Synthesis of isotopically labeled saturated fatty acids

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Abstract An approach to the synthesis of isotopically labeled saturated fatty acids is outlined which is based on the copper-catalyzed coupling of an ω-bromo acid with an isotopically labeled Grignard reagent. The method provides high yields of pure products and offers considerable flexibility in the type of isotopically enriched compound that can be prepared.— **DasGupta, S. K., D. M. Rice, and R. G. Griffin.** Synthesis of isotopically labeled saturated fatty acids. *J. Lipid Res.* 1982. **23:** 197–200.
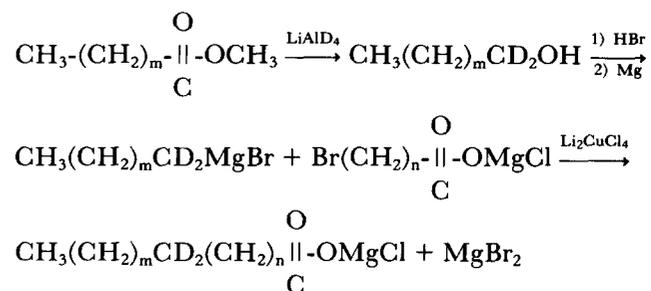
Isotopically labeled fatty acids are of interest for a number of different types of investigations. For example, tritium- and ^{14}C-labeled compounds have been employed extensively in radioactive tracer studies for many years. More recently, fatty acids, which have been enriched with stable isotopes (^{2}H and ^{13}C) and subsequently incorporated into phospholipids and glycolipids, have found extensive application in NMR, Raman, and neutron diffraction studies (1–3). In the first two cases, spectral lines from specifically labeled sites can be studied without undue interference from background signals, and in the last case, the differential scattering cross section between ^{1}H and ^{2}H permits accurate location of ^{2}H labels in a lipid bilayer.

Several methods for preparation of isotopically enriched fatty acids have been described and these have been recently reviewed (4). The two most general methods of synthesis (in the sense that they allow placement of ^{2}H or ^{13}C at an arbitrary point in a fatty acid chain) involve Kolbe anodic coupling or reduction of keto acids. The former method is known for its low yields and for by-products that are difficult to separate from the desired product (5), while the latter method is somewhat consumptive of deuterium. For example, in one scheme the keto acid is first reduced to a hydroxy acid with NaBD₄, converted to the tosylate, and then reduced again with LiAlD₄. The result is a tetradeuterated alcohol which must be reoxidized to the desired gem [^{2}H₂] fatty acid

(6). The tetradeuterated alcohol may be avoided by performing the reduction of the tosylate with NaBD₃CN, but a fourfold excess of the deuterated cyanoborohydride is required for good yields (7, 8). In this communication we describe an alternative approach to synthesis of isotopically enriched fatty acids which we have used extensively for preparation of ^{2}H-labeled compounds. The method is based on the copper-catalyzed coupling of a labeled Grignard reagent with the MgCl salt of an ω-bromoacid to produce the compound of interest (9). The advantages of the method are: *1*) the yields in the synthetic scheme are reasonably high in all steps, *2*) it offers considerable flexibility in the placement of ^{2}H or ^{13}C at an arbitrary point in the chain, and *3*) the starting materials are readily available. To date we have used the method exclusively for synthesis of saturated fatty acids, but it should also be applicable to unsaturated compounds. Although the coupling reaction (9), which is the basis for the synthetic scheme, was described some time ago, it does not seem to be widely known. For example, a discussion of it was omitted from the recent review referenced above.

The synthetic scheme is shown below and consists of three steps: *1*) preparation of ^{2}H-labeled alcohol, *2*) conversion of the alcohol to a bromide and subsequently to a Grignard reagent, and *3*) the copper-catalyzed coupling of the Grignard with the MgCl salt of an ω-bromo acid.

$$CH_3\text{-}(CH_2)_m\text{-}\overset{\displaystyle O}{\underset{\displaystyle C}{\|}}\text{-}OCH_3 \xrightarrow{\text{LiAlD}_4} CH_3(CH_2)_m CD_2OH \xrightarrow[\text{2) Mg}]{\text{1) HBr}}$$

$$CH_3(CH_2)_m CD_2MgBr + Br(CH_2)_n\text{-}\overset{\displaystyle O}{\underset{\displaystyle C}{\|}}\text{-}OMgCl \xrightarrow{\text{Li}_2\text{CuCl}_4}$$

$$CH_3(CH_2)_m CD_2(CH_2)_n \overset{\displaystyle O}{\underset{\displaystyle C}{\|}}\text{-}OMgCl + MgBr_2$$

The metal deuteride reduction used in the first step insures a high level of incorporation of ^{2}H into the final product. The analogous step in the Kolbe scheme generally involves deuterium exchange to produce an α-deuterated compound that is not always fully labeled. The

alcohol is next converted to a bromide and this may be accomplished by a number of standard procedures—HBr, PBr$_3$, etc. In the final step, the deuterated Grignard formed from the bromide is coupled with the MgCl salt of an ω-bromo acid to yield the desired product. The coupling reaction is based on the differential reactivity of Grignard reagents towards carboxylate anions and alkyl bromides. In particular, at low temperatures, Grignards react slowly with such anions, but they readily undergo copper-catalyzed coupling with alkyl bromides. This property, together with the high solubility of MgCl salts of ω-bromo acids in tetrahydrofuran at low temperatures, is the basis for the preparation of isotopically labeled fatty acids.

As mentioned above, we have used this method to prepare several specifically deuterated saturated fatty acids (primarily C16:0 acids but also C14:0 and C24:0 compounds) and the yield of the coupling step has generally been about 85%. However, we have noted that the reaction does work slightly better with relatively long chain deuterated Grignards and ω-bromo acids. Thus, in the synthesis of C16:0-4,4-d$_2$ from η-tridecyl magnesium bromide and ω-bromopropionic acid we obtained a 77% rather than an approximately 85% yield from the coupling step. This is a point noted by Baer and Carney (9) and for this reason the most efficient route to a 3,3-d$_2$ saturated fatty acid is via a malonic ester condensation (10) rather than a coupling with bromoacetic acid. Below we give a detailed procedure for the synthesis of C16:0-7,7-d$_2$.

Finally, there are three other practical points worth noting. First, in order to avoid the presence of impurities in the final product, it is important to have concentrations of the Grignards as nearly equal as possible. Thus, it is important to perform the titration with 1,10-phenanthroline mentioned below. Second, the reaction cannot be employed to prepare 5,5-d$_2$ compounds since the MgCl salt of ω-bromobutyric acid cyclizes to form the lactone.[1] This deficiency can be circumvented by using another organocopper (I)-ate coupling due to Bergbreiter and Whitesides (11) or two sequential malonic ester condensations. Third, it should be mentioned that this approach does offer considerable flexibility in the type of isotopically enriched compound that may be prepared. For example, a monodeuterated or tritiated fatty acid is available by reducing an aldehyde with NaBD$_4$ (or NaBT$_4$) in the first step of the scheme. Similarly, a [13]C- or ^{14}C-labeled fatty acid can be obtained by reducing a [13]C- or ^{14}C-labeled methyl ester with LiAlH$_4$.

In summary, we have outlined a scheme for synthesis of isotopically labeled saturated fatty acids. The method is straightforward and has provided high yields of pure

products in every case we have tried. Finally, it offers considerable flexibility in the type of isotopically enriched compound that can be prepared.

EXPERIMENTAL PROCEDURES AND RESULTS

Materials and methods

Methyl decanoate and 6-bromohexanoic acid were purchased from Aldrich Chemicals. The latter was dried under vacuum òver phosphorus pentoxide before use. Methyl-magnesium chloride (2.7 M) in tetrahydrofuran was purchased from Alfa Inorganics, Inc. Its concentration, and Grignard concentrations in general, were determined by the method of Watson and Eastham (12) using charged transfer complex with 1,10-phenanthroline (Frederick Smith Chemical Co.) as an indicator. Prior to use, tetrahydrofuran was distilled from lithium aluminium hydride and stored over molecular sieves, Linde type 5A. Lithium aluminium deuteride was purchased from Stohler or Merck and Co. Cupric chloride and lithium chloride, both of reagent grade, were extensively desiccated under vacuum over phosphorus pentoxide before use.

Thin-layer chromatography (TLC) was performed on Merck silica gel plates. Fatty acids and their esters were developed using a solvent system of petroleum ether (bp 60–80°C)–diethyl ether–acetic acid 8:2:0.1 (v/v) and petroleum ether–acetone 50:0.7 (v/v), respectively. Components on the plates were detected as fluorescent bands under ultraviolet light after spraying with a solution of Rhodamine 6G in acetone, or else they were visualized by spraying with a 5% solution of phosphomolybdic acid in methanol followed by baking on a hot plate at 200°C for 5 min.

Synthesis of (7,7-d$_2$) palmitic acid

(1,1-d$_2$) Decanol. Methyl decanoate (37.2 g, 200 mmol) diluted with 150 ml of dry ether (distilled over CaH$_2$) was added slowly under nitrogen to a chilled slurry of lithium aluminum deuteride (8.4 g, 200 mmol) in dry ether (200 ml). It was gently stirred for 10 hr at room temperature. The reaction mixture was chilled, stirred, and quenched by careful addition of Na$_2$SO$_4$·10 H$_2$O in small portions until there was no further evolution of gas. Finally, water (4 ml) was added. The mixture was filtered and the residue was washed with additional ether (3 × 50 ml). The combined filtrate was evaporated to dryness. The residue was distilled under reduced pressure, bp 120–121°C/12 mm (lit. 120°C/12 mm) (13) giving 29 g of (1,1-d$_2$) 1-decanol.

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[1] Baer, T. A. Private communication.

(1,1-d₂) *1-Bromodecane.* Following the procedure described in Organic Synthesis (14), (1,1-d₂) decanol (29 g, 181 mmol), heated to 100°C, was treated with a current of anhydrous hydrogen bromide until absorption ceased. The reaction mixture was cooled and extracted with ether (100 ml). The ether extract was thoroughly washed with water, a saturated solution of sodium bicarbonate solution (100 ml), and with water again, and was dried over anhydrous Na₂SO₄. Evaporation of the solvent yielded an oil which was dissolved in hexane (50 ml). It was filtered through a short column of silica gel-60 (70–230 mesh, 20 g). The column was further washed with an additional 300 ml of hexane. The combined hexane eluate was evaporated under reduced pressure to dryness. The residue was distilled to give (1,1-d₂) 1-bromodecane, bp 150°C/20 mm (lit. 103°C/6 mm) (15) weighing 34 g (yield 85%). Thin-layer chromatography (solvent, pentane–hexane) showed a single component that matched with bromodecane.

(1,1-d₂) *Decyl magnesium bromide (Grignard reagent).* A 300-ml three-necked flask, fitted with a condenser and a 150-ml dropping funnel, was flame-dried and then cooled under a pre-purified nitrogen atmosphere. It was charged with magnesium turnings for Grignard (1.6 g, 64 mmol) and covered with 10 ml of dry tetrahydrofuran. A trace of iodine was added. (1,1-d₂) 1-Bromodecane (15 g, 64 mmol) dissolved in dry tetrahydrofuran (30 ml) was added dropwise under nitrogen and stirring conditions until the reaction started. This was indicated by a quick discharge of the color of iodine. The rate of addition was then increased and the vigor of the reaction, as evidenced by the gentle boiling of the solvent, was maintained by a controlled addition of the bromide solution. After the addition was complete (1–2 hr), most of the magnesium was found to be in solution. The mixture was further heated at gentle reflux for an additional 15 min. The Grignard reagent was then carefully transferred under nitrogen with a syringe to a measuring flask, diluted to 50 ml, and stored under nitrogen. A careful titration under nitrogen of an aliquot (1 ml diluted with 3 ml of dry tetrahydrofuran) with a freshly prepared primary standard solution (0.1 M) of dry isopropanol (reagent grade, distilled over sodium) in dry xylene (reagent grade), using a very small crystal of 1,10-phenanthroline as an indicator, determined the concentration of the Grignard reagent (yield 62 mmol).

Coupling of the Grignard reagent with 6-bromohexanoic acid: (7,7-d₂) palmitic acid. A 300-ml three-necked flask containing a solution of 6-bromohexanoic acid (11.7 g, 60 mmol) in dry tetrahydrofuran (70 ml) was fitted with a dropping funnel, a magnetic stirrer, and an inlet for dry nitrogen. The following reaction was carried out under nitrogen atmosphere. The flask was

cooled to −30°C (liquid nitrogen and ethylene glycol bath). Methyl magnesium chloride (61 mmol; titrated as described earlier) dissolved in dry tetrahydrofuran was added carefully. After the gas (CH₄) evolution stopped, a solution of dilithium tetrachlorocuporate (1.2 mmol, prepared by dissolving 0.341 g of dry CuCl₂ and 0.170 g of dry LiCl in 10 ml of dry tetrahydrofuran) was added quickly from a syringe. The color of the reaction mixture changed from an intense blue to a light orange. The Grignard reagent earlier described (63 mmol) was carefully transferred under nitrogen to the dropping funnel, added with stirring to the cooled solution at −30°C over a period of 30–45 min, and stirred for an additional hour in the cold. It was then left at room temperature for 10 hr, when the solution became dark blue.

The reaction was quenched with a chilled solution of dilute sulfuric acid (5%), and, after dilution with another 200 ml of cold water, it was extracted with ether (3 × 100 ml). The combined ether extract was extensively washed with a saturated solution of NaCl and then with water again; the solvent was evaporated to dryness. The semisolid residue was dissolved in ether (200 ml) and washed with a chilled solution of potassium hydroxide (5%) in water (4 × 100 ml). The combined aqueous extract was carefully acidified in the cold giving the crude 7,7-d₂ palmitic acid, which, after filtering and air drying, weighed 15 g (crude yield 94%). Thin-layer chromatography of the above sample, on comparison with that of an authentic sample of palmitic acid, showed it was contaminated with a trace of another closely following component, presumably 6-bromohexanoic acid.

Purification of (7,7-d₂) palmitic acid. The crude (7,7-d₂) palmitic acid (5 g) was converted to its methyl ester either by the standard procedure of esterification with anhydrous methanol in the presence of sulfuric acid or by a treatment with a solution of diazomethane in ether. The yield was better than 95%. The crude methyl ester was filtered through a silica gel-60 (70–230 mesh, 40 g) column and the column was washed with 500 ml of a solution of hexane–benzene 85:15. The eluate was evaporated to dryness giving the crude methyl ester (single spot on TLC corresponding to methyl palmitate). It was hydrolyzed with a 10% methanolic potassium hydroxide (200 ml) by heating at reflux for 5 hr. The mixture was cooled, diluted with 250 ml of cold water, and carefully acidified in the cold with minimum amount of acid. The (7,7-d₂) palmitic acid crystallized in the cold; it was filtered and, after air drying, it weighed 4.2 g. The recovery was 82% over the two steps. Thin-layer chromatography showed a single spot having the same R_f value as that of an authentic specimen of palmitic acid. The overall yield of the final purified acid from methyl decanoate, involving four steps, was 58%.∎

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