SYNTHESIS OF [8,9,10,11-$^{13}C_4$]LEUKOTRIENE C_4.

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Summary.

A "one pot" reduction of ethyl [1,2-$^{13}C_2$]bromoacetate with diisobutylaluminium hydride in dichloromethane, followed by reaction with triphenylphosphine, then triethylamine, yields [1,2-$^{13}C_2$]formylmethylenetriphenylphosphorane. Consecutive Wittig reactions of [1,2-$^{13}C_2$] formylmethylenetriphenylphosphorane with methyl 5(S),6(R)-epoxy-6-formylhexanoate and subsequent Wittig reaction with Z-3-nonen-1-triphenylphosphorane yields [8,9,10,11-$^{13}C_4$]LTA_4 methyl ester, which is readily converted to [8,9,10,11-$^{13}C_4$]LTC_4.

Key words: [$^{13}C_4$]-labelling; leukotrienes; mass spectrometry.

Introduction.

The cysteine-containing leukotrienes (LTs) (LTC_4, LTD_4 and LTE_4) are formed by the enzymic metabolism of LTA_4, which is formed *via* the 5-lipoxygenase pathway from arachidonic acid[1]. These compounds are produced by many different cell types (including human neutrophils, eosinophils and macrophages) in response to a number of different stimuli. Their biological effects, elicited at very low concentrations, include smooth muscle contraction and hypersensitivity reactions[2].

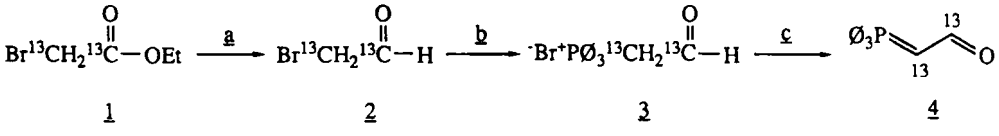
Quantification of cysteine-containing LTs is commonly achieved by bioassay or radioimmunoassay but the development of a procedure based on stable isotope dilution and mass spectrometry (MS) is desirable to provide reference data. MS analysis of intact cysteine-containing LTs may be achieved using desorption techniques such as fast atom bombardment[3]. Several recent reports describe the preparation of [^{2}H]-labelled analogues which may be suitable as internal standards[4]. Balazy and Murphy[5] have described a procedure based on gas chromatography (GC)/MS of a derivative of 5-hydroxyeicosanoic acid by hydrogenation and reduction of the cysteine-containing LTs. [$^{18}O_2$]-5-hydroxyeicosatetraenoic acid was used as the internal standard; [^{2}H]-labelled analogues were considered unsuitable because of the likelihood of isotopic exchange during catalytic hydrogenation. It is clear that the ideal internal standard for the GC/MS and other MS procedures would be a [^{13}C]-analogue, with sufficient labelled sites to avoid interference from natural isotopic variants in the MS detection of the internal standard. Here we describe the simple preparation of [8,9,10,11-$^{13}C_4$]LTC_4 *via* [1,2-$^{13}C_2$]formylmethylenetriphenylphosphorane.

0362–4803/91/030313–06$05.00

Received September 9, 1990
Revised October 15, 1990

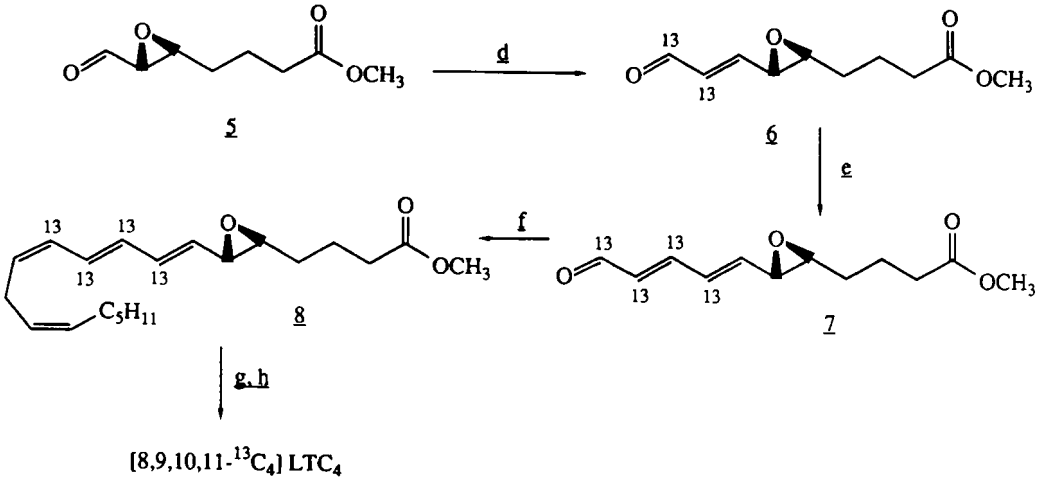
Results and Discussion.

The reduction of carboxylic esters generally leads to the formation of an alcohol; however, the aldehyde may be produced in high yield by partial reduction using selective agents and low temperatures[6]. Scheme 1 shows an outline of the synthetic scheme followed to produce [1,2-$^{13}C_2$]formylmethylenetriphenylphosphorane.



1 2 3 4

Scheme 1. a diisobutylaluminium hydride, dichloromethane, -78°C; b triphenylphosphine, acetonitrile, reflux; c triethylamine.



5 6 7 8

Scheme 2. d, [1,2-$^{13}C_2$]formylmethylenetriphenylphosphorane, benzene, reflux; e, [1,2-$^{13}C_2$]formylmethylenetriphenylphosphorane, toluene, reflux; f, Z-3-nonene-1-triphenyl-phosphorane, hexamethyl-phosphoramide, tetrahydrofuran; g, glutathione, triethylamine, methanol; h, lithium hydroxide.

The subsequent synthetic procedure to produce [8,9,10,11-$^{13}C_4$]LTC_4 is outlined in Scheme 2 and is a modification of the method reported by Rokach[9]. Incorporation of [1,2-$^{13}C_2$]formylmethylenetriphenylphosphorane into the synthesis of [8,9,10,11-$^{13}C_4$]LTA_4 methyl ester is readily achieved and the final product [8,9,10,11-$^{13}C_4$]LTC_4 is obtained in modest yield. The ultraviolet spectrum of [8,9,10,11-$^{13}C_4$]LTC_4 (λ_{max} 279, 280 and 290 nm) indicated the E,E,Z,Z geometry about the double bonds[10]. Incorporation of ^{13}C was assesed by tandem MS analysis. Narrow mass range parent ion scanning of the precursor ions of the product of m/z 308 (corresponding to the glutathione moiety) provided an indication of the isotopic composition of the lipid-derived portion of LTC_4. [$^{13}C_1$], [$^{13}C_2$] and [$^{13}C_3$] analogues acccounted for < 1% of [$^{13}C_4$]LTC_4 (ie > 97% [$^{13}C_4$]LTC_4).

Experimental.

General

Ethyl [1,2-$^{13}C_2$]bromoacetate (99% ^{13}C) was obtained from Sigma (St. Louis, MO). Diisobutylaluminium hydride (1M in dichloromethane) was obtained from Aldrich (Milwaukee, WI). Solvents were of HPLC grade (B and J brand, Houston, TX) and used as received (except that dichloromethane was distilled over calcium hydride before use). Proton and carbon 13 nmr spectra were obtained on a General Electric QE-300 NMR spectrometer (300 or 75 MHz) using deuterochloroform as solvent and tetramethylsilane as internal standard. Preparative and analytical HPLC were performed using two Waters 6000A pumps, a 660 gradient controller, and a 441 ultraviolet detector at 280 nm (Waters, Milford, MA). All mass spectra were run on a ZAB SEQ (VG Analytical, Manchester, UK).

[1,2-$^{13}C_2$]formylmethylenetriphenylphosphorane.

Ethyl [1,2-$^{13}C_2$]bromoacetate 1 (1.0 g, 5.9 mmol) was dissolved in dichloromethane (10 ml) and cooled to -78°C. Diisobutylaluminium hydride (6 ml, 1.0 M, 6 mmol) was then added over 2 minutes and the solution stirred at -78°C. After 30 minutes saturated ammonium chloride (0.75 ml) was added and the solution warmed to 23°C over 5 minutes. Acetonitrile (10 ml) was added, followed by triphenylphosphine (1.50 g, 5.7 mmol). The resultant solution was refluxed for 30 minutes, and triethylamine (2 ml, 14.4 mmol) and dichloromethane (35 ml) were then added. The resulting solution was washed with saturated sodium chloride (3 x 35 ml) and dried (sodium sulphate). Removal of the solvent under reduced pressure gave an orange solid (1.95g) which was purified by flash chromatography on silica (diethyl ether:methanol, 85:15) to give 0.93 g (3.06 mmol, 52%) of [1,2-$^{13}C_2$]formylmethylenetriphenylphosphorane 4[7,8].

^1H nmr (300 MHz, $CDCl_3$) δ: 8.97, ddd, J_{H2P}=39 Hz, J_{H2C2}=162.4 Hz, J_{H2C1}=27.8 Hz (E), 8.22, d m, J_{H2C2}=162.4 Hz (Z), 1H, (H2); 7.8-7.4, m, 15H (aromatic); 4.5-3.3 (E and Z), b m, 1H (H2).

Partial ^{13}C nmr (75 MHz, $CDCl_3$) δ: 181.61, d, J_{C2C1}=62.3 Hz, (C2); 54.82, J_{C1C2}=62.3 Hz, J_{C1P}=100 Hz (C1).

MS (FAB, glycerol matrix): $[M+H]^+$, m/z 307.

[8,9,10,11-$^{13}C_4$]-5(S), 6(R)-oxido-11-oxo-7(E), 9(E)-undecadienoate.

Methyl 5(S),6(R)-epoxy-6-formylhexanoate 5 (0.20 g, 1.16 mmol) and [1,2-$^{13}C_2$]formylmethylenetriphenylphosphorane 4 (0.36 g, 1.18 mmol) were refluxed in benzene (10 ml). After 30 minutes the reaction mixture was separated by flash chromatography on silica (diethyl ether:hexane 1:1) to give the α,β-unsaturated aldehyde 6 (0.106 g, 0.54 mmol, 46%).

^1H nmr (300 MHz, $CDCl_3$) δ): 9.57, ddd, $J_{H9,8}$=7.52 Hz, $J_{H9,C9}$=173.7 Hz, $J_{H9,C8}$=26.4 Hz, 1H, (H9); 6.7-6.08, b m, 2H, (H8 and 7); 3.69, s, 3H (OCH_3); 3.35, m, 1H (H6); 2.97, m, 1H (H5); 2.40, t, $J_{H2,3}$=7 Hz, 2H (H2), 1.6-1.9, m, 4H (H3 and 4).

MS (Chemical ionization (CI), methane, pressure measured at ion source housing: 10^{-4} mbar, 70eV): MH^+ m/z 201 (25%), -H_2O 183 (100%), -MeOH, 169 (95%).

The α,β-unsaturated aldehyde 6 (0.156 g, 0.79 mmol) and [1,2-$^{13}C_2$]formylmethylene-triphenylphosphorane 4 (0.24 g, 0.79 mmol) were refluxed in toluene (5 ml) for 30 minutes. The reaction products were again separated by flash chromatography (diethyl ether:hexane 1:1) to give [8,9,10,11-$^{13}C_4$]-5(S), 6(R)-oxido-11-oxo-7(E), 9(E)-undecadienoate 7 (0.073 g, 0.32 mmol, 41%).

^{1}H nmr (300 MHz, $CDCl_3$) δ: 9.58, ddd, $J_{H11,10}$=7.77 Hz, $J_{H11,C11}$=171.9 Hz, $J_{H11,C10}$=25.9 Hz, 1H, (H11); 7.4-5.8, b m, 4H, (H10,9,8 and 7); 3.69, s, 3H (OCH_3); 3.23, m, 1H (H6); 2.92, m, 1H (H5); 2.40, t, $J_{H2,3}$=7.2 Hz, 2H (H2), 1.6-1.9, m, 4H (H3 and 4).

MS (CI, methane, pressure measured at ion source housing: 10^{-4} mbar, 70eV): MH^+ 229 (34%), -H_2O 221 (35%), -MeOH, 197 (77%), 129 (100%).

[8,9,10,11-$^{13}C_4$]LTC_4.

Z-3-nonene-1-triphenylphosphonium iodide (67 mg, 0.13 mmol) in tetrahydrofuran (750 μl) was cooled to -55°C, n-butyllithium (75 μl, 1.5 m, 0.12 mmol) was added and the solution left for 10 minutes at -55°C. Hexamethylphosphoramide (190 μl, 1.1 mmol) in tetrahydrofuran (300 μl) was added followed by [8,9,10,11-$^{13}C_4$]-5(S), 6(R)-oxido-11-oxo-7(E), 9(E)-undecadienoate 7 (20 mg, 0.09 mmol) in tetrahydrofuran (1 ml). The solution was left for 10 minutes at -55°C, then warmed to 0°C. Aqueous sodium bicarbonate (2%, 4 ml) was then added to the solution which was extracted with diethyl ether (3 x 10 ml) and washed with 2% sodium bicarbonate and saturated sodium chloride (2 x 5 ml) before drying over sodium sulphate. Flash chromatography (diethyl ether:hexane:triethylamine, 1:4:0.01) gave [8,9,10,11-$^{13}C_4$]LTA_4 methyl ester 8 (17 mg, 0.151 mmol, 58%)[9,10].

[8,9,10,11-$^{13}C_4$]LTA_4 methyl ester 8 (1 mg, 3.0 μmol), glutathione (4 mg, 13 μmol) and triethylamine (4 μl) were stirred at 23°C for 3 hours in methanol (100 μl). Buffer (acetic acid 10 mM, pH adjusted to 5.7 with ammonium acetate) was added and the solution purified by reverse phase HPLC (C_{18} μBondapak, 7.8 mm x 30 cm; gradient, 50 to 100% methanol in buffer, 20 minutes; 4 ml/minute). The buffer was removed under reduced pressure to give [8,9,10,11-$^{13}C_4$]LTC_4 methyl ester, which was hydrolysed by lithium hydroxide (10% methanol, 100μl, 1 M) for 10 minutes, then purified by HPLC (C_{18} μBondapak, 3.9 mm x 30 cm; gradient, 5 to 100% methanol in buffer, 19 minutes; 1.5 ml/minute). Removal of the buffer gave [8,9,10,11-$^{13}C_4$]LTC_4 (226 μg, 359 nmol, 12 %).

MS (FAB, thioglycerol:2-hydroxyethyldisulphide (1:1) matrix): $[M+H]^+$ m/z 630. Tandem MS, precursor ion 630; product ions (relative abundance %) 612 (16%), 323 (20%), 308 (100%), 305 (23%), 191 (17%).

UV, λ_{max} 269, 280, 290 nm.

Conclusion.

In summary, the partial reduction of ethyl [1,2-$^{13}C_2$]bromoacetate 1 to [1,2-$^{13}C_2$]bromoacetaldehyde 2, reaction with triphenylphosphine, and subsequent deprotonation represents an efficient procedure for the preparation of [1,2-$^{13}C_2$]formylmethylene-triphenylphosphorane 4. This labelled phosphorane 4 has been incorporated into the synthesis

of [8,9,10,11-$^{13}C_4$]LTA_4 methyl ester 8, with subsequent conversion to [8,9,10,11-$^{13}C_4$]LTC_4. Equivalent procedures may be employed for the synthesis of other [$^{13}C_4$]-cysteine containing leukotrienes. These compounds are suitable as stable isotopically labelled internal standards in the quantification of the leukotrienes.

Acknowledgments.

We thank Drs. H. Kohn and A. Sayed (Department of Chemistry, University of Houston) for access to NMR facilities. This work was supported by the National Institutes of Health (grants AI 26916 and GM 34120).

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