JOURNAL OF LABELLED COMPOUNDS AND RADIOPHARMACEUTICALS
J Label Compd Radiopharm 2006; **49**: 675–682.
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jlcr.1094

Research Article

An expeditious procedure for the synthesis of isotopically labelled fatty acids: preparation of 2,2-d_2-nonadecanoic acid

Annalisa Guaragna[1,*], Mauro De Nisco[1], Silvana Pedatella[1], Vittoria Pinto[1,2] and Giovanni Palumbo[1]

[1] *Dipartimento di Chimica Organica e Biochimica, Università di Napoli Federico II, Via Cynthia 4, I-80126 Napoli, Italy*

[2] *Chiron Vaccines, Via Fiorentina 1 Research Center, Bldg 35 I-53100 Siena, Italy*

Summary

We describe a new synthetic path for the preparation of isotopically labelled saturated odd-numbered fatty acids by a homologation procedure for the direct conversion of carboxylic acids into their 2,2-dideuterated homologues. This process can be used to obtain both odd- and even-numbered 2,2-dideuterated fatty acids starting from carboxylic acids. Furthermore, the reiteration of this process can also be used for the synthesis of polydeuterated fatty acids in which the heavy atoms are placed on adjacent carbon atoms or differently arranged in the molecule. Copyright © 2006 John Wiley & Sons, Ltd.

Received 9 May 2006; Revised 13 May 2006; Accepted 13 May 2006

Key Words: deuterium; isotopically labelled; deuterated fatty acids

Introduction

There is a constantly increasing demand for the synthesis of commercially unavailable labelled starting materials, for use in studies investigating several major topics, such as the metabolic pathways of drugs and bioactive molecules in biological media,[1] or the order and dynamics of liquid crystals in their different mesophases.[1,2] Isotopically labelled fatty acids are of interest as biological tracers in various different types of investigation.[3] Indeed, ^{2}H and ^{13}C enriched fatty acids have been incorporated into phospholipids and

*Correspondence to: Annalisa Guaragna, Dipartimento di Chimica Organica e Biochimica, Università di Napoli Federico II, Via Cynthia 4, I- 80126 Napoli, Italy. E-mail: guaragna@unina.it

glycolipids with extensive applications in NMR, Raman, and neutron diffraction studies.[4,5] However, preparation of deuterated lipids has involved much more extensive chemical investigations than ^{13}C labelled lipids, due to the more numerous ways to introduce deuterium. Several syntheses of deuterated lipids have been made, for use in studies of fragmentation in mass spectrometry (MS).[6–8] Basically, only two methods for the introduction of deuterium into organic molecules are known: exchange and reduction.[9–11] More methods are available for the synthesis of deuterium labelled lipids, besides those just mentioned, including the use of chemical reactions to connect labelled segments to the non-labelled part of the molecule,[11,12] and biosynthesis.[13]

Over recent years particular attention has been devoted to the study of odd-numbered fatty acids, due to their presence in human tissues, in a concentration generally not exceeding 5% of total fatty acids.[14] Since the normal synthetic pathway for fatty acids leads to only even numbers of carbon atoms in the products, it may seem curious to find any odd-numbered chains present in human tissues. Fatty acids with odd numbers of carbon atoms are produced primarily by initiating the synthetic route with the three carbon compound, propionic acid. Vitamin B_{12} (cobalamin) is required for the conversion of propionate into succinate for oxidation in the central energy pathways.[15] As a consequence, deficiency of vitamin B_{12} leads to accumulation of propionate and subsequent build up of the odd-numbered fatty acids. The association between vitamin B_{12} and abnormal fatty acid synthesis provides a rationale for the neuropathy of cobalamin deficiency.[16,17] Vitamin B_{12} plays an important role in DNA synthesis and neurologic functions. Cobalamin deficiency can lead to a wide spectrum of haematologic and neuropsychiatric disorders that can often be reversed by early diagnosis and prompt treatment. Diagnosis of vitamin B_{12} deficiency is typically based on measurement of serum vitamin B_{12} levels; however, about 50% of patients with subclinical disease have normal B_{12} levels.[18,19] The detection of low concentrations of odd-numbered fatty acids could be a useful instrument to study pathologies associated with cobalamin deficiency. Usually, stable isotope tracers associated with mass spectrometry techniques are used to follow and quantify the biosynthesis and metabolism of fatty acids.

Results and discussion

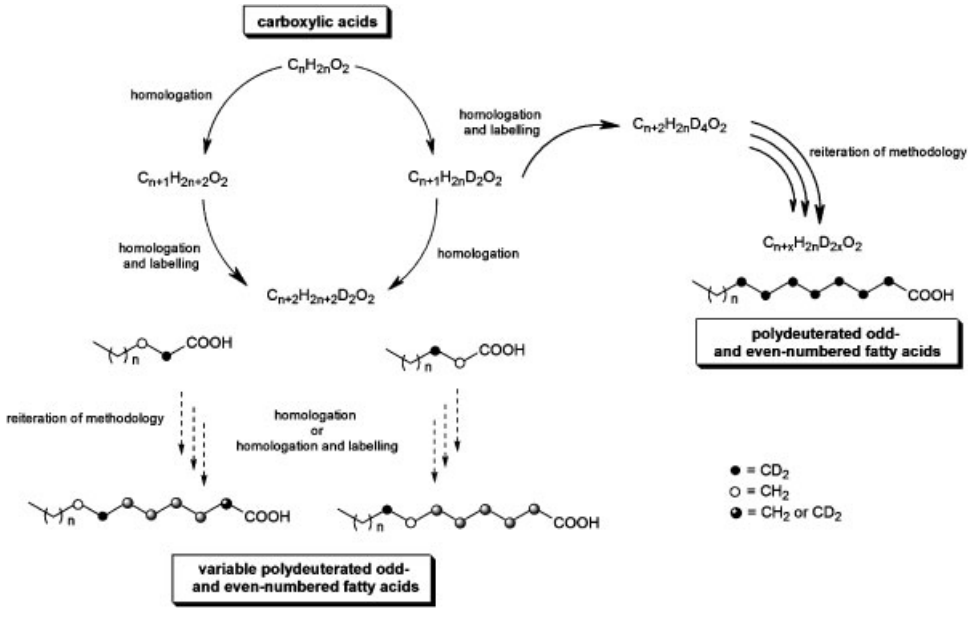
We report a new methodology for the synthesis of 2,2-dideuterated saturated odd-numbered fatty acids, which can also be applied to even-numbered fatty acids. The early literature[20] of deuterium chemistry features a large number of papers on the exchange behaviour H/D of carboxylic acids. Kinetic studies were most common, followed by combustion analysis, and neither enabled exchange to be definitively located in such compounds. Recently, there has

DOI: 10.1002/jlcr

been renewed interest in the liability of the α-protons of carboxylic acids and early exchange[21] reactions with D_2O. The latter make possible a one-step preparation of a wide variety of α-deutero carboxylic acids. Additional steps intended to introduce the second deuterium atom into α-position have proved to be more difficult and low yielding. These syntheses are in fact quite complex and not generally available on the large scale.

We have devised a new process for the direct conversion of carboxylic acids into their 2,2-dideuterated homologues under smooth, clean, quick conditions and on the large scale. This process can be used to obtain both odd- and even-numbered 2,2-dideuterated fatty acids starting from carboxylic acids. The reiteration of this process can also be used for the synthesis of polydeuterated fatty acids in which the heavy atoms are placed on adjacent carbon atoms or differently arranged in the molecule. Some of the isotopically labelled fatty acids which can be obtained are shown in Scheme 1.

In this paper, we describe the preparation of the 2,2-dideuterated nonadecanoic acid starting from stearic acid. The current path is based on the homologation of carboxylic acids by reduction and then substitution of the hydroxyl group of the corresponding primary hydroxyl function with a cyano group (Scheme 2). We began our synthesis of isotopically labelled compounds by treating stearic acid with methyl chloroformate: the corresponding *in situ* formed carbomethoxy anhydride was next reduced and labelled by means of $NaBD_4$ (98% atom D) in D_2O, affording a high yield (91%) of



Scheme 1.

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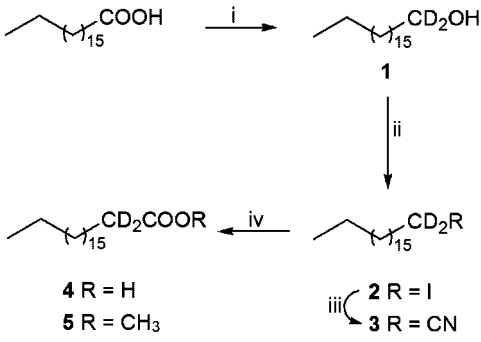

i) NMM, MeOCOCl in THF, then $NaBD_4$ in D_2O; ii) PPh_3, I_2, Im in CH_2Cl_2; iii) $Et_4^+CN^-$ in CH_2Cl_2; iv) aq HCl in dioxane or HCl(g) in CH_3OH

Scheme 2.

1,1-d_2-octadecanol (**1**) with a 98% deuterium incorporation (determined by ^{1}H NMR spectroscopy). On the basis of previously acquired knowledge,[22] the dideuterated alcohol **1** was then converted into its corresponding alkyl iodide **2** using a suspension of triphenyl phosphine polymer-bound/iodine complex (polystyryl diphenyl iodophosphonium iodide) in anhydrous acetone. The phosphine oxide, which under our conditions is the only by-product of the reaction, is linked to the polymeric matrix and can thus be easily separated by filtration. The triphenylphosphine polymer-bound/halogen complex is a Lewis acid and a dehydrating agent widely used in miscellaneous reactions[23] with low environmental impact. In fact it avoids contamination from by-products and use of non environmentally friendly solvents in the purification processes. Triphenylphosphine polymer-bound/iodine complex is an easy to prepare,[24] convenient, semicrystalline solid; when dried and kept properly, it can be stored for weeks at room temperature, under nitrogen atmosphere. The somewhat high cost of the starting triphenylphosphine polymer-bound does not actually represent a limitation of this procedure, if one considers that the polymer-linked phosphine oxide generally obtained from the reaction can be readily filtered off and reduced to the original phosphine form with trichlorosilane.[25] Compound **2** so obtained was directly employed in the next step, and the iodine atom was easily replaced by a cyano group in the presence of $Et_3N^+CN^-$. Finally, hydrolysis of nitrile **3** using conc. aq HCl in dioxane directly afforded, following the common purification procedures, the 2,2-d_2-nonadecanoic acid **4**. Furthermore, acid catalysed alcoholysis of **3** led to the corresponding 2,2-d_2-methylnonadecanoate **5**. It is noteworthy that the deuterium content percentage was maintained unchanged during the whole synthetic route, at a rate shown by MS spectrometry and ^{1}H NMR spectroscopy to be not less than 98%.

DOI: 10.1002/jlcr

Experimental

General

All moisture-sensitive reactions were conducted under dry nitrogen using oven-dried glassware. THF and diethyl ether were distilled from sodium/benzophenone immediately prior to use.

^{1}H and ^{13}C NMR spectra: Varian Gemini 300 MHz and Varian Inova 500 MHz spectrometers. MS (ESI) analyses were performed on Waters Micromass ZQ spectrometer. Reactions were monitored by TLC (precoated silica gel plate F254, Merck). Column chromatography: Merck Kieselgel 60 (70–230 mesh). Melting points are uncorrected and were determined with a capillary apparatus.

Triphenyl phosphine polymer-bound was purchased from Fluka Chemical Co. Deuterium oxide minimum isotopic purity 99.9 at.% D and $NaBD_4$ 98 at.% D were used.

The position of deuteration was established by ^{1}H and ^{13}C NMR experiments of compounds **2**, while the complete deuterium incorporation was determined by mass spectrometry.

*1,1-d_2-octadecanol (**1**)*

To a magnetically stirred solution of 4-methyl morpholine (NMM) (3.1 ml, 28.1 mmol) and stearic acid (4.0 g, 14.0 mmol) in anhydrous THF (80 ml), methyl chloroformate (MeOCOCl) (28.1 mmol, 2.17 ml) was added dropwise at 0°C.

After 40 min the solution was filtrated through a glass sinter funnel and the *N*-methylmorpholinium salt so obtained was washed with anhydrous THF (3×70 ml).

A suspension of $NaBD_4$ (1.2 g, 28.0 mmol) in D_2O (10 mL) was then added to the filtrate, under magnetic stirring and at 0°C. The reaction mixture was stirred for 10 min at room temperature and then evaporated under reduced pressure. The crude residue was diluted with ethyl acetate (200 ml) and washed with water (100 ml) until neutral. The organic layer dried (Na_2SO_4), and evaporated under reduced pressure afforded a solid residue whose chromatography on silica gel (1:15, $CHCl_3/CH_3OH = 95:5$) gave pure **1** (3.5 g, 91% yield); m.p. 57.0–59.0°C (from CH_3CN). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 0.89 (*t*, $J_{CH_3,CH_2} = 6.6$, 3 H, CH_3), 1.2–1.36 (*m*, 30 H, $15 \times CH_2$), 1.44–1.63 (*m*, 2 H, CH_2CD_2), 1.62 (*bs*, 1 H, OH), 3.61–3.67 (*m*, 0.04 H, CH_2OH); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 13.9 (CH_3), 22.5 (CH_2CH_3), 25.6 ($CH_2CH_2CD_2OH$), 29.4 ($12 \times CH_2$), 31.8 ($CH_2CH_2CH_3$), 32.5 (CH_2CD_2OH), 62.2 (CD_2). MS (ESI^+) *m*/*z* 271.42 (M-1).$^+$

*2,2-d_2-nonadecanonitrile (**3**)*

To a magnetically stirred suspension of dry polystyryl diphenyl phosphine (4.5 g, $\approx$ 13.4 phosphine units) in anhydrous CH_2Cl_2 (50 ml) at room

DOI: 10.1002/jlcr

temperature, a solution of I_2 (3.4 g, 13.2 mmol) in the same solvent (50 ml) was added dropwise in the dark and under dry nitrogen atmosphere. After 15 min, solid octadecanol-1,1-d_2 (**1**) (3.0 g, 11.2 mmol) was added in one portion to the suspension. TLC monitoring ($CHCl_3/CH_3OH$, 9:1) showed that the starting material was completely consumed within 30 min. The reaction mixture was then filtered through a glass sinter funnel and washed with CH_2Cl_2. The solvent was removed under reduced pressure to afford a crude residue which was used without purification in the next synthetic steps.

Conversion of compound **2** *into cyanide* **3**. To a magnetically stirred solution of the crude reaction product **2** in anhydrous CH_2Cl_2 (30 ml), at room temperature and under nitrogen atmosphere, solid tetraethylammonium cyanide (2.5 g, 14.6 mmol) was added in one portion. The mixture was refluxed for 4 h, until complete consumption of the starting product (TLC monitoring, petroleum ether/EtOAc = 7:3), then cooled and, after addition of silica gel (10 g), evaporated under reduced pressure. The solid residue was transferred onto a short silica gel (20 g) column and eluted with petroleum ether/EtOAc (5 → 20%) affording **3** as a crystalline white solid (2.8 g, yield 88%); m.p. 41.6–44.3°C (from CH_3CN). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 0.88 (*t*, 3 H, $J_{CH_3,CH_2} = 6.8$, CH_3), 1.20–1.36 (*m*, 28 H, 14xCH_2), 1.40–1.48 (*m*, 2 H, CH_2CD_2), 1.64 (*t*, 2 H, $J_{CH_2,CH_3} = 7.8$, CH_2CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 14.3 (CH_3), 22.9 (CD_2CN), 25.4 (CH_2CH_3), 28.8, 29.0, 29.5, 29.7, 29.9 (14xCH_2), 32.2 ($CH_2CH_2CH_3$), 119.9 (CN).

*2,2-d_2-nonadecanoic acid (***4***)*

A magnetically stirred solution of a pure **3** (1.9 g, 6.7 mmol) in dioxane (10 ml) and conc. aq hydrogen chloride (10 ml) was heated under reflux for 8 h. After cooling, the solution was diluted with water (25 ml) and extracted with Et_2O (3 × 30 ml). The organic layer was then washed with water until neutral, dried (Na_2SO_4), and evaporated under reduced pressure. The crude reaction product was recrystallized (CH_3CN) to afford **4** as a crystalline white solid (1.9 g, yield 93%); m.p. 69.0–71.0°C (from CH_3CN). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 0.89 (*t*, 3 H, $J_{CH_3,CH_2} = 6.5$, CH_3), 1.18–1.42 (*m*, 30 H, 15 × CH_2), 1.52–1.70 (*m*, 2 H, CH_2CD_2); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 14.0 (CH_3), 22.6 (CH_2CH_3), 24.4 ($CH_2CH_2CD_2$), 28.9, 29.1, 29.2, 29.3, 29.6, 31.8 (16 × CH_2 and CD_2), 31.8 ($CH_2CH_2CH_3$), 179.4 (C = O). MS (ESI^+) m/z 301.24 $(M+1)^+$.

*Methyl-2,2-d_2-nonadecanoate (***5***)*

A magnetically stirred solution of a pure **3** (0.8 g, 2.84 mmol) in anhydrous methanol (10 ml), at −15°C, is saturated with HCl (g) dry and then kept at room temperature for 36 h. To the solution are added few drops of water

DOI: 10.1002/jlcr

(76 mg, 4.26 mmol) and the solvent is evaporated with Et_2O (3×10 ml). The desired compound (**5**) is obtained as a crystalline white solid (0.8 g, yield 94%); m.p. 39.0–41.0°C (from CH_3CN); ^{1}H NMR (500 MHz, $CDCl_3$) δ: 0.95 (*t*, 3 H, $J_{CH_3,CH_2} = 6.8$, CH_3), 1.18–1.40 (*m*, 30 H, $15 \times CH_2$), 1.45–1.70 (*m*, 2 H, CH_2CD_2), 3.70 (*s*, 3 H, CH_3O). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 13.9 (CH_3), 22.6 (CH_2CH_3), 24.7($CH_2CH_2CD_2$), 28.9, 29.1, 29.2, 29.3, 29.6, 31.8 ($16 \times CH_2$ and CD_2), 31.8 ($CH_2CH_2CH_3$), 51.3 (CH_3O), 174.3 (C = O).

Conclusion

We have described the preparation of the 2,2-d_2-nonadecanoic acid, as a generic example, to be used as standard material for mass spectrometric analysis. The efficiency of our method lies in the wide flexibility of synthetic strategy: it is possible to choose how many heavy atoms to incorporate and in which positions, replacing $NaBD_4$ with $NaBH_4$ at the reduction step during the homologation process, thereby obtaining dideuterated or polydeuterated fatty acids. To date we have used the method exclusively for synthesis of saturated odd-numbered fatty acids, but it should also be applicable, as previously shown in Scheme 1, to even-numbered fatty acids and to unsaturated compounds.

Work is also in progress to prepare, by the same strategy, 2,2-dideuterated β-amino acids to be used as linker moieties in new isotope-coded affinity tags (ICAT) reagents for quantitative proteome analysis.

Acknowledgements

^{1}H NMR spectra were performed at Centro Interdipartimentale di Metodologie Chimico-Fisiche, Università di Napoli Federico II.

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DOI: 10.1002/jlcr

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DOI: 10.1002/jlcr