Research Article

Journal of Labelled Compounds and Radiopharmaceuticals

Received 9 October 2007, Accepted 8 November 2007 Published online 25 January 2008 in Wiley Interscience

(www.interscience.wiley.com) DOI: 10.1002/jlcr.1485

Synthesis of the lipid peroxidation product 4-hydroxy-2(E)-nonenal with ^{13}C stable isotope incorporation

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The aim of this work was to synthesize ^{13}C internal standards for the quantification of 4-hydroxy-2(E)-nonenal (HNE), a lipid peroxidation product, and of the etheno-adducts possibly formed by HNE damage to DNA nucleobases. We designed an eight-step synthesis starting from ethyl 2-bromoacetate and giving access to 4-[(tetrahydro-2*H*-pyran-2-yl)oxy]-2(E)-nonenal. This compound is a precursor of HNE. The scheme was then used to produce the ^{13}C precursor [1,2-$^{13}C_2$]-4-[(tetrahydro-2*H*-pyran-2-yl)oxy]-2(E)-nonenal. [1,2-$^{13}C_2$]-HNE was obtained by acid deprotection. All the intermediary and final compounds were fully characterized by IR, HRMS, ^{1}H and ^{13}C NMR. It is the first synthesis of HNE which enables the incorporation of two ^{13}C labels at determined positions.

Keywords: lipid peroxidation; 4-hydroxy-2(E)-nonenal; HNE; carbon 13; synthesis

Introduction

4-hydroxy-2(E)-nonenal (HNE) is one of the major α,β-unsaturated aldehyde arising from the lipid peroxidation of ω-6 fatty acids. It has cytotoxic and genotoxic effects and is probably implied in pathologies related to oxidative stress.

The measurement of HNE is a challenge since the molecule is highly reactive. Internal standards are needed for mass spectrometry analyses and quantification. Radio-labeled compounds have been developed as tracers for biotransformation studies,[1–4] but they do not have any utility as reference standards for mass spectrometry measurement. Stable isotopes can overcome this problem, and the labeling of HNE or metabolites has been used so far with deuterium derivatives.[5–8]

Indirect detection of metabolites has also held the attention. Metabolic activation could epoxide HNE. The epoxide reacts with DNA nucleobases to form ring-extended structures called etheno-adducts, which are likely to play a role in carcinogenesis process.[9] These structures can retain the whole aliphatic chain of HNE or be restricted to the two etheno carbons (Figure 1).

Therefore, only the adducts retaining the whole aliphatic structure are specific of the exposure to a defined chemical because the simple etheno-adducts (without side–Chain) can be generated by many bifunctional aldehydes coming from the exogenous exposure to synthetic chemicals as well as from endogenous lipid peroxidation products. Labeling by stable isotope has been used for the measurement by mass spectrometry (LC/MS, GC/MS) of simple etheno-adducts arising from various chemicals. To our knowledge, stable isotopes (^{13}C and ^{15}N) have been incorporated into nucleobases and into chemicals (^{13}C-haloacetaldehyde).[10,11]

Many different synthetic approaches have been described in the literature for the preparation of non-labeled stable aldehyde-protected HNE precursors,[12–16] but none of them was directly transposable with ^{13}C precursors, except Armanath's synthesis of HNE with one ^{13}C, which provided structural information on protein adducts using ^{13}C NMR spectrometry.[17]

Therefore, the measurement of HNE by ^{13}C labeling has not been reported yet. Besides, the *in vivo* quantification of specific etheno-adducts arising from HNE is lacking. The aim of our study was to design a synthetic scheme to the target [1,2-$^{13}C_2$]-4-hydroxy-2(E)-nonenal with stable labeling by two ^{13}C at defined positions. The product was needed in a few milligram quantity so that quantification methodologies by mass spectrometry could be initially developed for HNE measurement. We describe the ^{12}C test synthesis and the ^{13}C analog in eight steps.

Results and discussion

The structural analysis of HNE shows a nine carbon skeleton with three functions: an α,β-unsaturated aldehyde and an alcohol at the allylic position. This high degree of functionality on four carbon atoms makes the three functions highly reactive and the compound is known to decompose easily at room temperature. Using a retrosynthetic approach, disconnection between C3 and C4 seemed to be an interesting solution and it was described in the literature for the production of 4-[(tetrahydro-2*H*-pyran-2-yl)oxy]-2(E)-nonenal as an intermediary product in the synthesis of a prostaglandin.[18,19] Yet, this was not appropriate for the

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introduction of ^{13}C into the structure of HNE. The main limitation was to select a suitable ^{13}C commercial precursor, preferably with two labels on carbons C1 and C2. We thought that a Wittig reaction on a stabilized ylide would be the key of the carbon skeleton construction and the best way to easily introduce ^{13}C at the target position with ethyl [1,2-$^{13}C_2$]-2-bromoacetate as starting material.

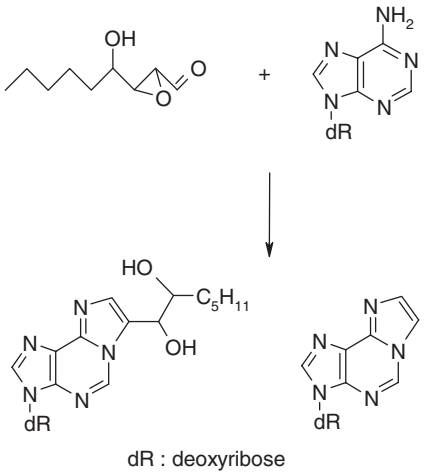
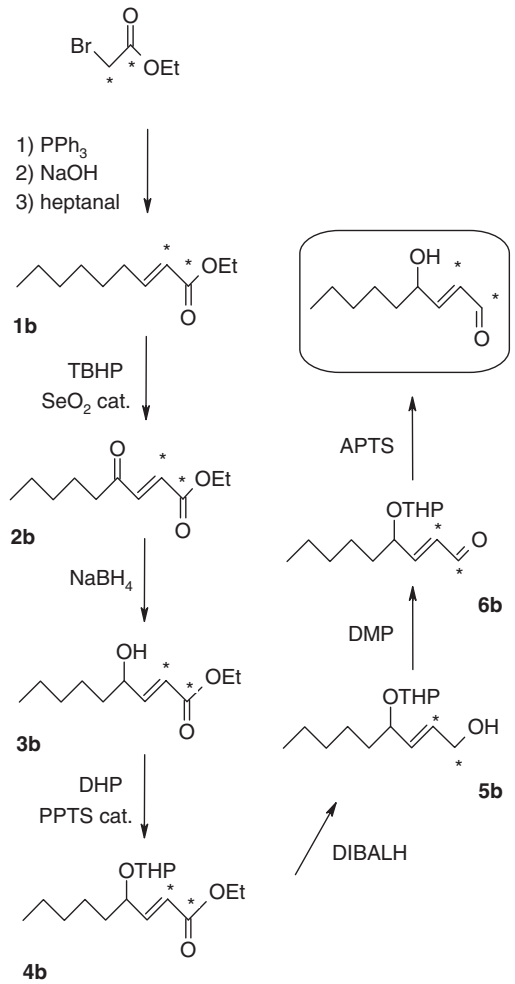


Figure 1. Etheno-adducts with and without HNE side–chain.



Asterisks indicate ^{13}C atoms.

Scheme 1

A retrosynthetic path based on the late disconnection of the olefin bond was then selected. It corresponded to a linear synthesis (Scheme 1, Asterisks indicate ^{13}C atoms). The synthetic procedures were initially developed with ^{12}C products (**1a**–**6a**).

The first steps of the synthesis were traditional Wittig chemistry. Ethyl 2-bromoacetate was converted to the phosphonium salt which gave the stabilized ylide in quantitative yield. The wittig reaction on heptanal gave the α,β-unsaturated ester **1a** with *E* stereochemistry. We then considered that the 4-hydroxy functionality could be introduced on this ester. Yet, allylic oxidation of the electro-deficient olefin was believed to be very difficult. Selenium oxide associated to *tert*-butyl hydroperoxide (TBHP) is the reagent of choice for allylic hydroxylation of sterically hindered olefines and allylic ketone are often observed. To our knowledge, this reaction has never been described on a deactivated alkene, although ketone **2a** was obtained by CrO_3-Oxidation.[20] **2a** was also synthesized from another starting material, a 2,4-dioxo-alkenoate, which was not suitable for our synthetic approach.[21]

Since we wanted to avoid the use of chromium oxide, we tried different reaction conditions and obtained the allylic ketone in low yield (25%) with excess TBHP and selenium oxide in catalytic amount, after three days at $+65^{\circ}C$. This ketone **2a** was then reduced to the alcohol **3a** with sodium borohydride in methanol at $0^{\circ}C$. This alcohol has been reported as an intermediary structure in the synthesis of lactones.[22] We then protected it as tetrahydro-2*H*-pyran (THP) to **4a**. Eventually, the ester function was changed to an aldehyde in two steps. Reduction with diisobutyl aluminum hydride (DIBALH) did not stop at the aldehyde but gave the alcohol **5a**; then oxidation with Dess–Martin periodinane (DMP)[23] led to the aldehyde **6a**. No degradation of the product occurred after at least one month as checked by NMR. The protective group was removed in methanol with catalytic amount of paratoluene sulfonic acid (APTS) and free HNE was obtained.

We then applied the same scheme starting from 1 g of ethyl [1,2-$^{13}C_2$]-2-bromoacetate and up to 51 mg of **6b** could be obtained. Acid deprotection with APTS on an aliquot afforded [1,2-$^{13}C_2$]-4-hydroxy-2(E)-nonenal. HRMS, ^1H and ^{13}C NMR of the crude material enabled analytical characterization and estimation of the purity (90%). Because the product started to decompose after 24 h, no efficient purification was possible and it has to be prepared just before use. Chemical shifts were in accordance with those of HNE and large $^1H/^{13}C$ coupling constants were observed. All the other ^{12}C and ^{13}C products were fully characterized by FT-IR, HRMS, ^1H and ^{13}C NMR spectrometries. Ester $^{13}C=O$ stretch band showed about 40 cm^{-1} negative shift compared with ester $^{12}C=0$ stretch band in IR spectra. The multiplicity and chemical shifts of the olefin protons in ^1H NMR analyses clearly reflect the chemical environment. THP introduces a chiral center in the structures and leads to the observation of diastereomeric mixtures. ^{13}C products show characteristic $^1H/^{13}C$ coupling constants.

Experimental

Caution: HNE is cytotoxic and ethyl bromoacetate is carcinogenic. They should be handled carefully with gloves under a well-ventilated hood.

Chemicals

Chemicals and solvents were purchased from Acrös Organics (Belgium) and were used without purification, except heptanal that was distilled before use.

Ethyl [1,2-$^{13}C_2$]-2-bromoacetate (99% atom ^{13}C) was from Eurisotop (France).

DMP was prepared from standard method.[23] Column chromatography was carried out on silica gel 60 (63–200 mesh, Merck, Germany) and silica gel 60F254 plates (20∗20 cm∗ 0.25 mm); the eluent was AcOEt /petroleum ether (1: 4, by vol.) unless otherwise stated.

Melting points were measured on a Tottoli apparatus and are uncorrected.

FT-IR spectra were recorded on a Perkin Elmer Paragon 1000 using KBr plates.

NMR spectra were recorded on a Bruker DRX Avance 600 equiped with a 5 mm TXI cryo-probe. Chemical shifts are reported in parts per million (ppm) relative to $CDCl_3$ (δ: 7.25 for ^1H, 77.0 for ^{13}C).

High-resolution accurate mass measurements were performed on a LTQ–Orbitrap (Thermo Fisher Scientific) equipped with an electrospray ionization probe and operated in the positive ion mode. Two microliter of analyte solutions (2 ng/μL) was injected in pure methanol at 15 μL/min. Measurements were made at a resolving power of 60 000 at *m/z* 400 in internal calibration mode using impurity ions at *m/z* 413.266230. The mass accuracies of all mass spectra were <1 ppm. The chemical formulas were obtained from the accurate *m/z* ions.

Phosphonium salt and ylide preparation

To 9.75 g (37.2 mmol) of triphenylphosphine in 30 mL of toluene was added dropwise 4.1 mL (1 equiv.) of ethyl bromoacetate. The white suspension was heated to 70°C with stirring for 2 h.

Heating was stopped, the white solid filtered and washed with toluene, then precipitation in diethyl ether afforded 15.485 g of carbethoxymethyl triphenylphosphonium bromide as a white solid. Yield: 97%. mp: 158–159°C (lit.: 158°C).

Carbethoxymethyl triphenylphosphonium bromide 15.485 g (36.1 mmol) was mixed with 60 mL of AcOEt/DCM (3:1, by vol.) and a concentrated solution of sodium hydroxide was added. When the phases had cleared, the organic phase was separated and dried with magnesium sulfate, then evaporated. Carbethoxymethylene triphenylphosphorane (12.56 g) was obtained as a white solid. Yield: 100%. mp: 126–127°C (lit.: 124–129°C).

*Ethyl 2(E)-nonenoate (**1a**)*

Five milliliter of heptanal (36.1 mmol) was diluted by 25 mL of toluene. Under stirring, 12.56 g of carbethoxymethylene triphenylphosphorane (1 equiv.) was added and the mixture was heated to reflux for 18 h.

Upon cooling, a white solid (Ph_3PO) was discarded after filtration. Chromatography afforded 6.3 g of **1a** as a colourless liquid. Yield: 75%.

IR (neat): 2930; 1723; 1655; 1466; 1368; 1307; 1267; 1191; 1174; 1127; 1044; 977.

HRMS: *m/z* 185.15368 (MH^+ [$C_{11}H_{21}O_2$] = 185.15361).

^1H NMR: in accordance with the literature.[21]

^{13}C NMR: 168.3; 151.1; 122.8; 61.7; 33.8; 33.1; 30.4; 29.5; 24.1; 15.8; 15.6.

*Ethyl 4–Oxo-2(E)-nonenoate (**2a**)*

TBHP 26 mL (8 equiv.) and 759 mg (0.2 equiv.) of SeO_2 were added to 6.3 g (34.2 mmol) of **1a**. The mixture was stirred and heated to 65°C for 72 h, and then cooled. The yellow oil was separated from the aqueous phase, then it was diluted with DCM, washed with brine and dried with magnesium sulfate. Purification by chromatography with diethyl ether/petroleum ether (1:9, by vol.) afforded 1.734 g of **2a** as a light yellow liquid. Yield: 25%.

IR (neat): 2932; 1753; 1735; 1650; 1638; 1459; 1366; 1253; 1195; 1033.

HRMS: *m/z* 221.11495 (MNa^+ [$C_{11}H_{18}O_3Na$] = 221.11482). ^1H NMR: in accordance with the literature.[21]

^{13}C NMR: 199.9; 165.6; 139.4; 130.7; 61.4; 41.5; 31.3; 23.4; 22.4; 14.1; 13.9.

*Ethyl 4-hydroxy-2(E)-nonenoate (**3a**)*

Sodium borohydride 133 mg (1 equiv.) was added to 650 mg (3.28 mmol) of **2a** in 2 mL of methanol. After stirring 1 h, water was added and the mixture was carefully acidified with HCl solution. The mixture was extracted with diethyl ether, washed with brine and dried with magnesium sulfate. Chromatography afforded 432 mg of **3a** as a light yellow liquid. Yield: 97%.

IR (neat): in accordance with the literature.[22]

HRMS: *m/z* 223.13062 (MNa^+ [$C_{11}H_{20}O_3Na$] = 223.13047).

^1H NMR: 6.93 (1H, dd, J = 4.99; 15.7 Hz, CH = C); 6.01 (1H, dd, J = 1.61; 15.7 Hz, =CH−CO_2Et); 4.28 (1H, dt, J = 1.33; 6.79 Hz, CH(OH)); 4.18 (2H, q, J = 7.13 Hz, CO_2–CH_2); 1.56 (2H, m, CH_2–CH(OH)); 1.30–1.20 (6H, m+3H, t, J = 7.11 Hz, CO_2–CH_2–CH_3); 0.87 (3H, t, J = 6.89 Hz, CH_3).

^{13}C NMR: 166.6; 150.3; 120.1; 71.3; 60.4; 36.6; 31.6; 24.9; 22.5; 14.2; 14.0.

*Ethyl 4-[(tetrahydro-2H-pyran-2-yl)oxy]-2(E)-nonenoate (**4a**)*

3a 190 mg (0.950 mmol) was diluted with 2 mL of DCM. DHP 0.43 mL (5 equiv.) and 39 mg (0.1 equiv.) of PPTS were added and the mixture was stirred for 3 h. After washing with brine, the mixture was chromatographied and afforded 169 mg of **4a** as a colorless liquid. Yield: 63%.

IR (neat): 2934; 1725; 1633; 1466; 1368; 1350; 1261; 1196; 1165; 1128; 1078; 1035; 984; 904; 870; 815.

HRMS: *m/z* 307.18803 (MNa^+ [$C_{16}H_{28}O_4Na$] = 307.18798).

^1H NMR: 6.95 (1H, dd, J = 5.39; 15.7 Hz, CH = C); 6.77 (1H, dd, J = 6.67; 15.7 Hz, CH = C); 6.05 (1H, dd, J = 1.53; 15.6 Hz, = CH–CO_2Et); 5.93 (1H, dd, J = 1.04; 15.7 Hz, = CH–CO_2Et); 4.70 (1H, t, J = 3.37 Hz, O–CH–O); 4.56 (1H, m, O–CH–O); 4.26 (2H, m, CH–OTHP); 4.18 (4H, m, CO_2–CH_2); 3.86 (2H, m, O–CH_2); 3.46 (2H, m, O–CH_2); 1.82 (2H, m, CH_2(THP)); 1.70 (2H, m, CH_2(THP)); 1.55–1.50 (8H, m, $(CH_2)_2$(THP)+4H, m, CH_2–CH-OTHP); 1.30–1.20 (12H, m, $(CH_2)_3$–CH_3+6H, m, CO_2–CH_2–CH_3); 0.88 (6H, m, CH_3).

^{13}C NMR: 166.7; 166.3; 149.1; 148.3; 122.1; 120.4; 97.3; 96.1; 75.0; 74.4; 62.4; 62.4; 60.5; 60.3; 35.2; 33.8; 31.8; 31.7; 30.7; 25.5; 25.4; 25.0; 22.5; 22.5; 19.4; 19.4; 14.25; 14.23; 14.04; 14.01.

*4-[(tetrahydro-2H-pyran-2-yl)oxy]-2(E)-nonenol (**5a**)*

4a (169 mg (0.595 mmol)) was cooled to 0°C. 1.18 mL (2 equiv.) of DIBALH (1 M solution in hexane) was added. After 2 h, methanol was added then 1 N HCl drop by drop until pH = 4. The

mixture was extracted with DCM, washed with brine and dried on magnesium sulfate. Chromatography with AcOEt/petroleum ether (1:2, by vol.) afforded **5a** as a colourless liquid (74 mg). Yield: 63%.

IR (neat): 3424; 2936; 1458; 1377; 1346; 1200; 1130; 1112; 1077; 980; 904; 860; 812.

HRMS: *m/z* 265.17739 (MNa^+ [$C_{14}H_{26}O_3Na$] = 265.17742).

^{1}H NMR: 5.80 (2H, 2td, J = 5.23; 15.6 Hz, CH = C); 5.73 (1H, dd, J = 6.64; 15.6 Hz, = CH–CH_2OH); 5.51 (1H, dd, J = 7.98; 15.6 Hz, = CH–CH_2OH); 4.67 (1H, m, O–CH–O); 4.65 (1H, t, J = 3.18 Hz, O–CH–O); 4.15 (2H, dd, J = 1.15; 4.44 Hz, CH_2–OH); 4.13 (2H, d, J = 5.92 Hz, CH_2–OH); 4.06 (2H, m, CH–OTHP); 3.87 (2H, m, O–CH_2); 3.47 (2H, m, O–CH_2); 1.82 (2H, m, CH_2(THP)); 1.69 (2H, m, CH_2(THP) + 2H, m, CH_2-CH-OTHP); 1.60–1.50 (8H, m, $(CH_2)_2$THP + 2H, m, CH_2-CH-OTHP); 1.30 (12H, m, $(CH_2)_3$–CH_3); 0.87 (6H, m, CH_3).

^{13}C NMR: 133.2; 132.1; 131.8; 129.7; 97.8; 94.9; 75.3; 63.3; 63.0; 62.7; 62.2; 35.8; 34.6; 31.9; 31.8; 30.9; 30.8; 25.6; 25.4; 25.2; 24.7; 22.6; 19.8; 19.5; 14.1; 14.0.

*4-[(tetrahydro-2H-pyran-2-yl)oxy]-2(E)-nonenal (**6a**)*

5a (34 mg (0.140 mmol)) was diluted with 3 mL of DCM. DMP (59 mg (1 equiv.)) was added and the suspension was heated to reflux for 2 h. After removal of the white solid by filtration, the filtrate was washed twice with saturated $NaHCO_3$ solution, once with brine and dried with magnesium sulfate. Chromatography on a silica gel plate afforded **6a** (21 mg as light yellow oil). Yield: 62%.

IR (neat): 2938; 1696; 1458; 1377; 1342; 1201; 1126; 1078; 1023; 979; 904; 870; 815.

HRMS: *m/z* 263.16187 (MNa^+ [$C_{14}H_{24}O_3Na$] = 263.16177).

^{1}H NMR: 9.57 (1H, d, J = 5.76 Hz, CHO); 9.56 (1H, d, J = 5.77 Hz, CHO); 6.84 (1H, dd, J = 5.28; 15.7 Hz, CH = C); 6.68 (1H, dd, J = 6.02; 15.7 Hz, CH = C); 6.31 (1H, dd, J = 7.97; 15.7 Hz, = CH–CHO); 6.21 (1H, dd, J = 7.89; 15.7 Hz, = CH–CHO); 4.70 (1H, m, O–CH–O); 4.56 (1H, t, J = 3.48 Hz, O–CH–O); 4.42 (1H, q, J = 6.27 Hz, CH–OTHP); 4.36 (1H, q, J = 5.40 Hz, CH–OTHP); 3.89 (1H, ddd, J = 2.92; 8.20; 11.2 Hz, CH_2–O); 3.80 (1H, ddd, J = 3.21; 8.23; 11.3 Hz, CH_2–O); 3.50 (1H, m, CH_2–O); 3.48 (1H, m, CH_2–O); 1.82 (2H, m, CH_2(THP)); 1.70 (2H, m, CH_2(THP) + 2H, m, CH_2–CH–OTHP); 1.60–1.50 (8H, m, $(CH_2)_2$THP + 2H, m, CH_2–CH–OTHP); 1.30 (12H, m, $(CH_2)_3$–CH_3); 0.88 (6H, t, CH_3).

^{13}C NMR: 193.9 (d, J = 10.6 Hz); 193.5 (d, J = 10.8 Hz); 158.4; 157.3; 132.3; 131.1; 98.0; 96.4; 75.5; 74.3; 62.7; 62.4; 35.0; 33.9; 31.7; 31.7; 30.7; 30.6; 25.4; 25.3; 25.0; 24.4; 22.5; 19.5; 19.3; 14.02; 13.99.

THP was removed in methanol with APTS (1 h). Concentration, addition of DCM and washing with brine afforded HNE (^{1}H NMR in accordance with the literature).

^{13}C syntheses

The same procedure was used starting from 1 g (5.92 mmol) of ethyl [1,2-^{13}C2]-2-bromoacetate to prepare 2.012 g of the corresponding ylide (97% yield).

*Ethyl [1,2-$^{13}C_2$]-2(E)-nonenoate (**1b**)*

Same procedure as for **1a** starting from 0.74 mL (5.31 mmol) of heptanal and 2.012 g (1 equiv.) of ylide.

Yield: 67% (738 mg, 3.97 mmol).

IR (neat): 2930; 1679; 1627; 1459; 1302; 1233; 1183; 1156; 1122; 1043; 975.

HRMS: *m/z* 209.14240 (MNa^+ [$^{13}C_2C_9H_{20}O_2Na$] = 209.14226).

^{1}H NMR: 6.96 (1H, dqd, J = 1.68; 6.87; 13.7 Hz, CH = ^{13}C); 5.80 (1H, dd, J = 15.6; 161 Hz, = ^{13}CH-$^{13}CO_2$Et); 4.18 (2H, dq, J = 2.99; 7.10 Hz, $^{13}CO_2$–CH_2); 2.18 (2H, p, J = 6.78 Hz, CH_2–CH = ^{13}C); 1.44 (2H, m, CH_2); 1.28 (6H, m, CH_2 + 3H, t, J = 7.09 Hz, $^{13}CO_2$–CH_2–CH_3); 0.89 (3H, t, J = 6.89 Hz, CH_3).

^{13}C NMR: **166.8** (d, J = 74.6 Hz, $^{13}CO_2$Et); 149.5 (d, J = 70.1 Hz); **121.2** (d, J = 74.6 Hz, **^{13}C**H–$^{13}CO_2$Et); 60.1; 32.2; 31.6; 29.8; 27.9; 22.5; 14.3; 14.1.

*Ethyl [1,2-$^{13}C_2$]-4–oxo-2(E)-nonenoate (**2b**)*

Same procedure as for **2a** starting from 715 mg (3.84 mmol) of **1b**, 3 mL (8 equiv.) of TBHP and 92 mg (0.2 equiv.) of SeO_2.

Yield: 18% (140 mg, 0.700 mmol).

IR (neat): 2959; 1774; 1682; 1602; 1466; 1366; 1288; 1267; 1245; 1222; 1159; 1030; 980; 865.

HRMS: *m/z* 223.12158 (MNa^+ [$^{13}C_2C_9H_{18}O_3Na$] = 223.12153).

^{1}H NMR: 7.05 (1H, ddd, J = 2.66; 6.63; 16.0 Hz, CH = ^{13}C); 6.66 (1H, ddd, J = 2.83; 16.0; 165 Hz, ^{13}CH-$^{13}CO_2$Et); 4.26 (2H, dq, J = 3.04; 7.10 Hz, $^{13}CO_2$–CH_2); 2.62 (2H, t, J = 7.38 Hz, CH_2–C(O)); 1.62 (2H, m, CH_2); 1.30 (6H, m + 3H, t, J = 7.12 Hz, $^{13}CO_2$–CH_2–CH_3); 0.89 (3H, t, J = 6.98 Hz, CH_3).

^{13}C NMR: 199.9; **165.6** (d, J = 73.9 Hz, **^{13}C**O_2Et); 139.4 (d, J = 69.7 Hz); **130.7** (d, J = 73.9 Hz, **^{13}C**H–$^{13}CO_2$Et); 61.4; 41.5; 31.3; 23.4; 22.4; 14.1; 13.9.

*Ethyl [1,2-$^{13}C_2$]-4-hydroxy-2(E)-nonenoate (**3b**)*

Same procedure as for **3a** starting from 110 mg (0.550 mmol) of **2b** and 21 mg (1 equiv.) of $NaBH_4$.

Yield: 91% (101 mg, 0.500 mmol).

IR (neat): 3450; 2933; 2860; 1678; 1629; 1489; 1364; 1293; 1250; 1154; 1077; 1039; 980.

HRMS: *m/z* 225.13726 (MNa^+ [$^{13}C_2C_9H_{20}O_3Na$] = 225.13718).

^{1}H NMR: 6.94 (2H, dddd, J = 2.66; 4.99; 6.86; 15.5 Hz, CH = ^{13}C) · 6.02 (1H, dddd, J = 1.57; 3.07; 15.7; 163 Hz, = ^{13}CH-$^{13}CO_2$Et); 4.30 (1H, m, CH(OH)); 4.20 (2H, dq, J = 2.99; 7.12 Hz, $^{13}CO_2$–CH_2); 1.56 (2H, m, CH_2–CH(OH)); 1.30–1.20 (6H, m + 3H, t, J = 7.11 Hz, $^{13}CO_2$–CH_2–CH_3); 0.89 (3H, t, J = 6.85 Hz, CH_3).

^{13}C NMR: **166.5** (d, J = 74.9 Hz, ^{13}**C**O_2Et); 150.1 (d, J = 71.5 Hz); **120.2** (d, J = 74.9 Hz, ^{13}**C**H–$^{13}CO_2$Et); 71.2; 60.5; 36.6; 31.7; 24.9; 22.5; 14.2; 14.0.

*Ethyl [1,2-$^{13}C_2$]-4-[(tetrahydro-2H-pyran-2-yl)oxy-2(E)-nonenoate (**4b**)*

Same procedure as for **4a** starting from 78 mg (0.386 mmol) of **3b**, 176 µL (5 equiv.) of DHP and 11 mg (0.1 equiv.) of PPTS.

Yield: 67% (74 mg, 0.259 mmol).

IR (neat): 2937; 1682; 1630; 1466; 1262; 1153; 1078; 1037; 980; 870; 815.

HRMS: *m/z* 309.19480 (MNa^+ [$^{13}C_2C_{14}H_{26}O_4Na$] = 309.19469).

^{1}H NMR: 6.95 (1H, m, CH = ^{13}C); 6.77 (1H, dtd, J = 2.38; 6.60; 15.6 Hz, CH = ^{13}C); 6.04 (1H, m, = ^{13}CH–$^{13}CO_2$Et); 5.92 (1H, ddd, J = 2.74; 15.7; 163 Hz, = ^{13}CH–$^{13}CO_2$Et); 4.71 (1H, m, O–CH–O); 4.57 (1H, m, O–CH–O); 4.23 (2H, m, CH–OTHP); 4.19 (4H, m, CO_2–CH_2); 3.83 (2H, m, O–CH_2); 3.47 (2H, m, O–CH_2); 1.82 (2H, m, CH_2(THP)); 1.70 (2H, m, CH_2(THP)); 1.60–1.50 (8H, m, $(CH_2)_2$THP + 4H, m, CH_2–CH-OTHP); 1.28 (12H, m, $(CH_2)_3$–CH_3 + 6H, m, CO_2–CH_2–CH_3); 0.87 (6H, m, CH_3).

^{13}C NMR: **166.7** (d, J = 74.8 Hz, ^{13}**C**O$_2$Et); **166.3** (d, J = 74.5 Hz, ^{13}**C**O$_2$Et); 149.1 (d, J = 71.5 Hz); 148.3 (d, J = 70.7 Hz); **122.1** (d, J = 74.5 Hz, ^{13}**C**H–^{13}CO$_2$Et); **120.4** (d, J = 74.8 Hz, ^{13}**C**H–^{13}CO$_2$Et); 97.3; 96.1; 75.0 (d, J = 6.29 Hz); 74.4 (d, J = 6.45 Hz); 62.40; 62.39; 60.4; 60.3; 35.2; 33.8; 31.8; 31.7; 30.74; 30.69; 25.6; 25.4; 25.0; 24.4; 22.5; 19.5; 19.4; 14.3; 14.06; 14.05.

*[1,2-$^{13}C_2$]-4-[(tetrahydro-2H-pyran-2-yl)oxy-2(E)-nonenol (***5b***)*

Same procedure as for **5a** starting from 50 mg (0.175 mmol) of **4b** and 350 µL (2 equiv.) of DIBALH.

Yield: 79% (34 mg, 0.139 mmol).

IR (neat): 3467; 3261; 2934; 1441; 1201; 1021; 810.

HRMS: *m/z* 267.18414 (MNa$^+$ [^{13}C$_2$C$_{12}$H$_{26}$O$_3$Na] = 267.18413).

^{1}H NMR: 5.93 (1H, m, CH = ^{13}C); 5.77–5.63 (2H, m, CH = ^{13}C, = ^{13}CH-^{13}CH$_2$OH); 5.51 (1H, m, = ^{13}CH–^{13}CH$_2$OH); 4.67 (1H, m, O–CH–O); 4.65 (1H, m, O–CH–O); 4.26 (2H, td, J = 4.46; 8.55 Hz, CH$_2$–OH); 4.13–4.01 (2H, m, CH$_2$–OH; 2H, m, CH–OTHP); 3.87 (2H, m, O–CH$_2$); 3.47 (2H, m, O–CH$_2$); 1.82 (2H, m, CH$_2$(THP)); 1.74–1.61 (2H, m, CH$_2$(THP)+2H, m, CH$_2$-CH-OTHP); 1.45–1.23 (8H, m, (CH$_2$)$_2$THP+2H, m, CH$_2$–CH–OTHP); 1.30 (12H, m, (CH$_2$)$_3$–CH$_3$); 0.87 (6H, t, J = 6.19 Hz, CH$_3$).

^{13}C NMR: 133.4; **132.1** (d, J = 45.9 Hz, =^{13}CH–^{13}CH$_2$OH); 132.1; **129.7** (d, J = 45.9 Hz, =^{13}CH–^{13}CH$_2$OH); 97.6; 94.9; 75.3; 63.2; 63.1 (t, j=45.5 Hz, =^{13}CH–^{13}CH$_2$OH); 63.0; 62.2; 35.8; 34.6; 31.9; 31.8; 30.9; 30.8; 25.6; 25.4; 25.3; 24.9; 22.6; 19.8; 19.5; 14.1; 14.0.

*[1,2-$^{13}C_2$]-4-[(tetrahydro-2H-pyran-2-yl)oxy-2(E)-nonenal (***6b***)*

Same procedure as for **6a** starting from 31 mg (0.127 mmol) of **5b** and 48 mg (1 equiv.) of DMP.

Yield: 62% (19 mg, 0.079 mmol).

IR (neat): 2945; 1653; 1440; 1388; 1342; 1201; 1127; 1077; 1023; 979; 906; 867; 814.

HRMS: *m/z* 265.16862 (MNa$^+$ [^{13}C$_2$C$_{12}$H$_{24}$O$_3$Na] = 265.16848).

^{1}H NMR: 9.59 (1H, ddd, J = 5.98; 7.70; 172 Hz, ^{13}CHO); 9.54 (1H, ddd, J = 5.68; 7.77; 172 Hz, ^{13}CHO); 6.84 (1H, ddd, J = 5.24; 9.19; 14.6 Hz, CH = ^{13}C); 6.68 (1H, ddd, J = 6.09; 9.04; 15.1 Hz, CH = ^{13}C); 6.31 (1H, ddd, J = 7.93; 15.7; 162 Hz, = ^{13}CH–^{13}CHO); 6.21 (1H, ddd, J = 7.82; 15.8; 162 Hz, = ^{13}CH–^{13}CHO); 4.70 (1H, m, O–CH–O); 4.56 (1H, t, J = 3.47 Hz, O–CH–O); 4.43 (1H, m, CH–OTHP); 4.36 (1H, m, CH–OTHP); 3.88 (1H, m, CH$_2$–O); 3.80 (1H, m, CH$_2$–O); 3.49 (1H, m, CH$_2$–O); 3.45 (1H, m, CH$_2$–O); 1.82 (2H, m, CH$_2$(THP)); 1.75–1.65 (2H, m, CH$_2$(THP)+2H, m, CH$_2$-CH-OTHP); 1.60–1.50 (8H, m, (CH$_2$)$_2$THP+2H, m, CH$_2$-CH-OTHP); 1.30–1.25 (12H, m); 0.87 (6H, m, CH$_3$).

^{13}C NMR: **193.9** (dd, J = 10.5; 53.2 Hz, ^{13}**C**HO); **193.5** (dd, J = 10.7; 53.0 Hz, ^{13}**C**HO); 158.5 (d, J = 68.8 Hz, CH =); 157.4 (d, J = 67.7 Hz, CH =); **132.3** (d, J = 53.1 Hz, ^{13}**C**H); **131.1** (d, J = 53.3 Hz, ^{13}**C**H); 98.1; 96.4; 75.5; 74.3; 62.74; 62.5; 35.0; 33.9; 31.8; 31.7; 30.8; 30.7; 25.4; 25.3; 25.0; 24.4; 22.5; 19.6; 19.4; 14.04; 14.02.

[1,2-$^{13}C_2$]-4-hydroxy-2(E)-nonenal: deprotection of **6b**

THP was removed in methanol with APTS (1 h). Concentration, addition of DCM and washing with brine afforded crude [1,2-^{13}C$_2$]-HNE (estimated purity: 90%).

HRMS: *m/z* 159.12900 (MH$^+$ [^{13}C$_2$C$_7$H$_{17}$O$_2$] = 159.12902).

^{1}H NMR: 9.58 (1H, ddd, J = 7.82; 25.8; 172 Hz, ^{13}CHO); 6.81 (1H, m, CH = ^{13}C); 6.31 (1H, ddd, J = 7.82; 15.6; 162 Hz, =^{13}CH–^{13}CHO); 4.44 (1H, m, CH–OH); 1.65–1.35 (4H, m, CH$_2$); 1.35–1.25 (4H, m, CH$_2$); 0.90 (3H, t, J = 6.83 Hz, CH$_3$).

^{13}C NMR: 193.5 (dd, J = 9.06; 53.3 Hz, ^{13}**C**HO); 130.7 (d, J = 53.3 Hz, ^{13}**C**H).

Conclusion

In summary, we have designed a synthetic scheme for the preparation of [1,2-^{13}C$_2$]-4-hydroxy-2(E)-nonenal. Because of its high reactivity, it has to be produced just before use by acid deprotection of the stable precursor: [1,2-^{13}C$_2$]-4-[(tetrahydro-2H-pyran-2-yl)oxy]-2(E)-nonenal.

The path was selected for two requirements: synthesis from a suitable commercial ^{13}C precursor and location of the stable isotope at the aldehyde end for the measurement of etheno-adducts. It is the only possibility so far reported to introduce two ^{13}C at determined positions. The ^{12}C isotope synthesis was initially developed and the ^{13}C analog was then obtained in eight steps in the acid labile hydroxy-protected form.

Our synthesis is not short, but the conditions are mild and neither require hazardous organometallic nor gas reagents. Yet, although the allylic oxidation was the key step in the scheme, our linear synthesis suffers from this low yield step. A convergent approach would avoid this step and improve the yield in high value ^{13}C product. Improvement of the synthetic scheme will be developed in future studies. The scheme opens not only to the synthesis of HNE derivatives (metabolites, adducts) but also to other alkenals implied in oxidative stress. We will report later on synthetic improvement and the development of mass spectrometry analyses with [1,2-^{13}C$_2$]-4-hydroxy-2(E)-nonenal as internal standard.

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