Large-Scale Preparation of Linoleic Acid-d_2-Enriched Triglycerides from *Crepis alpina* Seed Oil

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Catalytic deuteration of *Crepis alpina* seed oil provided a convenient one-step method for the direct synthesis of large quantities of triglycerides enriched with deuterium-labelled linoleic acid. *Crepis alpina* seed (19 kg) was crushed, and the oil [74% crepenynic acid (*cis*-9-octadecen-12-ynoic acid)] was extracted with hexane. After purification by column chromatography (silica gel), > 170-g batches of oil were deuterated with Lindlar catalyst and deuterium (D_2) gas. Purification (silica gel) resulted in > 150-g samples of triglyceride containing 74% *cis*-9, *cis*-12-octadecadienoic acid-12,13-d_2 (18:2-d_2) and 14% unlabelled linoleic acid. Pure (> 99%) tricrepenynin was recovered by further fractionation of the *Crepis alpina* triglycerides on silica gel. Deuteration of this sample produced deuterium-labelled trilinolein containing linoleic acid-d_2 of > 98% isotopic purity.

KEY WORDS: Acetylenic fatty acids and esters, C18 fatty acids and derivatives, crepis oil seeds and crepenynic acid, deuterium and deuteration, linoleic acid and esters.

Deuterium-labelled fatty acids have been useful as probes for investigation of fatty acid metabolism. They have been used to follow the incorporation of *cis* and *trans* positional fatty acid isomers, to study metabolic disorders and to trace metabolic pathways (1-6). Deuterium is nonradioactive; thus, deuterium-labelled fats can be fed to, and their metabolism studied in, humans. Blood samples taken over time can be analyzed by gas chromatography/mass spectrometry (GC/MS) to follow the absorption, conversion and incorporation of different fatty acid structures into various plasma lipid classes (7).

Two disadvantages of using deuterated fatty acids are that relatively large amounts are required and that many of the fatty acids of interest are not commercially available in deuterated form at a reasonable cost. In particular, to study the conversion of linoleic (18:2n-6) acid to arachidonic (20:4n-6) acid in several subjects required large amounts (100 + g) of the deuterated 18:2 substrate because of the low conversion in humans (6).

Published methods for synthesis of deuterium-labelled trilinolein of sufficient purity for human studies involve multiple synthetic steps, utilize tedious purification procedures and result in 10-20-g batches of material in overall yields of 30-70% (8-11). This paper describes the catalytic deuteration of *Crepis alpina* seed oil as a one-step method for the large-scale (100+ g) preparation of triglycerides containing *cis*-9,*cis*-12-octadecadienoic acid-12,13-d_2.

MATERIALS AND METHODS

Crepis alpina seed was obtained from plants grown at the USDA Agricultural Research Station (Corvallis, OR). This seed is available (USDA, NCAUR, Peoria, IL) and may be grown under contract. Lindlar catalyst, quinoline (both from Aldrich Chemical Co., Milwaukee, WI), silica gel (J.T. Baker Chemical Co., Phillipsburg, NJ) and deuterium gas, 99.9+% (Matheson Gas Products, Secaucus, NJ) were purchased from commercial sources.

Extraction and purification. Crepis alpina seed (19 kg) was ground in a hammer mill (W.J. Fitzpatrick Co., Troy, MI), and the oil was extracted batchwise with 15-20 L of hexane (bulk). The hexane was removed by rotary evaporator (Buchi Rotovapor 170; Buchi, Inc., Flawil, Switzerland) to yield 4.3 kg of crude oil. The crude oil was chromatographed on 1.7 kg of silica gel by eluting with 12 L of hexane to yield 2.1 kg of oil (yellow-orange color). This oil (see Table 1 for the fatty acid composition) was utilized in the large-scale deuterations. A smaller sample (30.9 g) of this oil was rechromatographed on a 7 cm × 61 cm glass column packed with 700 g silica gel. Elution with 2-4% diethyl ether (EE) in petroleum ether (PE) resulted in partial separation of triglycerides present in the *Crepis alpina* oil. The last fraction collected contained 5.1 g (> 99% pure) of tricrepenynin.

Deuteration procedure. Hexane (1 L) was added to a heat-dried (120°C) 2-L round-bottom flask, and the system was degassed by evacuation to 50 mm Hg and then flushing with deuterium gas. The evacuation-deuterium flush procedure was used a total of three times. Lindlar catalyst (8.5 g; 5% weight of substrate) and quinoline (freshly distilled; 8.5 mL) were added, and the system was degassed another three times. The slurry was stirred by magnetic stirrer under a D_2 atmosphere for 15 min, and 172.0 g of *Crepis alpina* oil was added. After the system was degassed three more times, stirring was resumed, and the D_2 uptake was monitored as described previously (12). When D_2 uptake had ceased, the slurry was filtered through a pad of Celite, and some of the hexane was removed by rotary evaporator. The solution (1.2 L total volume) was transferred with hexane to a 2-L separatory funnel and was washed with two 500-mL portions of 5% HCl and four 500-mL portions of water. The organic layer was dried over sodium sulfate for one hour, and the hexane was removed by rotary evaporator. The residue (169.2 g) was placed on a 10 cm × 85 cm glass column packed with 1200 g silica gel. Plasticizers (present in the original oil probably from shipping containers; identified by MS) were eluted with 100% PE, while 3-9% EE in PE was used to elute the oil. After evaporation of the solvent, 157 g (*ca.* 92% yield) of pale yellow oil was recovered. From 12-16 L of solvents were required to elute samples of this size. Tenox 26 (0.03%) and vitamin E (0.07%) were added as antioxidants, and the samples were stored at −25°C.

A small sample (1.6 g) of purified tricrepenynin was also reduced (35 mL hexane, 160 mg catalyst, 160 μL quinoline) with deuterium gas to yield, after purification by silica gel chromatography, 1.6 g (> 99% yield) of deuterium-labelled trilinolein (isotropic purity, 98%).

Analysis. Oil samples (before and after deuteration) were transesterified with 5% HCl in methanol, and the methyl esters were analyzed by GC (Varian 3400; 30 m × 0.25 mm SP2330 capillary column; helium as carrier gas, flame-ionization detector) and by GC/MS [30 m × 0.25

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TABLE 1

Fatty Acid Composition of Samples Before and After Deuteration with Lindlar Catalyst[a]

	Crepis alpina		Tricrepenynin	
Fatty acid	Seed oil	After deuteration	From crepis oil	Deuterated
14:0	1.2	0.9		
16:0	4.1	3.8		
18:0	1.0	1.1		
18:1	2.2	2.5		0.4
cis-9,*trans*-12-18:2	2.4	2.9		1.0
18:2	14.3	87.8	0.6	98.6[b]
Crepenynic	74.0	0.1	99.4	

[a]Methyl ester analyzed by gas chromatography (see Methods section).
[b]*cis*-9, *cis*-12-18:2-12,13-d_2.

mm Supelcowax 10 capillary column (both columns from Supelco, Inc., Bellefonte, PA); Hewlett-Packard model 5889 quadrupole mass spectrometer; positive chemical-ionization mode; isobutane as ionizing gas]. Methyl heptadecanoate (17:0) was added to samples as internal standard to confirm that fatty acid (FA) sample weights were accurate and that plasticizers and pigments, as well as other nonvolatile compounds, had been removed. Fourier transform infrared (FTIR) (Perkin-Elmer, Inc., Oak Brook, IL; Model 1750, equipped with a Model 7300 professional computer) was used in conjunction with GC and GC/MS to determine the amount of *trans* isomers present (13). A sample of the *trans* 18:2 isomer present in the *Crepis alpina* oil was isolated by HPLC [50 mm × 250 mm C18 RP column (Serva Feinbiochemica, Heidelberg, Germany; 5 micron particle size); sample size: 1.8 g; acetonitrile as solvent].

RESULTS AND DISCUSSION

The FA compositions of the oil and of a purified sample of tricrepenynin before and after deuterium atom incorporation are provided in Table 1. The *Crepis alpina* seed oil contained a *trans*-18:2 isomer, previously identified as 9-*cis*, 12-*trans*-octadecadienoic acid (14). The *trans*-18:2 isomer was present at 2.4 and 2.9%, respectively, in the oil before and after deuteration and was characterized by comparison of its GC retention time with that of an authentic *cis*-9, *trans*-12-18:2 standard, by GC/MS analysis for molecular weight and by FTIR absorption at 969 cm^{-1}. FTIR indicated the presence of a *trans* double bond.

The Lindlar-catalyzed reduction of monoacetylenic compounds was found to be highly stereospecific (>98% *cis* isomer); during our deuterations only 0.4–1.0% of the *cis*-9, *trans*-12-18:2-12,13-d_2 isomer was produced. The amount of *cis*-9, *trans*-12-18:2 isomer in the sample was therefore expected to increase only slightly during the reduction. We found this to be the case (2.4% before *vs.* 2.9% after reduction). In fact, excellent correlation is obtained if one compares the sums of the *cis*-9, *trans*-12-18:2, the *cis*-9, *cis*-12-18:2 and the crepenynate FA methyl ester percentages (Table 1) of the samples before and after deuteration (90.7% *vs.* 90.8%).

The mass analyses (deuterium distributions) for several samples of deuterated oil are presented in Table 2. The isotopic purities of *ca.* 80% for the 18:2-d_2 in the final products are due to the *ca.* 14% 18:2-d_0 present in the *Crepis alpina* oil. The presence of unlabelled 18:2 in the triglyceride containing the 18:2-d_2 does not cause a problem with the use of this material in metabolic experiments. For the purpose of these studies, the data are easily adjusted to correct for the unlabelled 18:2. After correction, the deuterium distribution of the labelled 18:2 (from the crepenynic acid component) is *ca.* 98% d_2. This is analogous to feeding a labelled fatty acid with 20% unlabelled FA added. The internal standard (17:0) data agreed with gravimetric data and indicated that the sample did not contain nonvolatile impurities that would not have been detected by GC analysis.

Normal, low-pressure silica gel chromatography can be used to isolate pure (>99%) tricrepenynin. The yield of 16.5% (see Methods section) is roughly half (50%) of the 36% tricrepenynin present in the oil after silica

TABLE 2

Mass Spectrometric Analysis of 18:2-d_2 from Samples After Deuteration[a]

	Number of deuterium atoms (%)						Average number of deuterated atoms per molecule[c]
Run #[b]	0	1	2	3	4	5	
I (175 g)	18.1	1.4	77.4	2.9	0.0	0.2	1.7 (2.0)
II (172 g)	19.0	0.0	79.7	1.1	0.0	0.2	1.6 (2.0)
III (31 g)	18.3	0.2	81.2	0.0	0.1	0.2	1.6 (2.0)
IV (1.6 g)[d]		1.4	97.6	1.0			2.0

[a]Analysis of methyl linoleate from transesterified samples of deuterated *Crepis alpina* oil.
[b]Weight of oil deuterated given in parentheses.
[c]Sum of the percentages times the number of deuterium atoms. The number in parentheses is a recalculated average and does not include the 18:2-d_0 data.
[d]Purified tricrepenynin.

purification (15). Deuteration of this material resulted in deuterium-labelled trilinolein composed of 98.6% all-*cis*-18:2-d_2, 1.0% *cis*-9, *trans*-12-18:2-d_2 and 0.4% *cis*-9- or *cis*-12-18:1-d_4. Freshly distilled quinoline was necessary to minimize over-reduction of the crepenynin to *cis*-9- or *cis*-12-18:1-d_4. When quinoline that had been stored on the shelf (or in the refrigerator) for several months was used, 10–15% over-reduction occurred. The freshly-distilled quinoline was colorless; the shelf quinoline tended to be yellow-brown to dark brown in color. Lindlar catalyst (Pd on $CaCO_3$, poisoned with lead) is heterogeneous and can thus be easily removed by filtration. It is also highly selective, resulting in >98% of the all-*cis*-18:2-12,13-d_2 isomer.

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REFERENCES

1. Emken, E.A., W.K. Rohwedder, R.O. Adlof, W.J. DeJarlais and R.M. Gulley, *Lipids 21*:589 (1986).
2. Hachey, D.L., M.R. Thomas, E.A. Emken, C. Garza, L. Brown-Booth, R.O. Adlof and P.D. Klein, *J. Lipid Res. 28*:1185 (1987).
3. Emken, E.A., R.O. Adlof, D.L. Hachey, C. Garza, M.R. Thomas and L. Brown-Booth, *J. Lipid Res. 30*:395 (1989).
4. Emken, E.A., R.O. Adlof, H. Rakoff and W.K. Rohwedder, in *Synthesis and Appliction of Isotopically Labelled Compounds*, proceedings from symposium held July 17–21, 1988, Innsbruck, Austria, edited by T.A. Baille and J.R. Jones, Elsevier Science Publishers, 1989, p. 713.
5. Emken, E.A., R.O. Adlof, H. Rakoff, W.K. Rohwedder and R.M. Gulley, *Biochem. Soc. Trans. 18*:766 (1990).
6. Emken, E.A., R.O. Adlof, H. Rakoff, W.K. Rohwedder and R.M. Gulley, *Nutrition 8*:213 (1992).
7. Rohwedder, W.K., E.A. Emken and D.J. Wolf, *Lipids 20*:303 (1985).
8. Tucker, W.P., S.B. Tove and C.R. Kepler, *J. Label. Comp. Radiopharm. 7*:11 (1971).
9. Adlof, R.O., and E.A. Emken, *Chem. Phys. Lipids 29*:3 (1981).
10. Rakoff, H., and E.A. Emken, *J. Am. Oil Chem. Soc. 60*:546 (1983).
11. Adlof, R.O., H. Rakoff and E.A. Emken, *Ibid. 68*:303 (1991).
12. Rohwedder, W.K., *Rev. Sci. Instr. 37*:1734 (1966).
13. Lanser, A.C., and E.A. Emken, *J. Am. Oil Chem. Soc. 65*:1483 (1988).
14. Gunstone, F.D., *The Lipid Handbook*, edited by F.D. Gunstone, J.L. Harwood and F.B. Padley, Chapman and Hall, London, 1986, p. 4.
15. Neff, W.E., R.O. Adlof, H. Konishi and D. Weisleder, *J. Am. Oil Chem. Soc. 70*:449 (1993).

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