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Biosynthesis of Uniformly Labeled ^{13}C - and ^{14}C -Arachidonic Acid in *Mortierella alpina*

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Abstract

Arachidonic acid (ARA) is one of the most abundant polyunsaturated fatty acids (PUFAs) in the mammalian brain. Many enzymatically- and nonenzymatically-produced metabolic products have important and potent pharmacological properties. However, uniformly isotope labeled forms of ARA are not commercially available for studying the metabolic fates of ARA. This study describes a simple and efficient protocol for the biosynthesis of U- ^{13}C -ARA from U- ^{13}C -glucose, and U- ^{14}C -ARA from U- ^{14}C -glucose by *Mortierella alpina*. The protocols yield approximately 100 nmol quantities of U- ^{13}C -ARA with an isotopic purity of 95 % from a 500 μl batch volume, and approximately 2 μCi quantities of U- ^{14}C -ARA with an apparent specific activity in excess of 1200 Ci/mole from a 250 μl batch volume.

Keywords

Mortierella alpina; arachidonic acid; carbon isotope labeling; micro-scale culture

1. Introduction

The production of arachidonic acid (ARA, 20:4 ω 6), a polyunsaturated fatty acid (PUFA), from *M. alpina* is an important industrial scale process that has been extensively studied and optimized (Shinmen et al., 1989; Bajpai et al., 1991; Dyal and Narine, 2005; Zhu et al., 2006; You et al., 2011; Ji et al., 2014a; Ji et al., 2014b; Li et al., 2015a; Li et al., 2015b). ARA is an “essential” dietary PUFA for terrestrial metazoans, who rely on single-cell oceanic organisms such as *M. alpina* as their ultimate source of PUFAs.

ARA is a biochemical precursor for enzyme-mediated synthesis of diverse eicosanoids, many of which have potent hormonal and signaling functions. It is also the source of chemically-reactive oxidative degradation products such as 4-hydroxy-2-nonenal and 4-oxo-2-nonenal when subjected to chemically-mediated (i.e. non enzymatically-mediated)

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Supplementary Data

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oxidative damage (Esterbauer et al., 1986; Schneider et al., 2001). Other products of chemically-mediated oxidative damage such as isoprostanes are relatively inert and are used as biomarkers of oxidative stress (Morrow et al., 1992; Marrow et al., 1994; Morrow et al., 1994).

Given the importance and diversity of ARA-derived biomolecules, especially in the brain where ARA is concentrated, isotope-labeled forms of ARA would be valuable for measuring turnover, for studying the metabolic fate of oxidation products, and for use as internal standards when quantifying these products by mass spectrometry. The biosynthetic production of ^{13}C -labeled ARA has been described previously, but an isotopic purity of only ~20% was achieved (Kawashima et al., 1995). The biosynthesis of a partially ^{13}C -labeled forms of the closely related PUFA, docosahexaenoic acid (DHA, 22:6 ω 3) has also been described (de Swaaf et al., 2003). However, for use as an internal standard for mass spectrometry, and to prepare ^{13}C -labeled derivatives, uniformly labeled forms of ARA are required.

A deuterated form of ARA is commercially available (*d8*-ARA, Cayman Chemical Company, Ann Arbor, MI, USA), and commonly used for mass spectrometric quantitation of ARA. However, the deuterium atoms are frequently lost during both chemical oxidation and enzymatic metabolism. Moreover, they are labile during saponification procedures. There are no ^{13}C -labeled forms of ARA commercially available at the time of this writing. A ^{14}C -labeled form with a single radiolabel in position 1 is available (Perkin-Elmer Inc., Wellesley, MA, USA), although labels in this position do not become part of some critically important degradation products (such as hydroxynonenal) that are derived from the distal carbon atoms (Schneider et al., 2001).

Therefore, procedures for the laboratory-scale preparation of ARA with a uniform distribution of carbon isotopes are of interest. This work describes the biosynthesis of $\text{U-}^{13}\text{C}$ -ARA and $\text{U-}^{14}\text{C}$ -ARA from isotope-labeled glucose on a laboratory scale, and with a level of purity that is suitable for *in vivo* use.

2. Materials and Methods

2.1. Materials

D-[U- ^{13}C]glucose (99% atom ^{13}C) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). D-[U- ^{14}C]glucose (300–360 Ci/mol, 1mCi/mL) was purchased from American Radiolabeled Chemicals (Saint Louis, MO, USA). Unlabeled ARA was purchased from Nu-Check Prep Inc. (Elysian, MN). *d8*-ARA was purchased from Cayman Chemical Company (Ann Arbor, MI). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). *Mortierella alpina* (ATCC 32222) was purchased from the American Type Culture Collection (Manassas, VA, USA). Glassware and pipette tips were sterilized by autoclave. All manipulations were performed in a biosafety hood using sterile technique.

2.2. Media

Media salt concentrations were adapted from Zhu et al. Seven stock solutions were prepared and mixed to yield the media indicated in Table 1. Solution A was 2gm of K_2HPO_4 , 1gm of

MgSO₄•7H₂O, and 1 gm KCl in 1000 ml water. Solution B1 was 2 gm of Na₂NO₃ in 50 ml water. Solution B2 was 0.25 gm of Na₂NO₃ in 50 ml water. Solution C1 was 20 gm of ordinary D-glucose in 100 ml water. Solution C2 was 2 gm of U-¹³C-glucose in 100 ml water. Solution D was 25 gm of CaCl₂ in 50 ml water. Solution E was 50 mg of FeCl₃, 1 gm of H₃BO₃, 150 mg of MnCl₂•4H₂O, 10 mg of ZnCl₂, and 5 mg of CoCl₂•H₂O in 100 ml water. All water was Millipore analytical grade. The pH of stock solutions A, C1, and C2 was adjusted to 7.0 with 1M NaOH or 1M HCl as needed. Stock solution E was sterilized by filtration through 0.22 µm Millex® syringe filters. All stock solutions were stored in the dark at 4°C. *M. alpina* stocks were kept in maintenance media (table 1) at ambient temperature and light. Inoculating suspensions were prepared by adding sterile glass beads to a 7-day-old culture and vortexing for 1–2 min to pulverize the mycelial mass. Subcultures were made by adding 200 µl of the inoculating suspension to 5 mL of maintenance media.

2.3. Glucose consumption and growth rates

Glucose concentrations in various media were measured after centrifugation for 15 min at 1600 × g with the DNS method (Miller, 1959). The DNS reagent consisted of 0.5 gm 3,5-dinitrosalicylic acid, 0.05 gm Na₂SO₃, and 0.5 gm NaOH in 50 ml of water. An equal volume of DNS reagent (300 µl) was mixed with an equal volume of the solution to be assayed (typically 300 µl) and incubated at 90 °C for 10 min. Following the incubation, 600 µl of quencher was added (2 gm of sodium potassium tartrate, NaKC₄H₄O₆•4H₂O in 50 ml water) and samples were cooled to room temperature. The absorbance at 540 nm was recorded and compared to a standard curve to determine concentrations. A concentration of 0.8 g/L in the medium typically yielded an absorbance of 1.10 AU at 540 nm, and the absorbance was proportional to concentration down to 0.2 g/L. Cultures starting with 5 g/L glucose were diluted 6× before the assay.

To measure growth rates, cultures were centrifuged for 15 minutes at 1600 × g, and the supernatant was discarded. The pellet was re-suspended in 1 or 2 mL of deionized water for cultures with initial volumes of 250 µl or 500 µl, respectively. Intact cells were evenly suspended by adding sterile glass beads to the solution and vortexing for 2 min to pulverize the mycelial mass. OD600 was measured on a Cary 400 Bio UV-vis spectrophotometer (Agilent, Santa Clara, CA) in lieu of cell dry weights so that growth could be monitored while the organisms were growing, and to avoid drying of the organisms which complicates PUFA extraction.

2.4. Lipid extraction and saponification

Lipid extractions were performed with the Bligh-Dyer (BD) method (Bligh and Dyer, 1959) modified by substituting dichloromethane for chloroform. Fungal cultures in 13×100 mm glass tubes were centrifuged for 15 min at 1600 × g, the supernatant was removed, and the mycelial mass was resuspended in 640 µL of water. This suspension was subjected to 3 freeze-thaw cycles (liquid nitrogen alternating with boiling water). 1600 µL of methanol and 800 µL of dichloromethane were then added and the mixture was tip-sonicated for 90 sec on ice with a model F60 sonic dismembrator (Fisher Scientific, Pittsburgh, PA). 800 µL of dichloromethane and 640 µL of water were then added to create separate phases, which were clarified by centrifugation at 110 × g for one minute. The lower (dichloromethane) phase

(~1.6 mL) was withdrawn, transferred to a new 13×100 mm glass tube, dried under argon, and saponified in 85% methanol in water with 0.5 M NaOH (2 mL final volume) at 85°C for 1 hour. Following saponification, samples were cooled to room temperature, acidified with 400 µL of 5M HCl, and extracted with 1 mL of isooctane three times. The three upper (isooctane) phases were combined in a Teflon tube, and evaporated under Argon gas. The dried residue was dissolved in 100 µL of ethanol and stored under argon at –80°C.

2.5. HPLC separation and mass spectrometry parameters

HPLC separations were performed with a 4.6 × 150 mm XDB-C18 column (Agilent, Santa Clara, CA) and a mobile phase pumped at 0.50 mL/min. Solvent A was 60% acetonitrile and 40% H₂O by volume, with 0.1% formic acid added; solvent B was 100% acetonitrile to which 0.1% formic acid was added. The gradient program was 40% B (0–10 min), 40–100% B (10–40 min), and 100% B (40–50 min), before returning to 40% B.

Non-radioactive extracts were analyzed on an ABI 4000 mass spectrometer (Sciex, Toronto, Canada) operating in negative ion multiple reaction monitoring (MRM) or Q1 mode. The MRM transitions monitored were m/z 303→259 for ¹²C-ARA with the neutral loss of CO₂, 323→279 for U-¹³C-ARA, and 311→267 for *d*₈-ARA.

NH₄OH (0.15 M) in 1:1 methanol: water at 100 µL/min was introduced into the post-column effluent via tee junction prior to the MS analysis to deprotonate the fatty acids. The DP was –75 V for both modes; the CAD was 7 psi and the CE was –20 V for MRM.

Both radioactive and non-radioactive extracts were collected in 30 second fractions. A small portion of each radioactive fraction was analyzed by scintillation counter to verify the location of peaks. The fractions corresponding to the elution time for ARA were collected, dried in a Teflon tube, reconstituted in ethanol, purged with Argon gas, and stored at –80°C.

2.6. Calculations

2.6.1. Isotopic Purity—ARA purified from fungal extracts eluted as a single peak with an m/z of 303 to 323, depending on the number of ¹³C atoms in the molecule. The isotopic purity (P_{iso}) of ¹³C-labeled ARA was calculated using Equation 1, where f_i is the integrated area of the peak at $m/z = i$.

$$P_{iso} = \frac{\sum_{303}^{323} \left(\frac{i-303}{20} \right) f_i}{\sum_{303}^{323} f_i} \quad \text{Equation 1}$$

2.6.2. Conversion Efficiency—A sample of *d*₈-ARA was calibrated with known concentrations of unlabeled ARA by MRM mass spectrometry, and aliquots were added to extracts to quantify the ¹³C-ARA recovered from each culture. For extracts of cultures grown in ¹³C-glucose, the efficiency of ¹³C conversion into ARA (i.e. the conversion efficiency) is given by

$$E = \frac{\text{moles U-}^{13}\text{C- ARA} * 20}{\text{moles U-}^{13}\text{C- glucose} * 6 * P_{iso}} \quad \text{Equation 2}$$

2.6.3. Specific Activity— ^{14}C -ARA purified from fungal extracts was quantified by scintillation counting. To estimate the mass of the radiolabeled material, U- ^{13}C -ARA was produced under the same conditions and quantified by MRM mass spectrometry. The specific activity of ^{14}C -ARA was then estimated using Equation 3, where A is the total activity of ^{14}C -ARA.

$$SA = \frac{A * P_{iso}}{\text{moles U-}^{13}\text{C- ARA}} \quad \text{Equation 3}$$

3. Results and Discussion

3.1. ^{13}C -ARA production

M. alpina did not grow, consume glucose, or yield ^{13}C -labeled ARA when cultivated in U- ^{13}C -glucose without nitrogen (data not shown). ARA yield has been reported to be optimal when the C/N ratio in the media is between 15–20 (Koike et al., 2001). Therefore, the media used in these experiments was made with a C/N ratio of 17.5.

In 500 μl batches of media containing 5 gm/L U- ^{13}C -glucose, glucose concentration declined almost linearly, reaching a minimum after 5 days (Fig. 1a). Fungal growth was first detected by OD600 on day 3, and continued almost linearly through day 7. The U- ^{13}C -ARA yield was measured by MRM mass spectrometry after adding a calibrated aliquot of *d8*-ARA to the final purified fraction. The yield reached a maximum on day 5 (Fig. 1b). The colony was a single white mass composed of mycelia with visible oil droplets.

Isotopic purity also reached a maximum of 95% on day 5, and then declined slightly. Extracted U- ^{13}C -ARA eluted between 25.0 and 26.5 minutes and a Q1 mass spectrum of this peak revealed quantifiable signals at *m/z* ratios ranging from 319–323, corresponding to ARA with 16–20 ^{13}C labels. U- ^{12}C -ARA (*m/z*=303) was not detected. Isotopically pure ^{13}C -ARA (U- ^{13}C -ARA, *m/z*=323) constituted 38% of the purified material. From three separate cultures, each containing 14 μmol of U- ^{13}C -glucose, an average of 110 ± 20 nmol of U- ^{13}C -ARA was recovered for a conversion efficiency of $2.8 \pm 0.6\%$ (Equation 2).

The isotopic purity of U- ^{13}C -ARA is important because the most sensitive approach to mass spectrometric quantitation (MRM), relies on complete isotope substitution in both parent ion and collision-induced fragments. The production of U- ^{13}C -ARA from U- ^{13}C -glucose has been reported previously (Kawashima et al., 1995). The yields in that work were higher than reported herein (14 % vs 2.8 % in this work). However, the isotopic purity that material was lower (78–83%), and the yield was estimated for the transesterified methyl ester. Yields would have probably been lower if the saponified product had been assayed.

Isotopic purity was maximized in the current experiments by omitting various ^{12}C sources in the growth media, such as yeast extract, and substituting an inorganic source of nitrogen. The stated isotopic purity of the commercially-prepared $\text{U-}^{13}\text{C}$ -glucose was 99%, although our measurements and calculations according to Equation 1 indicated it to be 97% (data not shown). Ultimately, the isotopic purity of recovered ARA was 95%.

3.2. ^{14}C -ARA production

$\text{U-}^{14}\text{C}$ -glucose is supplied with a maximum specific activity of 360 Ci/mole, and purchased in lots of 5 mCi. Therefore, one lot of $\text{U-}^{14}\text{C}$ -glucose, diluted into 25 ml of media, yielded sufficient complete media for 100 batches of 250 μl , each containing 50 μCi of ^{14}C -glucose and a maximum glucose concentration of 0.1 gm/L.

In this media, ^{14}C activity remaining in the supernatant after centrifugation declined almost linearly, reaching a minimum of $4.2 \pm 0.5 \mu\text{Ci}$ on day 4 (Fig. 2a), suggesting slightly faster glucose consumption than in the 5 gm/L cultures described above. OD600 estimates of fungal growth increased almost linearly for 3 days, reaching an apparent plateau between 3–5 days. ^{14}C activity recovered as ARA reached a maximum of $2.3 \pm 0.2 \mu\text{Ci}$ per batch on day 4 (Fig. 2b), for a conversion efficiency of $4.6 \pm 0.4 \%$. It is not surprising that the conversion efficiency in these experiments with 0.1 gm/L glucose in the media is higher than the efficiency in experiments with 5 gm/L glucose, given prior reports about efficiency and yield in the literature (Bajpai et al., 1991), although the concentrations of other media components also differed in our experiments.

To estimate the isotopic purity and specific activity of the radiolabeled material when its mass could not be measured, $\text{U-}^{13}\text{C}$ -ARA was produced under the same conditions (i.e. after 4 days in media containing 0.1 gm/L $\text{U-}^{13}\text{C}$ -glucose) and examined by Q1 and MRM mass spectrometry after adding a calibrated aliquot of *d8*-ARA. The Q1 scan revealed that the ARA contained 16–20 atoms of ^{13}C ($m/z = 319\text{--}323$) for an isotopic purity of 93% (Equation 1). There was $1.9 \pm 0.2 \text{ nmol}$ of $\text{U-}^{13}\text{C}$ -ARA recovered, corresponding to a 5.0% conversion efficiency. This efficiency (with 0.1 gm/L glucose in the media) is 1.8-fold greater than the efficiency reported above with 5 gm/L glucose, which may be attributed to metabolic shifts in the organism under nutrient-limited conditions. The yield suggests that the specific activity of ^{14}C -ARA may have been as high as 1210 Ci/mol (Equation 3), which is near the theoretical maximum of 62.4 Ci/mol/carbon for a 20-carbon molecule (i.e. 1248 Ci/mol). To the extent that the specific activity of $\text{U-}^{14}\text{C}$ -glucose is less than 360 Ci/mol, of course, the estimated specific activity for ^{14}C -ARA will be lower.

In addition to ARA, six other fatty acids were identified in fungal extracts by Q1 mass spectrometry and associated with measurable ^{14}C activity: linolenic acid (18:3 ω 3), linoleic acid (18:2 ω 6), eicosapentaenoic acid (20:5 ω 3), palmitic acid (16:0), oleic acid (18:1), and stearic acid (18:0) (Table 2). Notably, there was no docosahexanoic acid (22:5 ω 3) detected. The total activity recovered in these fractions was $2.0 \pm 0.2 \mu\text{Ci}$, making the overall conversion efficiency for $\text{U-}^{14}\text{C}$ -glucose into fatty acids equal to 8.6 %, of which 53 % was ARA. The latter result is similar to the fraction of ARA in total fatty acids reported by Koike (~40–50%) for a C/N range of 15–20 in flask cultures (Koike et al., 2001).

4. Conclusion

$U\text{-}^{13}\text{C}$ -ARA and $U\text{-}^{14}\text{C}$ -ARA can be produced efficiently from isotope-labeled glucose on a laboratory scale. The use of low glucose concentrations in the cultivation media facilitates efficient conversion of the carbon atoms in glucose into carbon atoms in ARA. Upon separation by HPLC, the free fatty acid form of ARA may be purified in a form that is suitable for *in vivo* (intracerebroventricular) injection (Furman et al., 2016). In addition, smaller quantities of 6 other uniformly-labeled free fatty acids are obtained.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Procedures for the laboratory-scale preparation of U-¹³C-ARA and U-¹⁴C-ARA are described.
- The yield of U-¹³C-ARA was >100 nmole with an isotopic purity of 95%
- The yield of U-¹⁴C-ARA was > 2 µCi with an estimated specific activity of >1200 Ci/mol
- Conversion efficiencies from glucose to ARA were 4–5%
- Smaller quantities of other isotope-labeled fatty acids were also obtained.

Fig. 1. Production in 5 gm/L ¹³C-glucose media. Averages of three separate batches ± standard deviations, (a) glucose consumption and growth curve, (b) U-¹³C-ARA yield and % ¹³C labels in all of recovered isotopes.

Fig. 2. Production in 0.1 gm/L ^{14}C -glucose media. Averages of three separate batches $\pm$ standard deviations, (a) glucose consumption and growth curves, (b) ^{14}C activity in ARA and % ^{14}C Activity ARA activity in total recovered activity.

Table 1

Media compositions

Stock Solution	Maintenance	5 gm/L ¹³ C-glucose	0.1 gm/L ¹² C-glucose	0.1 gm/L ¹³ C-glucose	0.1 gm/L ¹⁴ C-glucose
A	500 ml	5 ml	5 ml	5 ml	5 ml
B1	16 ml	160 µl	–	–	–
B2	–	–	32 µl	32 µl	32 µl
C1	250 ml	2.5 ml	50 µl	–	–
C2	–	–	–	50 µl	–
U- ¹⁴ C-glucose	–	–	–	–	2 mCi
D	5 ml	50 µl	50 µl	50 µl	50 µl
E	1 ml	10 µl	10 µl	10 µl	10 µl
Final Volume	1000 ml	10 ml	10 ml	10 ml	10 ml

Key Concentrations					
Glucose (mM)	28	28	0.55	0.55	0.55
	9.5	9.5	0.19	0.19	0.19
NO ₃ ⁻ (mM)					
C:N ratio	17.7	17.7	17.4	17.4	17.4

Table 2

Distribution of ^{14}C Activity in the Fatty Acids Produced from $[\text{U-}^{14}\text{C}]\text{glucose}$.

	Linolenic Acid	Arachidonic Acid	Linoleic Acid	Eicosapentaenoic Acid	Palmitic Acid	Oleic Acid	Stearic Acid
Activity (μCi) ^a	0.09 $\pm$ 0.02	2.29 $\pm$ 0.16	0.26 $\pm$ 0.13	0.14 $\pm$ 0.02	0.69 $\pm$ 0.30	0.50 $\pm$ 0.07	0.35 $\pm$ 0.02
Conversion (%)	0.18	4.6	0.51	0.28	1.4	1.0	0.70
Elution time (min)	21.82	25.77	27.56	30.38	34.02	35.19	43.55
<i>m/z</i> ratio	277	303	279	305	255	281	283

^aFatty acids extracted and purified from *M. alpina* after 4 days of growth in 0.1 gm/L ^{14}C -glucose media. Averages of three separate batches $\pm$ standard deviations.