

SYNTHESIS OF 11(*n*-8)<sub>L</sub> TRITIUM-LABELLED LINOLEIC ACID

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## SUMMARY

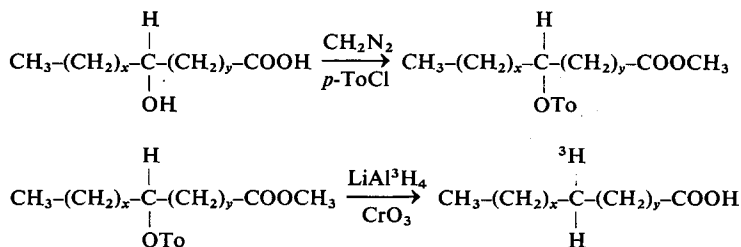
*n*-8-[11L<sub>5</sub>-<sup>3</sup>H]Linoleic acid has been prepared by incubation of [11L<sub>5</sub>-<sup>3</sup>H]stearic acid with the green algae *Chlorella vulgaris*. The stereospecifically tritium-labelled stearic acid has been synthesized starting from the lactone of 5D<sub>R</sub>-hydroxydodecanoic acid. The synthesis consists of:

- (1) hydrolysis of the lactone;
- (2) esterification of the free carboxyl function with diazomethane;
- (3) tosylation of the hydroxyl group with *p*-toluene sulphonyl chloride;
- (4) stereospecific reduction of the tosylate with LiAl<sup>3</sup>H<sub>4</sub>;
- (5) oxidation of the resulting *n*-[1,5L<sub>5</sub>-<sup>3</sup>H]dodecanol by CrO<sub>3</sub>;
- (6) chain elongation of [5L<sub>5</sub>-<sup>3</sup>H]dodecanoic acid by anodic coupling with monomethyl suberate.

## INTRODUCTION

In the framework of our studies about the enzyme lipoxxygenase (linoleate: oxygen oxidoreductase, EC 1.13.1.13), we became interested in the synthesis of stereospecifically 11(*n*-8) tritium-labelled linoleic acid.

Schroepfer and Bloch<sup>1</sup> and Hamberg and Samuelsson<sup>2</sup> described the synthesis of mono-tritium-labelled fatty acids starting from D- or L-hydroxy fatty acids *via* the reaction scheme shown below (where To = tosyl):



From a D-hydroxy fatty acid the L-<sup>3</sup>H-labelled fatty acid is formed. The availability of optically active hydroxy fatty acids in nature is rather limited. As a consequence, one is in most cases compelled to follow the tedious procedure of separation of syn-

thetic racemic mixtures for the preparation of the desired hydroxy acid. Tuynenburg Muys *et al.*<sup>3</sup> described the preparation of short chain D- or L-hydroxy fatty acids by incubation of keto fatty acids of the same chain length with micro organisms. The present paper describes the synthesis of mono-tritium-labelled fatty acids starting from 5D<sub>R</sub>-hydroxydodecanoic acid (lactone), which was prepared from 5-keto-dodecanoic acid by incubation with the yeast *Saccharomyces cerevisiae*. The applicability of this method, in view of the chain length attainable and the positioning of the tritium label, will be discussed.

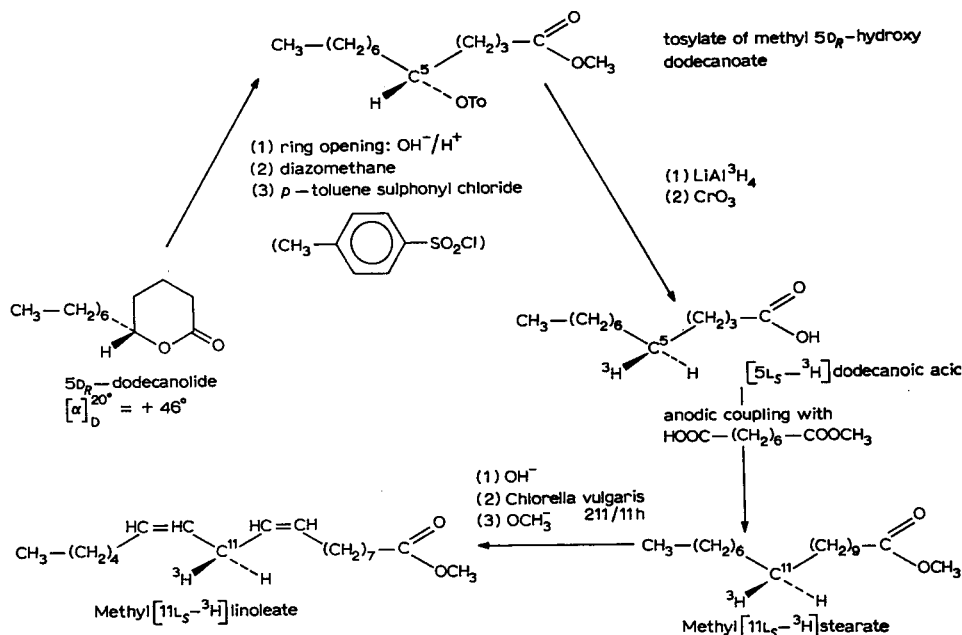
## EXPERIMENTAL

Pure 5D<sub>R</sub>-hydroxydodecanoic acid lactone (dodecanolide) was a gift from Unilever Research Laboratories, Vlaardingen/Duiven, The Netherlands. Optical rotatory dispersion curves of this substance have been published by Korver<sup>4</sup>. LiAl<sup>3</sup>H<sub>4</sub> was purchased from New England Nuclear Corp. (Boston Mass., U.S.A.), spec. act. 100 Ci/mole.

A culture of *Chlorella vulgaris* cells, (strain 211/11h from the Cambridge Collection of Algae and Protozoa) was obtained from Unilever Research Laboratories, Colworth/Welwyn, United Kingdom. Cells were stored on agar slopes, containing the "poor medium", according to Harris *et al.*<sup>5</sup>. Prior to incubation the cells were transferred to the "rich medium" (Harris *et al.*<sup>5</sup> and Bartels<sup>6</sup>) and were grown under sterile conditions in Roux bottles (250 ml), illuminated with 5 TL 40 W/33 tubes (Philips) at a distance of 30 cm. After 6–7 days, the algae were harvested by centrifugation (10 min at 1000 × g) and washed once with 0.2 M phosphate buffer, pH 7.4; the washed cells (6 g wet weight) were resuspended in the phosphate buffer, pH 7.4. Incubations were carried out with suspensions, containing 50 mg cells (wet weight) per ml buffer (pH 7.4)<sup>5,6</sup>. Thin-layer chromatography was carried out on 0.25-mm silica gel plates (DC silica gel 60 F 254, 20 cm × 20 cm, E. Merck A.-G., Darmstadt, Germany) or on 0.30-mm silica gel G plates (E. Merck) without indicator, if mass spectra were recorded of the purified substance. Silica gel column chromatography was carried out on 70–350 mesh silica gel (Mallinckrodt) columns (25 cm × 2 cm). 70 eV mass spectra were recorded with an AEI MS 9 mass spectrometer at an ion chamber temperature of 120 °C. Infrared absorption was measured with a Beckman IR 8 spectrophotometer. Optical rotation was measured with a Perkin Elmer 141 polarimeter (light pathway 10 cm). Radio-gas-liquid chromatography was carried out on a Hewlett Packard 5750 gas chromatograph, provided with a Nuclear Chicago gas chromatography counting system; 200 cm × 0.4 cm columns were used packed with 10% polyethyleneglycol adipate on chromosorb W or 2% polyethyleneglycol succinate on gaschrom Q. Scintillation counting was performed with a Packard Tricarb Scintillation spectrometer Model 3375. The absolute efficiency for <sup>3</sup>H was 37%.

## Syntheses

The reactions described are shown in Scheme I (where To = tosyl):



Scheme I. Synthesis of stereospecifically  $11L_5$  tritium-labelled linoleic acid.

*The tosylate of methyl  $5D_8$ -hydroxydodecanoate.* The lactone of  $5D_8$ -hydroxydodecanoic acid,  $[\alpha]_D^{20} = +46^\circ$ ,  $c$  1.4 in ethanol<sup>4</sup>, was saponified in 1 M NaOH. Acidification at  $0^\circ C$  gave a white precipitate, which was identified as  $5D_8$ -hydroxydodecanoic acid (sodium salt:  $[\alpha]_D^{20} = -8.2^\circ$ ,  $c$  3.3 in 1 M NaOH). The free acid was esterified with diazomethane in methanol-diethyl ether 1:3, v/v at  $0^\circ C$ . The methyl ester was freed from residual  $5D_8$ -hydroxydodecanoic acid lactone by dissolving in diethyl ether and extracting the solution with a 10% solution of  $NaHCO_3$  in water, pH 8.2. Infrared absorption spectra of the methyl ester showed absorptions at  $3440\text{ cm}^{-1}$  and  $1440\text{ cm}^{-1}$ , indicating the presence of a hydroxyl group.

1 g (4.3 mmole) methyl  $5D_8$ -hydroxydodecanoate was dissolved in 150 ml pyridine, which was freshly distilled from BaO. After cooling to  $-30^\circ C$ , 15 g (78.7 mmole) *p*-toluene sulphonyl chloride (crystallized from dry diethyl ether prior to use) was added. Thereafter the temperature was maintained at  $4^\circ C$  for 7 days. The crude tosylate of  $5D_8$ -hydroxydodecanoate was isolated by extraction with diethyl ether, after decomposition of the excess of *p*-toluene sulphonyl chloride with water. The combined ether extracts were washed with water and dried over anhydrous  $Na_2SO_4$ . The tosylate was purified first by silica gel column chromatography (see Experimental), then by thin-layer chromatography on silica gel G. The tosylate was eluted from the column with the solvent system light petroleum (b.p.  $60-80^\circ C$ )-diethyl ether (7:3, v/v); the silica gel G-coated plates were developed with light petroleum (b.p.  $60-80^\circ C$ )-diethyl ether (1:1, v/v). The tosylate fraction ( $R_F = 0.53$ ) was analysed by mass spectrometry, as is shown in Fig. 1.

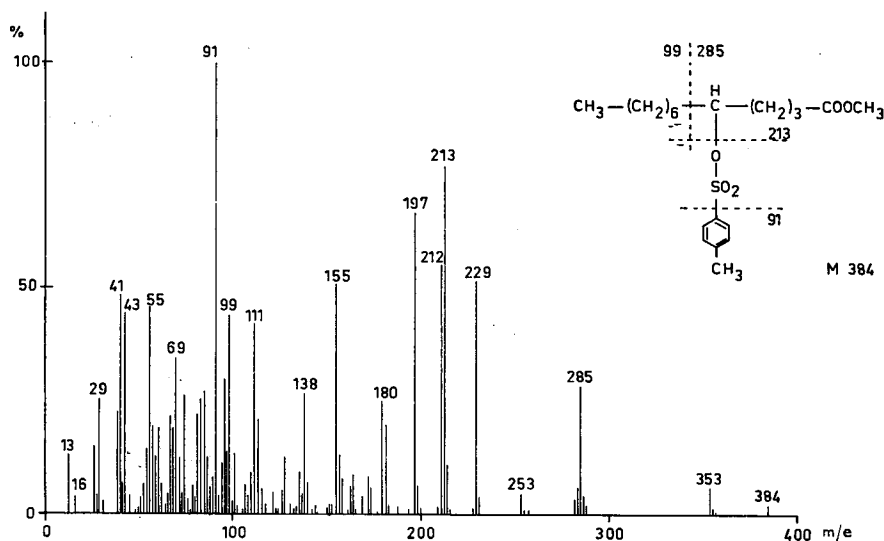


Fig. 1. Mass spectrogram of the tosylate of methyl 5D<sub>R</sub>-hydroxydodecanoate.

[5L<sub>S</sub>-<sup>3</sup>H]Dodecanoic acid. 26 mg (68 μmoles) tosylate of 5D<sub>R</sub>-hydroxydodecanoate was dissolved in 4 ml tetrahydrofuran, which was distilled from LiAlH<sub>4</sub> prior to use. 10.3 mg LiAlH<sub>4</sub> was added to the solution, immediately followed by 9.5 mg LiAl<sup>3</sup>H<sub>4</sub>, total activity 25 mCi. The final specific activity was 47.4 Ci/mole. The mixture was refluxed for 20 h in a closed system. The reaction mixture was treated first with moist tetrahydrofuran, then with water. Tritium-labelled hydrogen gas, liberated during reflux and hydrolysis of the excess of LiAl<sup>3</sup>H<sub>4</sub> was trapped by hydrogenation of oleic acid methyl ester, catalysed by palladium on carbon. 1 mCi crude *n*-[1,5L<sub>S</sub>-<sup>3</sup>H]dodecanol was obtained from the hydrolysate by extraction with diethyl ether. 6 mCi tritium were retained in the water layer, while 17.5 mCi tritium-labelled methyl stearate were formed from methyl oleate by hydrogenation. The *n*-[1,5L<sub>S</sub>-<sup>3</sup>H]-dodecanol was purified by thin-layer chromatography. The silica gel plates were developed with light petroleum (b.p. 60–80 °C)–diethyl ether (3:2, v/v). The purity of the tritium-labelled *n*-dodecanol was higher than 99%, as demonstrated by gas-liquid chromatography. Radioactivity and mass response of the radio-gas-liquid chromatographic recorder coincided completely. No other labelled compounds could be detected.

The labelled dodecanol was dissolved in 1 ml acetic acid. At 25 °C, a 0.3% solution of CrO<sub>3</sub> in 95% acetic acid (5% water) was added in 50-μl portions for 60 min until a slight excess of CrO<sub>3</sub> over the alcohol was reached. After 2 h, residual CrO<sub>3</sub> was removed by adding 50 μl methanol. The reaction mixture was diluted with water and after the addition of a few drops of 1 M HCl, it was extracted with diethyl ether. [5L<sub>S</sub>-<sup>3</sup>H]dodecanoic acid was obtained from the extracts after purification on silica gel plates, developed with light petroleum (b.p. 60–80 °C)–diethyl ether–acetic acid (50:50:1, v/v/v).

Methyl[11L<sub>S</sub>-<sup>3</sup>H]stearate. Without further purification, [5L<sub>S</sub>-<sup>3</sup>H]dodecanoic acid was dissolved in 1 ml methanol, containing 56 mg (298 μmoles) monomethyl suberate and 25 μmoles sodium methoxide. Monomethyl suberate was prepared from

suberic acid and dimethyl suberate according to Soffer *et al.*<sup>7</sup> A current of 75 mA was maintained between two platinum electrodes ( $0.25\text{ cm}^2$ ) for 40 min. The temperature of the reaction mixture was kept below  $30^\circ\text{C}$ . The slightly basic reaction medium was diluted with water and extracted with diethyl ether. Methyl $[1\text{H}_5\text{-}^3\text{H}]\text{stearate}$  was isolated from the combined extracts by thin-layer chromatography on silica gel plates, developed with light petroleum (b.p.  $60\text{--}80^\circ\text{C}$ )–diethyl ether (4:1, v/v). The labelled methyl stearate appeared to contain 10% tritium-labelled heptadecanoate, and 2% tritium-labelled hexadecanoate, when analysed by radio-gas-liquid chromatography. Therefore the stearate was subjected to reversed phase-partition chromatography on kieselguhr (E. Merck) plates, impregnated with paraffin oil. The plates were developed with the upper phase of the solvent system acetone–water–paraffin oil (9:2:1, v/v/v). The purity of the stearate was analysed by radio-gas-liquid chromatography and proved to be 99%. The specific activity was 48 Ci/mole. The recovery of tritium label in the stearate was  $280\text{ }\mu\text{Ci}$ .

The position of the tritium label in the stearate was determined by applying the oxidative degradation method according to Murray<sup>8</sup>.  $10\text{ }\mu\text{Ci}$  of the tritium-labelled methyl stearate was diluted with 2.6 mg ( $8.8\text{ }\mu\text{moles}$ ) pure methyl stearate and saponified in 5 ml 3% NaOH in methanol. After dilution with water and acidification to pH 3 with 1 M HCl, the tritium-labelled free acid was extracted with diethyl ether. The acid was chromatographed on silica gel plates, developed with light petroleum (b.p.  $60\text{--}80^\circ\text{C}$ )–diethyl ether–acetic acid (60:40:1, v/v/v). The purified tritium-

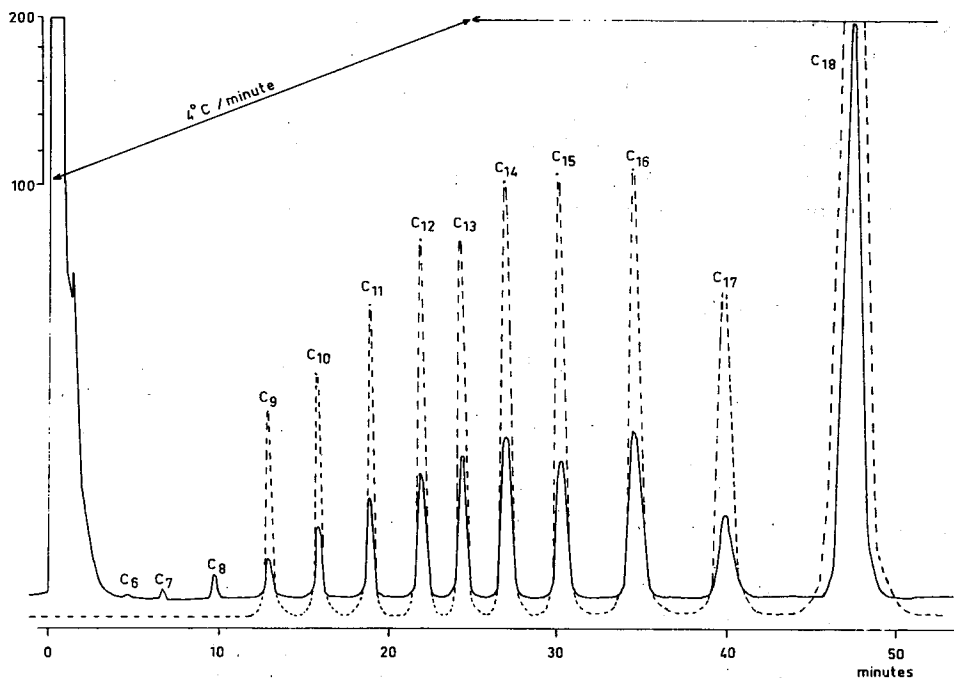


Fig. 2. Gas-liquid chromatogram of C 6 to C 18 carboxylic acid methyl esters, obtained after oxidative chain degradation of  $[1\text{H}_5\text{-}^3\text{H}]\text{stearic acid}$  according to Murray<sup>8</sup>. —, mass response; ---,  $^3\text{H}$  activity response.

labelled stearic acid was dissolved in 1 ml acetone and then refluxed for 6 h with 150 mg powdered  $\text{KMnO}_4$ . The acetone was removed under a stream of nitrogen gas. Unreacted  $\text{KMnO}_4$  and  $\text{MnO}_2$  were reduced with a saturated solution of  $\text{K}_2\text{S}_2\text{O}_5$  in water after adding 1 ml 10%  $\text{H}_2\text{SO}_4$ . The mixture of free fatty acids was extracted with diethyl ether. The combined extracts were washed with a dilute solution of  $\text{NaHCO}_3$  in water and then with water until the washings were free of acid. The mixture of fatty acids was esterified with diazomethane in diethyl ether and analysed by radio-gas-liquid chromatography. The resulting chromatogram is shown in Fig. 2. The complete absence of tritium activity in the octanoate and the fact that the relative specific activities of the fatty acid methyl esters longer than nonanoate are not higher than the relative specific activity of this fatty acid ester, indicate that the tritium label was located only at the correct position (carbon atom 11,  $n-8$ ).

*Conversion into [ $11\text{L}_5$ - $^3\text{H}$ ]linoleate.* [ $11\text{L}_5$ - $^3\text{H}$ ]stearate was saponified in 3% NaOH in methanol. The labelled stearic acid was incubated with a suspension of *C. vulgaris* cells in 0.2 M phosphate buffer, pH 7.4, (50 mg cells (wet weight)/ml phosphate buffer; see Experimental), for 24 h at 27 °C. The mixture was illuminated and stirred continuously in order to prevent sedimentation of the cells. After incubation, the cells were isolated by centrifugation at  $1000 \times g$  for 10 min and were washed once with 0.2 M phosphate buffer, pH 7.4. Lysis of the cells was accomplished by dissolving them in methanol. After 30 min, insoluble material was spun down at  $1000 \times g$  and extracted twice with chloroform-methanol (1:1, v/v) and once with methanol. The lipid extracts were combined and fatty acid methyl esters were recovered by methanolysis in 4% sodium methoxide in methanol-benzene (3:2, v/v) according to Glass<sup>9</sup>. [ $11\text{L}_5$ - $^3\text{H}$ ]Methyl linoleate was isolated from the mixture of fatty acid methyl esters by thin-layer chromatography on  $\text{AgNO}_3$ -impregnated (10%, w/w) silica gel G plates, developed with light petroleum (b.p. 60–80 °C)-diethyl ether (9:1, v/v). About 40% of the original radioactivity was recovered in the isolated linoleic acid methyl ester. The specific activity had dropped to 0.2 Ci/mole because of endogenous linoleic acid. Analysis with radio-gas-liquid chromatography showed that during incubation, the label remained exclusively in the 18 carbon chain fatty acids.

## CONCLUDING REMARKS

The labelled linoleic acid, synthesized as described above, has been used in combination with [ $1$ - $^{14}\text{C}$ ]linoleic acid (purchased from The Radiochemical Centre, Amersham, U.K.), as substrate for the enzyme lipoxygenase, in order to study the stereospecific removal of the 11( $n-8$ ) hydrogen atom from linoleic acid in the oxygenation reaction, catalysed by this enzyme. (Egmond *et al.*<sup>10</sup>). It has been shown that lipoxygenases from soya beans and corn germs remove the  $11\text{L}_5$  and  $11\text{D}_R$  hydrogen atoms, respectively.

It should be noted that tritium labelling can only be carried out before chain elongation by anodic coupling, because: (1) the free  $5\text{D}_R$ -hydroxydodecanoic acid does not permit anodic coupling for chain elongation, and (2) a suitable starting material for chain elongation,  $5\text{D}_R$ -acetoxydodecanoic acid could not be prepared because of lactonisation of  $5\text{D}_R$ -hydroxydodecanoic acid, when treated with an acylating agent.

According to the method presented in this paper, a wide range of D or L tritium-labelled fatty acids can be prepared. The application of biological reduction of  $\gamma$ - or  $\delta$ -keto acids, as has been published by Tuynenburg Muys *et al.*<sup>3</sup>, enables the stereospecific introduction of a tritium label at any carbon atom from carbon at  $n-1$  to carbon atom  $n-10$  (only  $\gamma$ - or  $\delta$ -keto fatty acids with chain length varying from 5 to 14 carbon atoms are reduced by the microorganisms). The ultimate chain length of the tritium-labelled saturated fatty acid is determined by the choice of the dicarboxylic acid mono ester for the anodic coupling reaction.

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