

## In vivo oxidation of [9-<sup>14</sup>C] cyclic fatty acids derived from linolenic acid in the rat

Lionel BRETILLON<sup>a\*</sup>, Olivier LOREAU<sup>b</sup>, Jean-Louis SÉBÉDIO<sup>c</sup>,  
Frédéric TARAN<sup>b</sup>

<sup>a</sup> Institut National de la Recherche Agronomique, UMR FLAVIC, équipe Œil et Nutrition, Dijon, France

<sup>b</sup> Commissariat à l'Énergie Atomique, Service des Molécules Marquées, Saclay, Gif-sur-Yvette, France

<sup>c</sup> Institut National de la Recherche Agronomique, Unité du Métabolisme Protéino-Energétique, Clermont-Ferrand, France

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**Abstract** – Heating oils and fats may lead to cyclization of polyunsaturated fatty acids, as for example linolenic acid. Cyclohexenyl and cyclopentenyl fatty acids are subsequently present in some edible oils and these are suspected to induce metabolic disorders. In a previous experiment using [1-<sup>14</sup>C] labeled molecules, we published that these cyclic fatty acids are beta oxidized to the same extent as linolenic acid, at least for the first cycle of beta oxidation. However, it is possible that the presence of a ring could alter the ability of the organism to fully oxidize the molecule. In order to test this hypothesis, we assessed the oxidative metabolism of cyclic fatty acids carrying a <sup>14</sup>C atom at the vicinity of the ring. For this purpose, rats were force-fed from 1.1 to 1.3 MBq of a representative fraction of dietary cyclohexenyl cyclic fatty acid monomers of [9-<sup>14</sup>C] 9-(6-propyl-cyclohex-3-enyl)-non-8-enoic acids and <sup>14</sup>CO<sub>2</sub> production was monitored for 24h. The animals were then necropsied and the radioactivity was determined in different tissues. No consistent radioactivity was recovered as <sup>14</sup>CO<sub>2</sub> 24h after administration of the molecules. Sixty percent of the radioactivity was recovered in the urine and 30% in the gastrointestinal tract. By combining our previous data on the oxidation of [1-<sup>14</sup>C] cyclic fatty acids and the present results, we suggest that cyclohexenyl fatty acids are first beta oxidized in a similar way as linolenic acid and that the remaining molecule carrying the ring is detoxified and eliminated in the urine and feces.

**cyclic fatty acid monomers / linolenic acid / oxidation**

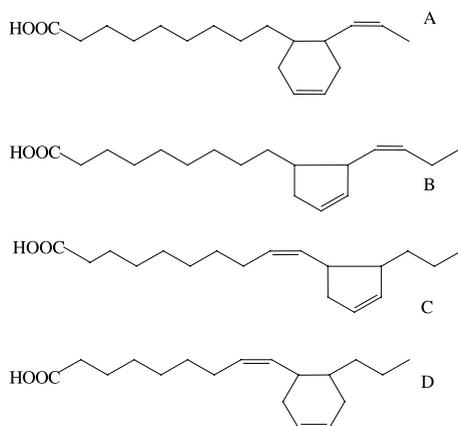
### 1. INTRODUCTION

Heating oils and fats result in alterations of the structure of the fatty acids and in the formation of new compounds [1]. Among them, cyclic fatty acids which are mainly formed from polyunsaturated fatty acids (linoleic and linolenic acids) are suspected to be the most toxic compounds formed in heated oils [2–4]. Cyclic Fatty Acid Monomers (CFAM) are mainly formed from the

C18 polyunsaturated fatty acids of edible oils (linoleic and linolenic acids) [5]. Cyclization of linolenic acid gives rise to sixteen dienoic fatty acids with a mixture of C5- and C6-membered ring structures (Fig. 1) [6].

Studies carried out on animals have shown that CFAM-18:3 cross the gastrointestinal border [7, 8] and may induce adverse effects including high mortality of rat neonates [9]. We previously showed that

\* Corresponding author: bretillon@dijon.inra.fr



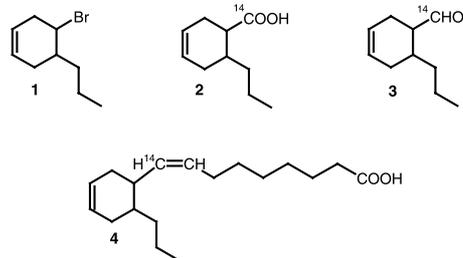
**Figure 1.** Chemical structures of cyclic fatty acid monomers formed by cyclization of  $\alpha$ -linolenic acid. Each of the 4 structures gives rise to 4 isomers, depending on the cis or trans configuration of the ring and on the Z- or E- configuration of the double bond along the lateral chain.

linolenic acid-derived cyclic fatty acids are metabolized *in vivo* via the  $\beta$ -oxidation process to the same extent as linolenic acid, at least for the first cycle of  $\beta$ -oxidation [10]. However, it remains unknown whether these molecules are fully oxidized. In order to answer this question, a representative fraction of dietary cyclohexenyl CFAM consisting of [9- $^{14}\text{C}$ ] 9-(6-propyl-cyclohex-3-enyl)-non-8-enoic acids were chemically synthesized (D-CFAM structure, Fig. 1) and orally administered to rats.  $^{14}\text{C}$  recovery as  $^{14}\text{CO}_2$  was monitored for 24 h and the distribution of the radioactivity in some organs was also determined. These molecules account for 25% of the total CFAM isomers formed by cyclization of linolenic acid [6].

## 2. MATERIALS AND METHODS

### 2.1. Chemical synthesis

The synthesis of [9- $^{14}\text{C}$ ] 9-(6-propyl-cyclohex-3-enyl)-non-8-enoic acid (4, Fig. 2)



**Figure 2.** Chemical structures of the intermediates in the synthesis of [9- $^{14}\text{C}$ ] 9-(6-propyl-cyclohex-3-enyl)-non-8-enoic acid (compound 4) corresponding to D-CFAM in Figure 1.

was carried out using 4-bromo-5-propylcyclohexene (1, Fig. 2) as the key intermediate for labeling. Compound 1 was prepared from 1,4-cyclohexadiene using a series of epoxidations with 3-chloroperoxybenzoic acid, reaction with propylmagnesium chloride in the presence of CuI [11] and bromination with dibromotriphenylphosphorane [12]. Formation of the Grignard reagent derived from 1, under  $^{14}\text{CO}_2$  atmosphere, furnished the labeled 6-propylcyclohex-3-enecarboxylic acid 2 which was reduced into its corresponding alcohol with  $\text{LiAlH}_4$ . Then [ $^{14}\text{C}$ ]-6-propylcyclohex-3-enecarbaldehyde 3 was obtained using the tetrapropylammonium perruthenate/N-methylmorpholine N-oxide system as the oxidant [13]. The Wittig reaction between aldehyde 3 and (7-methoxycarbonyl-heptyl)-triphenylphosphonium bromide (prepared as described by Awl and Frankel [14]) followed by saponification with LiOH gave [9- $^{14}\text{C}$ ] 9-(6-propyl-cyclohex-3-enyl)-non-8-enoic acid 4. The specific activity of 4 ( $2.035\text{GBq}\cdot\text{mmol}^{-1}$ ) was determined by mass spectrometry (Mariner Biospectrometry Workstation (ESI/TOF)). Its radiochemical purity was found to be greater than 98% by reversed phase high performance liquid chromatography (column: Zorbax SB C18; eluant: ethanol/water/trifluoroacetic acid (70/30/0.1)) and thin layer chromatography (silica gel 60F254, Merck; solvent: pentane/diethyl ether/acetic acid, 85/15/0.1). A  $^1\text{H}$ -nuclear

magnetic resonance spectrum was recorded on a Bruker AC 300 spectrometer using  $\text{CDCl}_3$  as the solvent (data not shown) and was consistent with the one obtained by Awl and Frankel on the methyl ester derivative [14].

## 2.2. Animal experiment

The protocol was conducted following the *Guidelines for the Care and Use of Experimental Animals* and approved by the local ethical committee.

Male Wistar rats (Janvier, Le Genest Saint Isle, France) weighing  $280 \pm 30$  g (mean  $\pm$  SEM) were housed in individual cages under controlled conditions of temperature ( $22 \pm 1$  °C) and relative humidity (55–60%). A 12 h light-dark cycle (lights on 7:00 am–7:00 pm) was maintained. The animals were fed ad libitum with sterilized commercial pellets (Harlan, France) and had free access to tap water. The day before the experiment, they had access to only 10 g of commercial pellets in order to be partially fasted. All the experiments started at the same time of the day (oral load of the radioactive fatty acid at 9:00 am) in order to avoid diurnal variations.

The fatty acids were dissolved in triolein (Sigma Chemicals, L'Isle d'Abeau, France) and then administered to rats by gastric tubing (approximately 200 mg, 1.2 MBq per rat).

After tubing, the rats were immediately installed in an airtight Plexiglas metabolic chamber as previously described [15]. The  $^{14}\text{CO}_2$  expired was trapped in a bottle containing 500 mL of a  $\text{CO}_2$  trapping agent (Carbosorb, Packard, Groningen, The Netherlands). The rats were fasted during the following 24 h.

Aliquots of the trapping agent were removed 30 min, 1, 2, 3, 5, 8 and 24 h after gastric tubing. Nine milliliters of a scintillation cocktail (Permafluor E, Packard) were added to each sample and the radioactivity was determined using a TriCarb 2900 liquid scintillation counter (Packard).

At the end of the 24 h experimental period, the animals were anaesthetized and blood was withdrawn into a heparinized syringe. Urine was collected during the whole 24 h-experiment. Aliquots of 1 mL were mixed with 10 mL of scintillation cocktail (Hionic Fluor, Packard) and the radioactivity was determined by liquid scintillation counting as described above. Tissues (brain, heart, liver, *Gastrocnemius* muscle, kidneys, adipose tissue) were removed from the carcass, blotted on filter paper and weighed. The gastrointestinal tract (from the stomach to rectum including the feces) was analyzed separately. The carcass of each animal was weighed before homogenization. Three aliquots of each tissue (70–80 mg each) and 5 aliquots of carcass and of the gastrointestinal tract (110–130 mg) were digested at 50 °C for 5 h using 1 mL Soluene (Packard). When the digestion was achieved, 10 mL of scintillation cocktail (Hionic Fluor, Packard) were added and the radioactivity was determined by liquid scintillation counting as described above. Two hundred microliters of blood were mixed with 500  $\mu\text{L}$  of propan-2-ol and 500  $\mu\text{L}$  of Soluene and heated at 50 °C for 30 min. Five hundred microliters of 25%  $\text{H}_2\text{O}_2$  were added and digestion was achieved for 15 min at 50 °C. Ten milliliters of scintillation cocktail (Hionic Fluor, Packard) were added and the radioactivity was determined by liquid scintillation counting as described above.

## 3. RESULTS AND DISCUSSION

Due to the presence of a ring, one may hypothesize that cyclic fatty acids would be poor substrates for metabolic pathways, especially oxidation. Our previous results using  $[1-^{14}\text{C}]$  molecules demonstrated that they are efficiently metabolized by the first cycle of  $\beta$ -oxidation, to the same extent as linolenic acid (more than 70%) [10]. Based on this result, we demonstrated that the carboxylic end moiety of the ring is shortened.

**Table I.**  $^{14}\text{C}$  distribution observed 24 h after oral administration of [9- $^{14}\text{C}$ ] 9-(6-propyl-cyclohex-3-enyl)-non-8-enoic acids to the fasting rat.

|  | Recovery<br>(% of the radioactivity<br>administered) |
|--|--|
| Urine  | 58.0 ± 4.6   |
| Gastrointestinal tract<br>including feces              | 33.4 ± 6.2   |
| Carcass*   | 6.6 ± 4.1  |
| Liver  | 1.5 ± 0.08   |
| Kidneys  | 0.3 ± 0.08   |
| Blood  | 0.14 ± 0.11  |
| CO <sub>2</sub> (final value, 24 h<br>after oral load) | 0.04 ± 0.01  |
| Heart  | 0.03 ± 0.01  |
| Peri-renal adipose tissue                              | 0.03 ± 0.02  |
| <i>Gastrocnemius</i> muscle                            | 0.02 ± 0.01  |
| Brain  | 0.005 ± 0.001  |

The results are means ± SD ( $n = 3$ ) of the percentage of the radioactivity administered to the rat recovered as  $^{14}\text{CO}_2$  and in the organs, blood and stools. \* Corresponds to the whole body of the animal minus the organs removed and cited in the table.

The present data using [9- $^{14}\text{C}$ ] 9-(6-propyl-cyclohex-3-enyl)-non-8-enoic acids clearly show that  $\beta$ -oxidation does not reach the ring. Indeed, less than 0.04% of the radioactivity administered was collected in expired breath as  $^{14}\text{CO}_2$  24 h after oral load of the cyclic fatty acids (Tab. I).

Most of the radioactivity was found in urine (58.0 ± 4.6% Tab. I) and in the gastrointestinal tract including the feces (33.4 ± 6.2%, Tab. I). Considering that these values were observed 24 h after an oral load of the fatty acids, which is a longer period of time than that required for the molecules to cross the gastrointestinal border, we assume that this value accounts for most of the non-digestible part of the molecules.

Enhanced excretion of urinary and fecal glucuronides has previously been reported in animals fed either linolenic acid-derived cyclic fatty acid fractions [16] (Grandgirard, unpublished data) or cyclic fatty acid

enriched-thermopolymerized linseed oil [17]. Although we did not elucidate the nature of the radioactivity in the gastrointestinal tract and stools, we assume that this value corresponded to glucurono-conjugates of chain shortened cyclic fatty acids.

In conclusion, the results of this study and of our previous one [10] give a clear understanding of the metabolism of cyclic fatty acids derived from linolenic acid, at least for C6-ring molecules, during the first 24 h post ingestion. Cyclic fatty acids first undergo  $\beta$ -oxidation (70% oxidation over 24 h) [10] and the remaining molecule is then excreted in the urine (60%) and feces (30%). These data do emphasize that the metabolic pathways of oxidation and elimination are powerful processes for the excretion of cyclic fatty acids. In addition, considering that only minor amounts of cyclic fatty acids are found in the diet [5, 18], one may suggest that cyclic fatty acids are not a major concern for human safety.

## ACKNOWLEDGMENTS

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