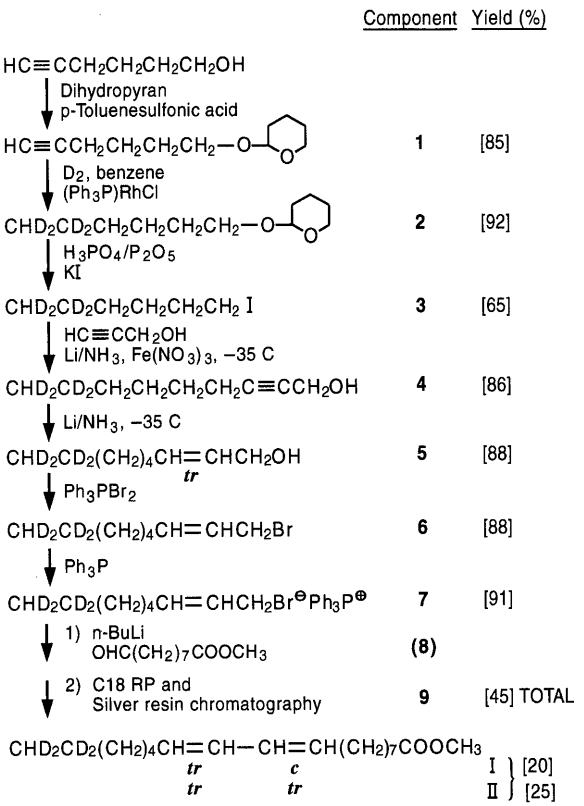

Fig. 1. Synthetic scheme for preparation of methyl *cis*-9, *trans*-11- and *trans*-9, *trans*-11-octadecadienoate-17,17,18,18-d_4. Compound numbers are underlined; yields for each step are given in brackets.

Preparation: 2-(5-hexynyloxy)tetrahydropyran, 1. The tetrahydropyranyl (THP) ether of 5-Hexyn-1-ol was prepared [dihydropyran, *p*-toluenesulfonic acid in diethyl ether (EE)] in 85% yield as described previously (Adlof and Emken, 1978) (b.p. 73–76°C/0.15 mmHg).

Preparation: 2-(5,5,6,6-tetradeutero-hexyloxy) tetrahydropyran, 2. Compound 1 (47.6 g; 550 mmol) in benzene (1000 ml) was treated with deuterium (D_2) gas at atmospheric pressure in the presence of tris-(triphenylphosphine) chlororhodium (8 g) in the manner described previously for a 5-carbon analogue (Adlof and Emken, 1981). The residue was eluted with petroleum ether (PE) through a 5×50 cm glass column packed with silica gel. Removal of the solvent by rotary evaporator yielded a colorless oil (92 g; 92% yield). Deuterium distribution by GC/MS: 2.0% d_0, 1.3% d_3, 96.7% d_4.

Preparation: 1-Iodohexane-5,5,6,6-d_4, 3. The halide was prepared from compound 2 using phosphoric acid and potassium iodide (KI) as described previously for a 5-carbon analogue. (Adlof and Emken, 1981). Purification by distillation through a short-path column (b.p. 50–51°C at 3.0 mmHg) gave compound 3 (30.9 g; 143 mmoles) in a yield of 65%.

Preparation: 2-Nonyn-1-ol-8,8,9,9-d_4, 4. Liquid ammonia (approx. 180 ml) was added to a 500 ml, three-neck flask equipped with a mechanical stirrer, a dry ice/acetone cooled condenser, and immersed in a dry ice/acetone bath (temperature maintained at approx. −40°C). As catalyst for the formation of lithium amide, $Fe(NO_3)_3 \cdot 9H_2O$ (0.06 g) was added. Lithium metal (1.0 g; 140 mmol) was added in small pieces over 15 min. After the grey slurry appeared, 2-propynol (3.9 g; 69 mmol) in tetrahydrofuran (THF; 40 ml) was added dropwise over 15 min. Compound 3 (10 g; 46 mmol) in THF (35 ml) was added dropwise over 15 min. The slurry was stirred for 1 h at −35°C, then allowed to warm slowly to room temperature (22°C) over 3.5 h. The slurry was then warmed to 30°C, 100 ml of THF was added, and stirring was continued (under argon atmosphere) at room temperature for 16 h. The flask was cooled in an ice bath and water (50 ml) was added dropwise. The reaction mixture was trans-

ferred to a round-bottomed flask and the THF removed by rotary evaporation. The residue was transferred to a separatory funnel with EE (150 ml) and the layers were separated. The water layer was acidified with 5N sulfuric acid (H_2SO_4) and extracted with 4×50 ml EE. The EE fractions were combined and washed with 50 ml saturated ammonium chloride (NH_4Cl), 50 ml of 5N H_2SO_4, 2×50 ml water and dried over sodium sulfate (Na_2SO_4). After removal of the drying agent and ether, the residue was eluted through 100 g silica gel with PE, then 75/25 PE/EE to give 4 (5.8 g; 86% yield).

Preparation: 2-*Trans*-nonen-1-ol-8,8,9,9-d_4, 5. Compound 5 was prepared by modification of a procedure described in Otsuki et al. (1986). Compound 4 (2.0 g; 14 mmol) in 40 ml THF was added to a 200 ml 3-neck round-bottomed flask equipped with a thermometer, argon inlet, mechanical stirrer, dry ice/acetone reflux condenser, and a drying tube (calcium chloride; $CaCl_2$). The flask was cooled in a dry ice/acetone bath at -35°C. Liquid ammonia (approx. 100 ml) was introduced slowly, with stirring, and then lithium metal (1.0 g; 140 mmol) was added in small pieces over 15 min. The blue-black solution was stirred at reflux conditions (-33°C) for 3 h, then slowly warmed to room temperature over 1.5 h. The slurry was heated to 40°C, stirred for another hour, then cooled to 10°C and water (20 ml) was slowly added via syringe. After the vigorous evolution of gas had ceased, the slurry was acidified with 50 ml 6 M hydrochloric acid (HCl) and transferred to a separatory funnel with 100 ml EE. The layers were separated and the water layer was extracted with 4×25 ml EE. The EE fractions were combined and dried over Na_2SO_4. After removal of the drying agent and EE, the residue was eluted through 15 g silica gel with 95/5 PE/EE to give 5 (1.8 g; 88% yield).

Preparation: 1-Bromo-*trans*-2-nonen-8,8,9,9-d_4, 6. Triphenylphosphine (7.6 g; 29 mmol) was dissolved in methylene chloride (20 ml) in a 100 ml 3-neck flask equipped with a mechanical stirrer, a burette, a thermometer, an inert gas inlet and a calcium chloride drying tube. The apparatus was flushed with a stream of argon and immersed in an ice bath. Bromine (4.6 g; 29 mmol) was added at a temperature of 10°C. Compound 5 (4.2 g; 29 mmol) dissolved in methylene chloride (5 ml) was added over 15 min at a temperature of 10°C. The cooling bath was removed and the solution was stirred at room temperature for 0.5 h. PE (35 ml) was added and the slurry was chromatographed on 25 g silica gel (PE as solvent). Removal of the solvent resulted in 5.1 g of 6 (99% pure; 88% yield).

Preparation: 8,8,9,9-Tetradeutero-*trans*-2-nonenyltriphenylphosphonium bromide, 7. Compound 6 (2.5 g; 12 mmol), triphenylphosphine (3.9 g; 15 mmol) and ACN (50 ml) were combined in a 100 ml, 1-neck round bottomed flask equipped with a reflux condenser and an argon inlet. The mixture was heated to reflux (oil bath) and stirred magnetically for 23 h. The homogeneous solution was cooled to room temperature, the acetonitrile was removed on a rotary evaporator and the viscous residue was triturated several times with fresh portions of EE. After crystallization, the residue was isolated by vacuum filtration and dried in a vacuum oven to give compound 7 (5.1 g; 91% yield). Compound 7 had a m.p. (uncorrected) of 166–169°C.

Preparation: Methyl 9-oxo-nonenoate, 8. This compound [prepared by ozonolysis of methyl *cis*-9-octadecenoate and subsequent zinc/acetic acid reduction (Pryde et al., 1960)] was freshly distilled before use.

Preparation: Methyl *cis*/*trans*-9, *trans*-11-octadecadienoate-17,17,18,18-d_4, 9. Compound 7 (5.1 g; 11 mmol) was dissolved in 80 ml hexamethylphosphoramide/THF (1:9 v/v) in a 200 ml 3-neck flask equipped with an argon inlet, a mechanical stirrer, a low temperature thermometer, and a $CaCl_2$ drying tube. The apparatus was cooled to -74°C with a dry ice/acetone bath and *n*-butyl lithium (2.0 M in hexanes; 6.5 ml; 13 mmol) was added by syringe over 25 min. The orange-brown slurry was stirred at -74°C for 1.5 h, then compound 8 (90% pure; 2.5 g; 13 mmol) was added dropwise over 0.5 h. The slurry was stirred at -74°C for another 5 h, the dry ice/acetone bath was removed, and the slurry was allowed to warm to room temperature overnight. Saturated sodium chloride solution (40 ml) was added and the slurry was transferred to a separa-

tory funnel with water. The layers were separated and the water layer was extracted with 50 ml PE. The organic layers were combined, washed with 2×50 ml water, then dried over Na_2SO_4. Removal of the drying agent and solvents provided the residue 9 (3.6 g). Residue 9 was purified by a combination of reversed-phase and silver resin chromatography (see Section 2 for details).

Initial purification was accomplished on a C18 reversed-phase chromatography column (6.5 ml ACN/min; 1.9 g sample size per injection; see Section 2 for details) to yield a 1.75 g mixture of the isomer pair (44% *cis*/*trans*; 54% *trans*/*trans*). The isomer pair were separated by silver resin chromatography (7 ml methanol/min; 500 mg sample size per injection; see Section 2 for details.) The isolated yields of the isomers were: 687 mg *cis*-9, *trans*-11- (97% pure; 20% yield; 2.5% *trans*/*trans* and 0.5% *trans*/*cis*) and 808 mg 9-*trans*, 11-*trans*- (25% yield; 99% pure).

4. Results and Discussion

Several points of interest were noted during this synthesis. 5-Hexynol was converted to the THP ether (1) to eliminate the possibility of hydrogen exchange during deuteration. The THP ether could also be readily converted to the iodide. The lower-than-expected yield of 3 was due to some loss of material during solvent removal. The deuterium atoms were located on the 17- and 18-positions to minimize potential isotope effects. The deuterium atoms may be located from carbon-14 to carbon-18, the number of deuterium atoms varied from d_2 (olefinic alcohol precursor) to d_6 ('ene-yne-ol' precursor). The overall CLA-d_4 yield of 12% was accomplished without pre-purification of solvents, etc.

We had previously found that use of the iodide rather than the bromide (6) for preparation of the phosphonium salt resulted in more easily purified phosphonium salts and higher yields of product in the subsequent Wittig coupling reaction (Adlof and Emken, 1985). Our initial synthesis of CLA thus included conversion of the olefinic bromide 6 to the iodide (NaI in acetone, reflux conditions). The triphenylphosphonium salt of the iodide was

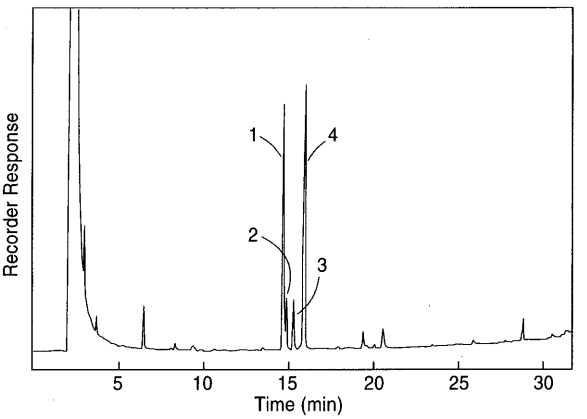

Fig. 2. GC analysis of synthesized CLA-d_4 using an SP2340 capillary column (see Section 2 for details). [Methyl *cis*-9, *trans*-11- (Peak # 1); *trans*-9, *cis*-11- (# 2); cis-9, *cis*-11- (# 3); *trans*-9, *trans*-11-(# 4) octadecadienoate-17,17,18, 18-d_4].

readily isolated (m.p. 134–136°C, uncorrected) and used in the subsequent Wittig coupling reaction. Conditions for the Wittig reaction were identical to those described for the preparation of 9. The reaction proceeded more rapidly than when the bromide was used and total CLA isomer yields were comparable for the bromide and iodide salts. When product 9 (prepared using the iodide salt precursor) was analyzed by GC, two by-products were noted (Fig. 2). Eluting just after the methyl *cis*-9, *trans*-11-octadecadienoate-17,17,18,18-d_4 (peak 1) and before the methyl *trans*-9, *trans*-11-octadecadienoate-17,17,18,18-d_4 (peak 4), they are tentatively identified (by coinjection of GC standards) as methyl *trans*-9, *cis*-11-octadecadienoate-17,17,18,18-d_4 (peak 2) and methyl *cis*-9, -3-*cis*-11-octadecadienoate-17,17, 18,18-d_4 (peak 4). The two components respectively comprised 9.2% and 8.5% of the total CLA mixture. While the *cis*-9, *cis*-11- isomer could be separated by silver resin chromatography, the *trans*-9, *cis*-11- isomer could not be separated from the *cis*-9, *trans*-11- isomer by reversed-phase or silver resin chromatography. Dual-column Ag-HPLC (10 μg sample size; 0.35% ACN in hexane; see Section 2 for details) was also unsuccessful in separating these isomers. Methyl *trans*-9, *cis*-11-octadecadienoate-

17,17,18,18-d_4 and methyl *cis*-9, *cis*-11-octadecadienoate-17,17,18,18-d_4 were present at only 0.3 and 0.8%, respectively, when the alkenyl phosphonium bromide (6), rather than the iodide, was used in the final Wittig coupling step.

Wittig coupling reactions between a moderate (semi-stabilized), α-unsaturated phosphonium salt with a saturated aldehyde (*n*-BuLi catalyst; THF as solvent; -30°C) tend to produce high (50% or more) percentages of the *trans* isomer at the newly formed double bond and to cause some isomerization at the existing double bond (approx. 20% conversion of *trans* to *cis*) (Ideses and Shani, 1989). Applying the composition data provided in Ideses and Shani (1989) to our Wittig coupling reactions, the calculated theoretical ratio of the CLA isomers should be 40% *cis*/*trans*, 9% *trans*/*cis*, 9% *cis*/*cis* and 40% *trans*/*trans*. This data is in excellent agreement with our observed ratio of 40.0, 9.2, 8.5 and 42.3% when the α-unsaturated phosphonium iodide salt was used. While both our Wittig reactions were run at -74°C, use of the less reactive bromide salt yielded a CLA mixture with the more favorable ratio of 46.6, 0.3, 0.8 and 52.3% respectively. A single synthesis may thus be used to prepare roughly equal amounts of two CLA isomers (labelled with four deuterium atoms) in high isotopic and chemical purity.

Acknowledgements

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