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Research Article

[6, 7, 10, 11-$^{13}C_4$]-Labelled leukotriene B_4 synthesis: standard for mass spectrometry determination and metabolic studies

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Summary

The mass spectrometry quantification and metabolic studies of leukotriene B_4 (LTB_4) in biological fluids require standards labelled with stable isotopes. This paper describes the synthesis of LTB_4 labelled with ^{13}C in positions 6, 7, 10 and 11 (**1**). These labelled carbons come from $^{13}C_2$-acetylene. A labelled LTB_4 is obtained with an isotopic enrichment of 99%. Copyright © 2001 John Wiley & Sons, Ltd.

Key Words: quantification LTB_4; ^{13}C labelled standard; GC/MS; LTB_4 metabolism; Pd°/Cu coupling reactions; stereoselective reduction of alkynes

Introduction

The leukotrienes discovered at the beginning of the 1980s are high biological activity compounds which come from the transformation of arachidonic acid by 5-Lipoxygenase.[1] These lipid mediators, often present in biological fluids in extremely low concentrations, exert a

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variety of biological actions and are thought to play roles in many diseases.[2–6] Highly sensitive methods are required for the analysis of these mediators. Until now, their quantification and studies in biological fluids has remained difficult and generally used immunoassay[7,8] or HPLC with prostaglandins B_2 as internal standards or LTB_4 as external standard.[9,10] However, interference between biological matrix molecules can compromise the interpretation of the results. Measuring techniques must be sensitive and specific, so mass spectrometry associated with gas chromatography (GC–MS) will be a particularly good method as long as adequate internal standards are used. Labelled leukotrienes incorporating stable isotopes are potentially ideal standards.

The use of GC/MS with $^{18}O_2$-LTB_4 as an internal standard allowed Wescott *et al.*[11] to measure natural LTB_4 in a number of biological fluids. But he observed that when the standard was introduced in the biological sample, the ^{18}O atoms in the $^{18}O_2$-LTB_4 slowly back exchanged with the ^{16}O in the water medium, thereby affecting the results. Oda *et al.* made measurements using $^2H_4$-LTB_4 and liquid chromatography tandem mass spectrometry.[12] Nevertheless, the ^2H were dispersed by the fragmentation of the parent ion. In so doing, the advantages of ^2H tetra-labelling were lost. That compromised the use of the $^2H_4$ labelled standard LTB_4 for quantification studies.

To improve on this type of study, the synthesis of **1** was undertaken (Figure 1). Using ^{13}C stable isotopes will ensure that no back exchange takes place and present several advantages.

First of all, the chromatography characteristics of the molecule are very close to those of natural LTB_4. It also has the same mass spectrometry fragmentation mechanisms, which allows analytical studies using GC/MS. With a difference in mass of four units, the isotopic profile of the labelled derivative and the natural one are clearly defined. Consequently, there is no risk of interference between the signals due to natural LTB_4 ions and those from the labelled standard. Finally, by placing the ^{13}C-atoms on positions 6, 7, 10, 11, the difference in mass is completely preserved on the fragments which are

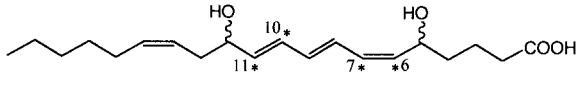

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Figure 1. [6, 7, 10, 11-$^{13}C_4$]LTB_4

characteristic of the molecule being studied. This is true for any mode of ionization used. Moreover, this standard is also interesting for metabolic studies.

An asymmetric synthesis would be unnecessary, because it has been shown in previous experiments[13] that it is possible to separate the diastereoisomers by flash chromatography. Natural LTB_4 is the 5S-12R enantiomer, consequently the 5S-12R, 5R-12S enantiomeric mixture can be used for analytical purposes since the chromatographic phases used in GC/MS do not allow enantiomeric resolutions.

Results and discussion

The retrosynthetic route (Figure 2) developed for the synthesis of chiral LTB_4[14] was again used for this research. The (*E*,*E*,*Z*) conjugated trienic system is constructed from two propargylic alcohols **3** and **2** condensed with (trimethylsilyl)acetylene using tetrakis(triphenylphosphine)palladium [$(Ph_3P)_4Pd$] as a coupling catalyst reagent.

The insertion of the ^{13}C-atoms during the synthesis of propargylic alcohols is achieved by opening precursory aldehydes **4** and **5** using monolithium $^{13}C_2$-acetylene. (Figures 3 and 4).

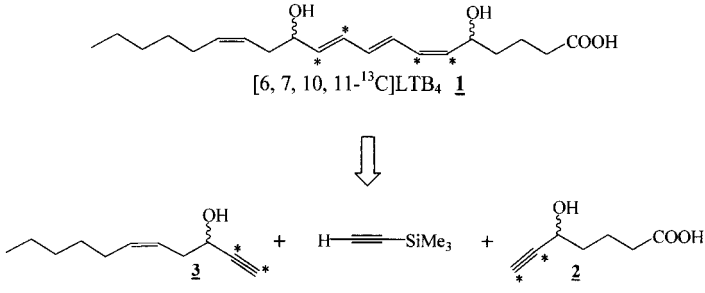


Figure 2. Retrosynthetic route for $^{13}C_4$-LTB_4 synthesis

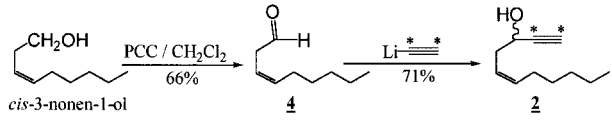


Figure 3. Synthesis route of synthon 2

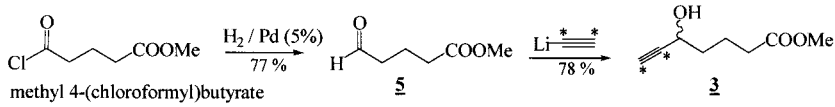
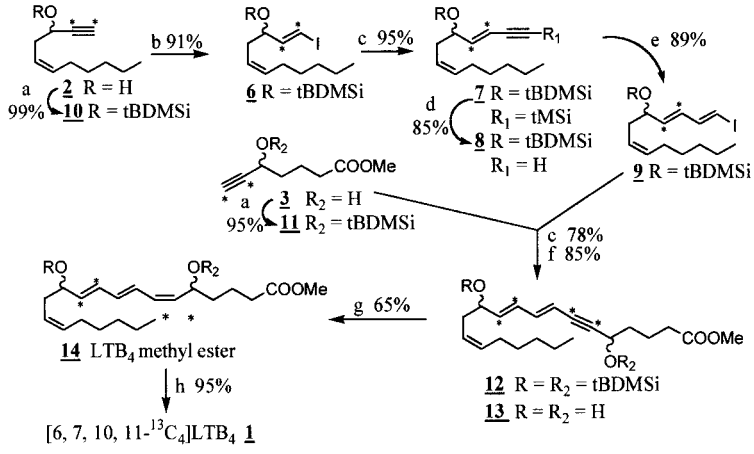


Figure 4. Synthesis route of synthon 3



Key : (a) *t*-BDMSiCl, imidazole, DMF, 35°C; (b) $Cp_2Zr(Cl)H$, C_6H_6, 40°C, I_2 ; (c) $HC{\equiv}CSiMe_3$, $(Ph_3P)_4Pd$ - CuI, C_6H_6, *n*-$BuNH_2$; (d) $AgNO_3$, $EtOH/H_2O$, 30°C ; KCN, H_2O ; (e) $Cp_2Zr(Cl)H$, C_6H_6/THF, 35°C, I_2 ;(f) Bu_4NF, THF ; CH_2N_2 ; (g) Zn, $MeOH/H_2O$, 30°C; (h) K_2CO_3, $MeOH/H_2O$.

Figure 5. Synthesis of labelled LTB_4 1

Commercially available *cis*-3-nonen-1-ol was oxidized to the corresponding aldehyde **4** with pyridinium chlorochromate (PCC). Synthon **2** results in the opening of this aldehyde with monolithium $^{13}C_2$-acetylene at −78°C in dried tetrahydrofuran.

Aldehyde **5** is the result of catalytic hydrogenation of methyl 4-(chloroformyl) butyrate and is used freshly distilled. After treatment with labelled monolithium acetylene it afforded the propargylic alcohol **3**.

The other feature of this route was the production of a pure triene system. This was accomplished by using the couple $(PPh_3)_4Pd/CuI$ (Pd°/Cu) as a catalyst in the condensation of the terminal acetylenic derivatives **2** or **3** with the vinyl halides **6** or **9**.[15,16] The formation of the C_9–C_{10} and C_7–C_8 bonds and the details of the synthesis are illustrated in Figure 5. The geometry of the triene system (*E*, *E*, *Z*) was accurately reproduced.

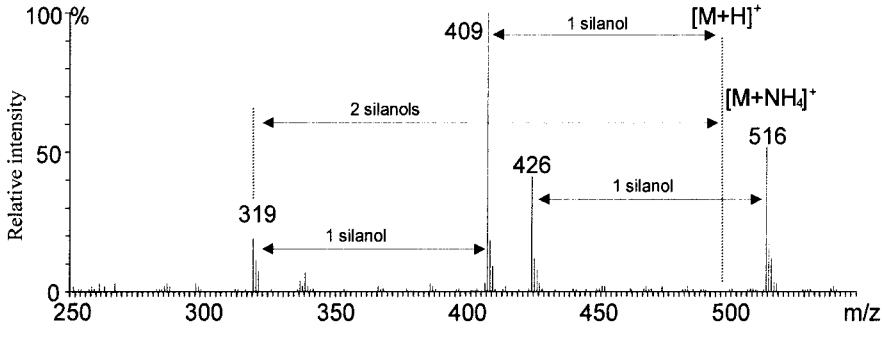
Vinyl iodide **6** was produced in a 91% yield from **10** by hydrozirconation using Bis(cyclopentadienyl)zirconium chloride hydride ($Cp_2Zr(Cl)H$; Schwartz's reagent) followed by iodination of the intermediate organometallic.[17] The C_9–C_{10} bond was formed by condensing **6** with (trimethylsilyl)acetylene in the presence of Pd°/Cu. The enyne **8** was obtained by specific desilylation of the triple bond of compound **7**.[18]

Hydrozirconation followed by iodination of **8** afforded the vinyl iodide **9**, which was condensed with the terminal acetylene group of compound **11**, thereby forming the C_7–C_8 bond. The dienyne **13** was obtained by desilylation using tetrabutylammonium fluoride (Bu_4NF) followed by a partial reduction using activated zinc[19] to give the methyl ester of $^{13}C_4$-LTB_4 **14**. The four-isomer mixture of labelled LTB_4 methyl ester **14** was obtained. Then the enantiomer pair 5S-12R, 5R-12S was isolated by flash chromatography[13]. Finally, the standard **1** was released by hydrolysis of the methyl ester **14** using K_2CO_3.

Conclusion

This research led to the production of 14 mg of $^{13}C_4$-LTB_4. All the reactions utilized conformed to the results obtained during the development of the asymmetric synthesis of LTB4.[14] The yields and the physico-chemical characteristics of the labelled compounds obtained are identical, with the exception of the ^{1}H-NMR and mass spectra.

The use of this standard with GC/MS (chemical ionization with ammonia) led to a spectrum which met our expectations: all the ions had an excess mass of four units (Spectrum 1). Thus, the pseudomo-



Spectrum 1. GC/MS of TMS derivative of $^{13}C_4$-LTB_4 methyl ester

lecular ion $[M + NH_4]^+$ was observed at m/z 516 instead of at 512 which corresponds to the natural product. The spectrum also showed the ions at m/z 409, 426 and 319 corresponding to the loss of one or two silanols, instead of 405, 422 and 315.

The isotopic purity was checked with a FAB spectrum because this ionization technique allows one to obtain better ionic statistics than with a GC/MS-CI. According to the supplier specifications of the labelled acetylene, the isotopic enrichment is 99%. We now have an ideal standard for analytical studies of LTB_4 in biological fluids.

Experimental section

All protocols for the synthesis of chiral LTB_4 have been published.[14] Only new compounds and spectrometric characteristics (^{1}H-NMR and mass spectra) are presented in this paper. The ^{1}H-NMR spectra were recorded in $CDCl_3$ at 300 MHz. Exact mass and GC/MS studies were carried out with a ZAB 2F instrument (VG-analytical, Manchester, UK). It was connected to a DANI 3800 chromatograph equipped with a 25 m × 0.25 mm i.d. OV-1 coated fused silica column (spiral, Dijon, France).

*Methyl 4-formylbutyrate (**5**).* 640 mg of Pd/C (5%) was saturated with hydrogen in a solution of 4.2 ml of 2,6-lutidine and 130 ml dried THF. Then, 5.25 g (32 mmol) of methyl 4-chloroformylbutyrate was introduced. The suspension was vigorously stirred under hydrogen pressure until consumption stopped. After elimination of hydrogen under reduced pressure, the suspension was filtered, concentrated and distilled (75°C/10 mm Hg) in the presence of 5 mg of *para*-toluene sulfonic acid. The resultant aldehyde **5** was obtained as a colorless oil (3.2 mg, 24.5 mmol, 77%) which was immediately used in the next step.

*$^{13}C_2$-Methyl 5-hydroxy-6-heptynoate (synthon **3**).* Anhydrous THF (50 ml) was placed in a thoroughly dried, round-bottomed flask under argon and cooled to −78°C. With continuous stirring, 500 ml (22.3 mmol) of labelled acetylene was added. A 13.9 ml (22.3 mmol) portion of *n*BuLi (1.6 M/hexane) was then added dropwise. After stirring for 20 min at −78°C, the aldehyde **5** (3.2 g, 24.5 mmol) in 10 ml of anhydrous THF was added slowly. After 20 min of additional stirring at −78°C, the temperature of the mixture was allowed to rise to room temperature. The mixture was hydrolysed with 70 ml of 5% aqueous NH_4Cl and then extracted with ether (3 × 100 ml). The combined

organic phases were washed with 50 ml of H_2O and 50 ml of saturated NaCl, dried, and concentrated. A yellowish oil was obtained. After chromatography (150 g silica; CH_2Cl_2/ethyl acetate (95 : 5) v/v; $R_f = 0.36$) 3 g (19 mmol, 78%) of compound **3** was isolated as a colorless oil; IR (film) ν 3500 3300 (s), 3400 (s), 2100 (w), 1730 (s) cm^{-1}; ^{1}H-NMR δ 5.64 (1H, dtt, $J = 10.9$, 6, 1.5 Hz, H_6), 5.45 (1H, dtt, $J = 10.9$, 6.5, 1.5 Hz, H_5), 4.38 (1H, td, $J = 6.5$, 2 Hz, H_3), 2.5 (2H, ddd, $J = 6.5$, 1.5 Hz, H_4), 2.45 (1H, d, $J = 2$ Hz, H_1), 2.06 (2H, tdd, $J = 6$, 1.5 Hz, H_7), 1.8 (1H, s, OH), 1,3 (6H, m, H_{8-9-10}), 0.87 (3H, t, $J = 6.8$ Hz, H_{11}); MS (CI-NH_3) $[M + NH_4]^+$ m/z 176; High-resolution MS (EI) of TMS derivative; $[M\text{-}15]^+$ found m/z 215.1025, calculated for $^{13}C_2$ $^{12}C_8H_{17}O_3Si$ 215.1014.

*(3Z)-Non-3-enal (**4**)*. The highly unstable compound **4** was used immediately for the following reaction. Only its IR spectrum was recorded: IR (film) ν 3020 (w), 2720 (m), 1730 (s) cm^{-1}. A single peak was observed by GC.

*$^{13}C_2$-5-Undecen-1-yn-3-ol (synthon **2**)*. ^{1}H-NMR δ 2.45 (1H, ddd, $J_{H1-C1} = 247{,}5$ Hz, $J_{H1-C2} = 51{,}25$ Hz, $J_{H1-H3} = 2$ Hz, H_1); High-resolution MS (CI–NH_3) $[M + NH_4]^+$ found m/z 186.1768, calculated for $^{13}C_2$ $^{12}C_9H_{22}NO$ 186.17698.

*$^{13}C_2$-3-[(tert-Butyldimethylsilyl)oxy]-5-undecen-1-yne (**10**)*. ^{1}H-NMR idem synthon **2**; MS (CI-NH_3) $[M + NH_4]^+$ at m/z 300 (100);

*$^{13}C_2$-1-Iodo-3-[(tert-butyldimethylsilyl)-oxyl]-1,5-undecadiene (**6**)*. ^{1}H-NMR δ 6.30 (2H, m, H_1 and H_2), 4.05 (1H, tdd, $J = 6$, 1.2 Hz, H_3).

*$^{13}C_2$-5-[(tert-Butyldimethylsilyl)oxy]-1-(trimethylsilyl)-3,7-tridecadien-1-yne (**7**)*. ^{1}H-NMR δ 6.23 (1H, dddd, $J_{H4-C4} = 150$ Hz, $J_{H4-H3} = 16.8$ Hz, $J_{H4-H5} = 6$ Hz, $J_{H4-C3} = 2.5$ Hz, H_4), 5.6 (1H, dddd, $J_{H3-C3} = 140$ Hz, $J_{H3-H4} = 16.8$ Hz, $J_{H3-C4} = 6$ Hz, $J_{H4-H5} = 2.5$ Hz, H_3), 4.16 (1H, m, H_5), 2.23 (2H, m, H_6); MS (CI-NH_3) $[M + H]^+$ $m/z = 381$; High-resolution MS (EI) found for $[M\text{-}C_4H_9]^+$ m/z 323.2142, calculated for $^{13}C_2$ $^{12}C_{16}H_{33}OSi_2$ 321.2138.

*$^{13}C_2$-5-[(tert-Butyldimethylsilyl)oxy]-3,7-tridecadien-1-yne (**8**)*. ^{1}H-NMR δ 6.24 (1H, dddd, $J_{H4-C4} = 165$ Hz, $J_{H4-H3} = 16.8$ Hz, $J_{H4-H5} = 6$ Hz, $J_{H4-C3} = 2.5$ Hz, H_4), 5.65 (1H, m, $J_{H3-C3} = 177$ Hz, H_3), 4.17 (1H, m, H_5), 2.85 (1H, m, H_1), 2.25 (2H, m, H_6); High-resolution MS (CI-NH_3) found for $[M + NH_4]^+$ m/z 326.2771, calculated for $^{13}C_2$ $^{12}C_{17}H_{38}NOSi = 326.2791$.

*$^{13}C_2$-1-iodo-5-[(tert-Butyldimethylsilyl)oxy]-1,3,7-tridecatriene (**9**)*. ^{1}H-NMR δ 7 (1H, m, H_2), 6.09 (1H, m, H_3), 5.70 (1H, m, H_4), 4.12 (1H, m, H_5).

6, 7, 10, 11 ^{13}C labeled leucotriene B_4 synthesis

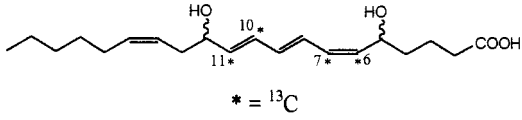


Figure 6. 6, 7, 10, 11 ^{13}C labeled leucotriene B_4 synthesis

*$^{13}C_2$-Methyl 5-[(tert-Butyldimethylsilyl)oxy]-6-heptynoate (**11**).* ^{1}H-NMR δ 4.4 (1H, m, H_6), 2.43 (1H, ddd, $J_{H7-C7} = 247.5$ Hz, $J_{H7-C6} = 51.4$ Hz, $J_{H7-H5} = 2.1$ Hz, H_7).

*$^{13}C_4$-Methyl 5,12-Bis[(tert-Butyldimethylsilyl)oxy]eicosa-8,10,14-trien-6-ynoate (**12**).* ^{1}H-NMR δ 6.56 (1H, m, H_9), 6.18 (1H, m, H_{10}), 5.77 (1H, m, H_{11}), 5.55 (1H, m, H_8),

*$^{13}C_4$-Methyl 5,12-Dihydroxy-8,10,14-eicosatrien-6-ynoate (**13**).* ^{1}H-NMR (300 MHz) idem **12**. High-resolution **MS of the di-TMS derivative (CI–NH_3)** found for $[M + NH_4]^+$ m/z 514.3566, calculated for $^{13}C_4\ ^{12}C_{23}H_{52}NO_4Si_2 = 514.3571$;

*$^{13}C_4$-Methyl 5,12-Dihydroxy-6,8,10,14-eicosatetraenoate (**14**).* ^{1}H-NMR (300 MHz) idem **12**. High-resolution **MS of the di-TMS derivative (CI–NH_3)** found for $[M + NH_4]^+$ m/z 516.3733, calculated for $^{13}C_4\ ^{12}C_{23}H_{54}NO_4Si_2 = 516.3728$;

*$^{13}C_4$-5,12-Dihydroxy-6,8,10,14-eicosatetraenoic acid ($^{13}C_4$-LTB$_4$ **1**).* ^{1}H-NMR: for H_8, H_9, H_{10}, H_{11} data was identical to literature values obtained after irradiation of ^{13}C atoms. Figure 6.

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