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In Situ Metabolism of Cinnamyl Alcohol in Reconstructed Human Epidermis: New Insights into the Activation of This Fragrance Skin Sensitizer

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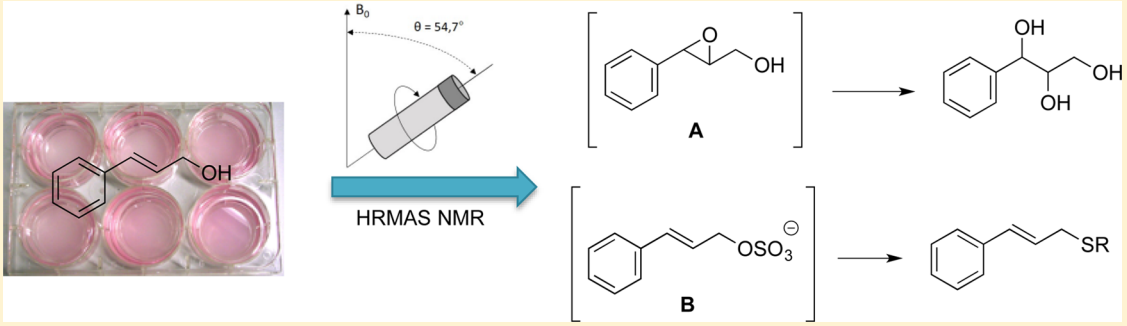


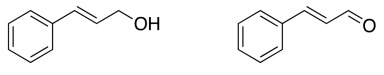
ABSTRACT: Chemical modification of epidermal proteins by skin sensitizers is the molecular event which initiates the induction of contact allergy. However, not all chemical skin allergens react directly as haptens with epidermal proteins but need either a chemical (prehaptens) or metabolic (prohaptens) activation step to become reactive. Cinnamyl alcohol has been considered a model prohapten, as this skin sensitizer has no intrinsic reactivity. Therefore, the prevailing theory is that cinnamyl alcohol is enzymatically oxidized into the protein-reactive cinnamaldehyde, which is the sensitizing agent. Knowing that reconstructed human epidermis (RHE) models have been demonstrated to be quite similar to the normal human epidermis in terms of metabolic enzymes, use of RHE may be useful to investigate the *in situ* metabolism/activation of cinnamyl alcohol, particularly when coupled with high-resolution magic angle spinning nuclear magnetic resonance. Incubation of carbon-13 substituted cinnamyl derivatives with RHE did not result in the formation of cinnamaldehyde. The metabolites formed suggest the formation of an epoxy-alcohol and an allylic sulfate as potential electrophiles. These data suggest that cinnamyl alcohol is inducing skin sensitization through a route independent of the one involving cinnamaldehyde and should therefore be considered as a skin sensitizer on its own.

INTRODUCTION

In addition to its physical barrier function,[1] the skin is a major immunocompetent[2] and xenobiotic-metabolizing organ.[3] Indeed, prolonged or repeated dermal exposure to chemicals can lead in some cases to a hypersensitivity immune response such as skin allergy/allergic contact dermatitis (ACD), a very common inflammatory skin disease.[4,5] Currently, in the western world, 15−20% of the population are allergic to at least one chemical present in their environment.[6] In most dermatological centers, ACD to fragrance chemicals ranks second after nickel as the most common source of allergy. Cinnamyl derivatives are among the fragrance materials present in Fragrance Mix I (amyl cinnamal, cinnamal, cinnamyl alcohol, eugenol, isoeugenol, geraniol, hydroxycitronellal, and oak moss) and Fragrance Mix II (hydroxyisohexyl-3-cyclohexene carboxaldehyde, citral, farnesol, coumarin, citronellol, and α-hexyl cinnamal), both included in the European baseline patch test series.[7,8] A response rate of 6−11% to Fragrance Mix I (FM I) and of 2.1−4.6% to Fragrance Mix II (FM II) has been reported in dermatological patients suffering from ACD.[9,10]

Cinnamyl alcohol **1** and cinnamaldehyde **2** (Chart 1) occur naturally as components of cinnamon and in a variety of essential oils (hyacinth, myrrh, Bulgarian rose, patchouli, and other plants) and are extensively used as fragrances and flavouring agents (worldwide use: ∼100−1000 metric tonnes per annum).[11,12] Both **1** and **2** are frequent allergens causing

Chart 1



Cinnamyl alcohol **1** Cinnamaldehyde **2**

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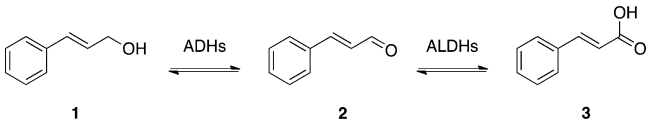
ACD in a substantial number of individuals and considered for a long-time as top-ranking fragrance allergens with a response rate of 0.6 and 1.0%, respectively.[13]

Cinnamyl alcohol **1** has been considered a prohapten, as this skin sensitizer has no intrinsic reactivity. It is hypothesized to be enzymatically oxidized into the protein-reactive cinnamaldehyde **2** (Scheme 1) to become sensitizing.[14−16] Within the epidermis, such bioactivation could presumably be performed by cutaneous oxido-reductase enzymes, such as alcohol dehydrogenases (ADHs), which are predominantly present and active in human skin, especially in the epidermis.[17,18] Cinnamaldehyde **2** could then be metabolized through aldehyde dehydrogenases (ALDHs) into the nonreactive cinnamic acid **3**. More recently, it has been suggested that cinnamyl alcohol may be also oxidized into cinnamaldehyde **2** through an abiotic process, by air exposure.[19] This metabolic explanation was mainly supported by the fact that, at a clinical level, a high level of concomitance was observed with patients sensitized to both **1** and **2**.[13,20] Indeed, many patients sensitized to cinnamyl alcohol also have positive patch-tests to cinnamaldehyde and vice versa. However, several clinical studies have demonstrated that a significant number of patients (about one-third) sensitized to cinnamyl alcohol **1** do not react when patch tested to cinnamaldehyde **2**.[13,20,21] This observation suggests that at least one alternative metabolic pathway can activate cinnamyl alcohol **1** into so far unidentified protein-reactive intermediates.

Scheme 1. Potential Oxidations and Reductions of Cinnamyl Derivatives in the Epidermis Mediated by Alcohol Dehydrogenases (ADHs) and Aldehyde Dehydrogenases (ALDHs)



Recently, we have developed a methodology combining the use of reconstructed human epidermis (RHE) and high-resolution magic angle spinning (HRMAS) nuclear magnetic resonance (NMR) to observe *in situ* and in a noninvasive way chemical interactions taking place between reactive skin sensitizers and nucleophilic residues on amino acids.[22−24] Knowing that RHE models have been demonstrated to be quite similar to the normal human epidermis in terms of metabolic enzymes, we have been interested in using this approach to investigate the *in situ* metabolism/activation of cinnamyl alcohol **1**. It has indeed been shown that even if the different models available differ in terms of morphology, the general metabolic properties of RHE are comparable to those of normal human skin.[3,25−28] It was thus shown that alcohol (ADHs) and aldehyde (ALDHs) dehydrogenases, cyclooxygenases (COXs), flavin monooxygenases (FMOs), gluthatione *S*-transferases (GSTs), and *N*-acetyltransferases (NATs) were expressed in RHE at a significant basal level, while CYP1, 2, and 3 families were expressed only at a low basal level.[27] Investigations on the catalytic activities of these xenobiotic-metabolizing enzymes have demonstrated that skin models are similar to normal human skin in terms of metabolic functionality toward xenobiotics.[27,28]

We now report our results on the *in situ* metabolism/activation of cinnamyl alcohol **1** in RHE by means of the noninvasive HRMAS NMR technique.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents. 1- and 2-(^{13}C)Acetonitrile and 1-(^{13}C)benzaldehyde were purchased from Euriso-Top (Saint Aubin, France). All other chemicals were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). Deionized water was obtained by filtration on ion exchanges cartridges (R3, M3, and ORC, Fisher Bioblock Scientific, Illkirch, France). Dichloromethane and tetrahydrofuran were dried by passing through active alumina under an argon pressure using Glasse Technology GTS100 devices. Acetonitrile was dried over Linde 4 Å. Benzaldehyde was classically distilled under nitrogen, at reduced pressure. Diisopropylamine was refluxed several minutes over potassium hydroxide and then distilled. All other chemicals were used as received. Air- or moisture-sensitive reactions were carried out in flame-dried glassware under an argon atmosphere. All extractive procedures were performed using technical grade solvents, and all aqueous solutions used were saturated. Analytical thin layer chromatography (TLC) was performed on precoated silica gel plates (Merck 60 F_{245}, Darmstadt, Germany) with visualization by UV light (254 nm) or sprayed with a solution containing *o*-anisaldehyde (0.5 mL), *p*-anisaldehyde (0.5 mL), sulfuric acid (5 mL), acetic acid (100 mL), and methanol (85 mL), followed by heating. Column chromatography was carried out using 40−63 μm silica gel Geduran Si 60 (Merck, Darmstadt, Germany), and the procedures included the subsequent evaporation of solvents under reduce pressure. ^{1}H and ^{13}C NMR spectra were recorded on Bruker Avance 300 or 500 MHz spectrometers. Chemical shifts (δ) are reported in part per million (ppm). The residual solvent peak was used as reference values. For ^{1}H NMR, $CDCl_3$ = 7.26 ppm. For ^{13}C NMR, $CDCl_3$ = 77.16 ppm. Data are presented as follows: chemical shift, multiplicity (s = single, d = doublet, t = triplet, q = quartet, m = multiplet, and br = broad), integration, and coupling constant (J in Hz). Assignments were performed either on the basis of unambiguous chemical shifts or coupling patterns. DEPT-135, COSY, HSQC, or HMBC experiments were performed when necessary to complete spectral attribution for related compounds.

Synthesis of Cinnamaldehyde 2: General Procedure. To a solution of lithium diisopropylamide (48.6 mmol, 4 equiv), previously prepared by addition at −40 °C of *n*-butyllithium (1.52 M in hexane, 32.0 mL, 48.6 mmol, 4 equiv) to a solution of distilled diisopropylamine (6.83 mL, 48.6 mmol, 4 equiv) in dry tetrahydrofuran (40 mL), was added at −70 °C a solution of dry acetonitrile **4** (1.25 mL, 24.3 mmol, 2 equiv) in dry tetrahydrofuran (20 mL). The mixture was stirred for 30 min at −70 °C, then warmed up at 0 °C, and a solution of diethyl chlorophosphate (3.51 mL, 24.3 mmol, 2 equiv) in dry tetrahydrofuran (20 mL) was added. The reaction medium was stirred for 1 h at 0 °C, then a solution of distilled benzaldehyde **5** (1.24 mL, 12.2 mmol, 1 equiv) in dry tetrahydrofuran (20 mL) was added. The mixture was stirred for an additional 3 h at room temperature and then quenched with water (200 mL). The aqueous layer was extracted with diethyl ether (3 × 200 mL) and the combined organic layers washed with brine (150 mL), dried over magnesium sulfate, filtered, and concentrated *in vacuo* to give the crude cinnamonitril **6** as an orange oil. To a solution of crude **6** (1.58 g) in dry dichloromethane (120 mL) at −70 °C was added dropwise a solution of diisobutylaluminum hydride (1 M in dichloromethane, 48.6 mL, 48.6 mmol, 4 equiv). The mixture was stirred for 15 min at −70 °C, then warmed up at room temperature and stirred for an additional 3 h. The reaction medium was quenched by controlled addition of aqueous ammonium chloride (280 mL) and then extracted with dichloromethane (4 × 120 mL). The organic layers were combined, washed with brine (360 mL), dried over magnesium sulfate, filtered, and concentrated *in vacuo*. The crude product was purified by column chromatography (R_f = 0.33, pentane/diethyl ether, 80:20 v/v) to give cinnamaldehyde **2** (0.511 g, 3.87 mmol, 32%) as a yellow oil (E/Z > 96%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.70 (dd, 1H, $^{3}J_{HH}$ = 15.9 Hz, $^{3}J_{HH}$ = 7.7 Hz, H2), 7.40−

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7.45 (m, 3H, 2 x *meta*-ArH and *para*-ArH), 7.47 (d, 1H, $^{3}J_{HH}$ = 15.9 Hz, H3), 7.53−7.59 (m, 2H, *ortho*-ArH), 9.70 (d, 1H, $^{3}J_{HH}$ = 7.7 Hz, H1); ^{13}C NMR (75 MHz, $CDCl_3$) δ 128.5 (2C, *meta*-ArCH), 128.6 (C2), 129.2 (2C, *ortho*-ArCH), 131.4 (*para*-ArCH), 134.1 (*ipso*-ArC), 152.9 (C3), 193.7 (C1).

1-(^{13}C)Cinnamaldehyde ***2a***. The title compound was prepared according to the general procedure described above starting from 1-(^{13}C)acetonitrile **4a** (1.26 mL, 24 mmol, 2 equiv) and benzaldehyde **5** (1.22 mL, 12 mmol, 1 equiv) to give **2a** (0.732 g, 5.50 mmol, 46% yield) as a yellow oil (E/Z > 96%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.70 (ddd, 1H, $^{3}J_{HH}$ = 16.0 Hz, $^{3}J_{HH}$ = 7.7 Hz, $^{2}J_{HC}$ = 1.3 Hz, H2), 7.40−7.47 (m, 3H, 2 x *meta*-ArH and *para*-ArH), 7.48 (d, 1H, $^{3}J_{HH}$ = 16.0 Hz, H3), 7.52−7.58 (m, 2H, *ortho*-ArH), 9.67 (dd, 1H, $^{1}J_{HC}$ = 172.8 Hz, $^{3}J_{HH}$ = 7.7 Hz, H1); ^{13}C NMR (75 MHz, $CDCl_3$) δ 128.5 (2C, *meta*-ArCH), 128.6 (d, $^{1}J_{CC}$ = 55.4 Hz, C2), 129.2 (2C, *ortho*-ArCH), 131.4 (*para*-ArCH), 134.0 (d, $^{3}J_{CC}$ = 7.6 Hz, *ipso*-ArC), 152.8 (d, $^{2}J_{CC}$ = 4.3 Hz, C3), 193.6 (^{13}C1).

2-(^{13}C)Cinnamaldehyde ***2b***. The title compound was prepared according to the general procedure described above starting from 2-(^{13}C)acetonitrile **4b** (1.36 mL, 26 mmol, 2 equiv) and benzaldehyde **5** (1.32 mL, 13 mmol, 1 equiv) to give 2-(^{13}C)cinnamaldehyde **2b** (0.727 g, 5.46 mmol, 42% yield) as a yellow oil (E/Z > 96%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.71 (ddd, 1H, $^{1}J_{HC}$ = 160.2 Hz, $^{3}J_{HH}$ = 15.9 Hz, $^{3}J_{HH}$ = 7.7 Hz, H2), 7.40−7.44 (m, 3H, 2 x *meta*-ArH and *para*-ArH), 7.47 (dd, 1H, $^{3}J_{HH}$ = 15.9 Hz, $^{2}J_{HC}$ = 1.5 Hz, H3), 7.55−7.57 (m, 2H, *ortho*-ArH), 9.70 (dd, 1H, $^{2}J_{HC}$ = 25.7 Hz, $^{3}J_{HH}$ = 7.7 Hz, H1); ^{13}C NMR (125 MHz, $CDCl_3$) δ 128.6 (^{13}C2), 128.6 (2C, *meta*-ArCH), 129.1 (2C, *ortho*-ArCH), 131.4 (*para*-ArCH), 134.0 (*ipso*-ArC), 152.9 (d, $^{1}J_{CC}$ = 69.0 Hz, C3), 193.8 (d, $^{1}J_{CC}$ = 54.8 Hz, C1).

3-(^{13}C)Cinnamaldehyde ***2c***. The title compound was prepared according to the general procedure described above starting from acetonitrile **4** (1.46 mL, 28 mmol, 2 equiv) and 1-(^{13}C)benzaldehyde **5c** (1.42 mL, 14 mmol, 1 equiv) to give 3-(^{13}C)cinnamaldehyde **2c** (0.751 g, 5.64 mmol, 40%) as a yellow oil (E/Z > 96%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.74 (dd, 1H, $^{3}J_{HH}$ = 16.0 Hz, $^{3}J_{HH}$ = 7.7 Hz, H2), 7.40−7.44 (m, 3H, 2 x *meta*-ArH and *para*-ArH), 7.46 (dd, 1H, $^{1}J_{HC}$ = 153.4 Hz, $^{3}J_{HH}$ = 16.0 Hz, H3), 7.51−7.59 (m, 2H, *ortho*-ArH), 9.67 (dd, 1H, $^{3}J_{HH}$ = 7.7 Hz, $^{3}J_{HC}$ = 1.0 Hz, H1); ^{13}C NMR (75 MHz, $CDCl_3$) δ 128.4 (d, 2C, $^{3}J_{CC}$ = 2.1 Hz, *meta*-ArCH), 128.5 (d, $^{1}J_{CC}$ = 68.5 Hz, C2), 129.2 (d, 2C, $^{2}J_{CC}$ = 4.5 Hz, *ortho*-ArCH), 131.4 (*para*-ArCH), 134.2 (d, $^{1}J_{CC}$ = 55.8 Hz, *ipso*-ArC), 152.9 (^{13}C3), 193.8 (d, $^{2}J_{CC}$ = 4.7 Hz, C1).

Synthesis of Cinnamyl Alcohol 1: General Procedure. To a solution of cinnamaldehyde **2** (170 mg, 1.29 mmol, 1 equiv) in methanol (5 mL) at 0 °C was added sodium borohydride (49 mg, 1.29 mmol, 1 equiv). The mixture was stirred for 15 min at 0 °C, then warmed up at room temperature and stirred for an additional 2 h. The reaction medium was quenched with water (10 mL), concentrated *in vacuo*, and dichloromethane (10 mL) was added. The organic layer was separated, washed with brine (10 mL), dried over magnesium sulfate, filtered, and concentrated *in vacuo* to give cinnamyl alcohol **1** (160 mg, 1.19 mmol, 92% yield, E/Z > 96%) as a white solid: ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.21 (br., 1H, − OH), 4.32 (dd, 2H, $^{3}J_{HH}$ = 5.6 Hz, $^{4}J_{HH}$ = 1.4 Hz, H1), 6.36 (dt, 1H, $^{3}J_{HH}$ = 15.8 Hz, $^{3}J_{HH}$ = 5.6 Hz, H2), 6.62 (dt, 1H, $^{3}J_{HH}$ = 15.8 Hz, $^{4}J_{HH}$ = 1.4 Hz, H3), 7.23−7.41 (m, 5H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 63.5 (C1), 126.5 (2C, *ortho*-ArCH), 127.7 (*para*-ArCH), 128.4 (C2), 128.6 (2C, *meta*-ArCH), 131.0 (C3), 136.8 (*ipso*-ArC).

1-(^{13}C)Cinnamyl Alcohol ***1a***. The title compound was prepared according to the general procedure described above starting from 1-(^{13}C)cinnamaldehyde **2a** (500 mg, 3.76 mmol) to give 1-(^{13}C)-cinnamyl alcohol **1a** (395 mg, 2.92 mmol, 78% yield, E/Z > 96%) as a white solid: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.51 (br., 1H, − OH), 4.33 (ddd, 2H, $^{1}J_{HC}$ = 142.6 Hz, $^{3}J_{HH}$ = 5.7 Hz, $^{4}J_{HH}$ = 1.5 Hz, H1), 6.32−6.42 (m, 1H, H2), 6.63 (ddt, 1H, $^{3}J_{HH}$ = 15.8 Hz, $^{3}J_{HC}$ = 7.0 Hz, $^{4}J_{HH}$ = 1.5 Hz, H3), 7.22−7.41 (m, 5H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 63.9 (^{13}C1), 126.6 (2C, 2 x *ortho*-ArCH), 127.9 (*para*-ArCH), 128.6 (d, $^{1}J_{CC}$ = 45.9 Hz, C2), 128.7 (2C, 2 x *meta*-ArCH), 131.3 (C3), 136.8 (d, $^{3}J_{CC}$ = 5.4 Hz, *ipso*-ArC).

2-(^{13}C)Cinnamyl Alcohol ***1b***. The title compound was prepared according to the general procedure described above starting from 2-(^{13}C)cinnamaldehyde **2b** (157 mg, 1.18 mmol) to give 2-(^{13}C)-cinnamyl alcohol **1b** (135 mg, 1.00 mmol, 85% yield, E/Z > 96%) as a white solid: ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.02 (br., 1H, − OH), 4.32 (m, 2H, H1), 6.37 (ddt, 1H, $^{1}J_{HC}$ = 151.9 Hz, $^{3}J_{HH}$ = 16.1 Hz, $^{3}J_{HH}$ = 5.5 Hz, H2), 6.62 (d, 1H, $^{3}J_{HH}$ = 16.1 Hz, H3), 7.24−7.40 (m, 5H, ArH); ^{13}C NMR (125 MHz, $CDCl_3$) δ 63.7 (d, $^{1}J_{CC}$ = 27.9 Hz, C1), 126.6 (d, 2C, $^{3}J_{CC}$ = 2.7 Hz, *ortho*-ArCH), 127.8 (*para*-ArCH), 128.6 (^{13}C2), 128.7 (2C, 2 x *meta*-ArCH), 131.2 (d, $^{1}J_{CC}$ = 43.6 Hz, C3), 136.8 (*ipso*-ArC).

3-(^{13}C)Cinnamyl Alcohol ***1c***. The title compound was prepared according to the general procedure described above starting from 3-(^{13}C)cinnamaldehyde **2c** (375 mg, 2.82 mmol) to give 1-(^{13}C) cinnamyl alcohol **1c** (330 mg, 2.48 mmol, 88% yield, E/Z > 96%) as a white solid: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.49 (br., 1H, − OH), 4.32−4.35 (m, 2H, H1), 6.32−6.42 (m, 1H, H2), 6.62 (ddt, 1H, $^{1}J_{HC}$ = 153.0 Hz, $^{3}J_{HH}$ = 15.6 Hz, $^{4}J_{HH}$ = 1.7 Hz, H3), 7.22−7.41 (m, 5H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 63.8 (C1), 126.6 (d, 2C, $^{2}J_{CC}$ = 1.9 Hz, *ortho*-ArCH), 127.8 (*para*-ArCH), 128.5 (d, $^{1}J_{CC}$ = 72.5 Hz, C2), 128.7 (d, 2C, $^{3}J_{CC}$ = 4.4 Hz, *meta*-ArCH), 131.2 (^{13}C3), 136.8 (d, $^{1}J_{CC}$ = 55.5 Hz, *ipso*-ArC).

Reconstructed Human Epidermis. For this study, the large 4 cm^2 SkinEthic RHE model (SkinEthic, Lyon, France, http://www.skinethic.com) was selected. This 3D epidermal skin model is composed of keratinocytes cultured on a polycarbonate filter at the air−liquid interface for 17 days. SkinEthic RHE were received on day 18, aseptically removed from the transport medium, and preincubated for 2 h in a growth culture medium (SkinEthic, Lyon, France) at 37 °C, 5% CO_2, and under humidified atmosphere, according to SkinEthic's protocol.

Treatment of RHE with 1-, 2-, or 3-(^{13}C)Cinnamaldehyde and 1-, 2-, or 3-(^{13}C)Cinnamyl Alcohol. SkinEthic RHE were topically and separately treated with 1-, 2- or 3-(^{13}C)cinnamaldehyde and 1-, 2-, or 3-(^{13}C)cinnamyl alcohol in acetone (0.4 M, 100 μL) and incubated for 1, 8, and 24 h, respectively. RHE negative controls were either untreated or treated with acetone (100 μL) and incubated for 24 h. After incubation, the RHE were rinsed with deionized water, separated from the polycarbonate filter using a treatment with Dispase II (neutral protease, grade II, Roche, Mannheim) in HEPES (Lancaster Synthesis, Pelham, United States) buffer solution, washed with deionized water, and stored at −80 °C pending NMR sample preparation.

Rotor Preparation and Data Acquisition by HRMAS NMR. The rotor preparation and the data acquisition by HRMAS NMR were carried out according to the methodology already described.[22] Briefly, each sample was prepared at −20 °C by introducing 15 to 20 mg of frozen RHE completed with D_2O into a disposable 30 μL KelF inserts. Shortly before HRMAS analysis, the inset was placed into a standard 4 mm ZrO_2 rotor and closed with a cap. The HRMAS experiments were performed at 3 °C. Upon completion of the analysis, the inset was taken out of the rotor and stored back at −80 °C for further complementary NMR analysis at a later stage.

HRMAS spectra were recorded on a Bruker Avance III 500 spectrometer (Hautepierre University Hospital, Strasbourg) operating at a proton frequency of 500.13 MHz, equipped with a 4 mm double resonance (^{1}H, ^{13}C) gradient HRMAS probe. The conditions of acquisition and processing for the 1D ^{1}H experiments using standard one pulse and Carr−Purcell−Meiboom−Gill (CPMG) pulse sequences coupled with water presaturation and for the 2D ^{1}H−^{13}C g-HSQC (gradient Heteronuclear Single Quantum Coherence) experiments using echo-antiecho gradient selection were those described in the detailed methodology. All spectra were referenced by setting the lactate doublet chemical shift to 1.33 ppm in ^{1}H and to 22.7 ppm in ^{13}C.

RESULTS

Synthesis of Carbon-13 Substituted Chemicals. In order to increase the sensitivity of the method and be able to

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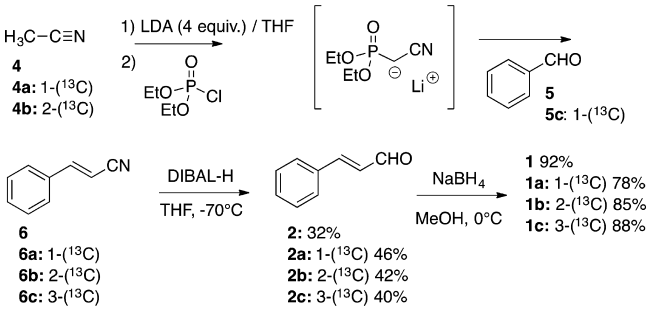
follow metabolism/chemical modifications taking place on cinnamyl alcohol **1**, it was decided to individually carbon-13 substitute the 3 positions of the alkyl chain (Scheme 2). A

Scheme 2. Synthesis of Carbon-13 Substituted at Positions 1, 2, and 3 of Cinnamaldehyde 2 and Cinnamyl Alcohol 1 Derivatives



general synthetic approach was thus designed to access the targeted chemicals using a common sequence and only changing the carbon-13 substituted precursors. Thus, the anion derived from acetonitrile **4** in the presence of an excess of lithium diisopropylamide was condensed in THF with diethyl chlorophosphate to form *in situ* a Horner−Wadsworth−Emmons intermediate that was reacted at 0 °C with benzaldehyde **5** to give a crude cinnamonitrile **6** used without further purification in the subsequent reduction step. The crude **6** was thus reacted in THF with an excess of diisobutylaluminum hydride at −70 °C, to give after hydrolysis and purification cinnamaldehyde **2** in an overall yield of 32% as a mixture of stereoisomers (E/Z > 96%). Carbon-13 substitution can thus be easily achieved using 1-(^{13}C)acetonitrile **4a**, 2-(^{13}C)-acetonitrile **4b**, or 1-(^{13}C)benzaldehyde **5c** to give 1-(^{13}C)-cinnamaldehyde **2a**, 2-(^{13}C)cinnamaldehyde **2b**, or 3-(^{13}C)-cinnamaldehyde **2c** in overall yields ranging from 40 to 46%. Cinnamyl alcohol **1** and its isotopomers **1a**−**c** were directly obtained from cinnamaldehyde **2** and its isotopomers **2a**−**c** by a simple reduction with sodium borohydride in methanol at 0 °C.

Oxidation and Reduction Processes Taking Place in RHE. In order to confirm the presence and activity of ADHs and ALDHs in the selected RHE model and the ability of the HRMAS NMR technique to evidence *in situ* metabolic transformations, cinnamaldehyde **2a** was chosen as the substrate since it undergoes both oxidation and reduction.[29,30]

After 1, 8, and 24 h of incubation and subsequent 1D ^{13}C-HRMAS NMR analysis, a signal at 200.6 ppm corresponding to the aldehyde function and two new signals at 64.7 and 178.2 ppm corresponding to the formation of cinnamyl alcohol **1a** and cinnamic acid **3a**, respectively, were observed (Figure 1a). First, this evidenced efficient (already present after 1 h of incubation) concomitant reduction and oxidation of cinnamaldehyde **2** and therefore the presence of active ADHs and ALDHs in RHE. Second, this demonstrated that HRMAS NMR is a valuable tool to observe *in situ* metabolic transformations. When cinnamyl alcohol **1a** was incubated on RHE for 1, 8, and 24 h and analyzed by 1D ^{13}C-HRMAS NMR, a large signal at 64.7 ppm corresponding to the allylic alcohol position was observed. No signal at 200.6 ppm, which would correspond to cinnamaldehyde **2a**, could be detected even after 24 h of incubation (Figure 1b). It should be mentioned that a very tiny signal at 178.2 ppm that could correspond to cinnamic

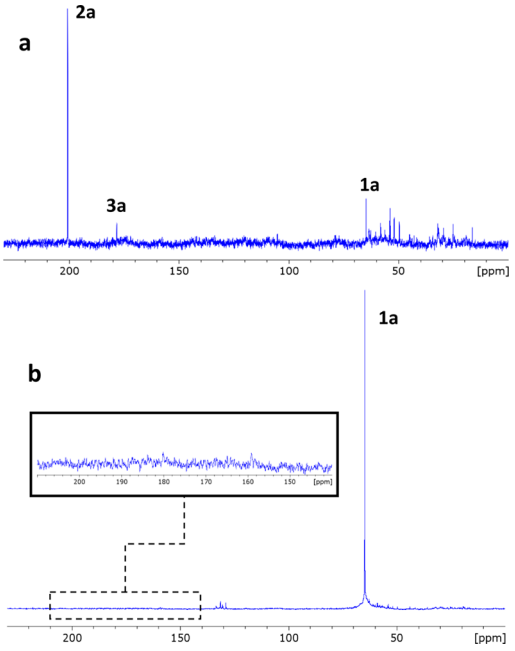


Figure 1. 1D ^{13}C HRMAS MNR spectra of reconstructed human epidermis treated with cinnamaldehyde **2a** or cinnamyl alcohol **1a**. (a) Spectrum of RHE treated with cinnamaldehyde **2a** after 24 h of incubation showing the formation of cinnamyl alcohol **1a** and cinnamic acid **3a**. (b) Spectrum of RHE treated with cinnamyl alcohol **1a** after 24 h of incubation showing the absence of cinnamaldehyde **2a**.

acid **3a** was observed after 1 h of incubation but was not observable after 8 and 24 h.

Metabolism/Activation of Cinnamyl Alcohol 1 in RHE. As no obvious transformation of cinnamyl alcohol **1** into cinnamaldehyde **2** could be detected and thus explain its sensitizing potential, we then investigated metabolism/activation that could occur on the alkyl chain of **1**. RHE were thus separately treated with **1**, **1a**, **1b**, and **1c**, incubated for 1, 8, and 24 h and then analyzed by HRMAS 2D ^{1}H−^{13}C g-HSQC NMR experiments. In addition to changes in the metabolome of the epidermis following exposure to **1** used as control (Figure 2a), additional signals associated with the specific substituted positions (**1a**, **1b**, and **1c**) were detected (Figure 2b,c,d). Thus, when RHE were treated with **1a**, in addition to a large signal at 4.65/67.6 ppm corresponding to the substituted allylic alcohol position, two new signals at 3.97/67.0 ppm and 3.98/45.6 ppm, corresponding to a −CH_2− position, were observed (Figure 2b). When RHE were treated with **1b**, in addition to the signals at 6.68/130.7 and 6.47/130.7 ppm corresponding to the Z and E isomers of **1b**, respectively, a new signal at 6.24/126.4 ppm corresponding to a vinylic −CH- and a new signal at 3.93/71.6 ppm corresponding to a non vinylic −CH- were observed (Figure 2c). Finally, when RHE were treated with **1c**, in addition to the signals at 6.65/133.9 and 6.45/133.8 ppm corresponding to the E and Z isomers of **1c**, respectively, a new signal at 6.56/136.1 ppm corresponding to a vinylic −CH- and a new signal at 4.85/73.9 ppm corresponding to a nonvinylic −CH- were observed (Figure 2d). It should be mentioned that in these 2D ^{1}H−^{13}C g-HSQC NMR experiments signals that would arise from the formation of cinnamaldehyde **2** by oxidation of **1** were never detected, irrespective of the incubation time (1, 8, and 24 h), thus confirming the 1D ^{13}C NMR experiments reported above.

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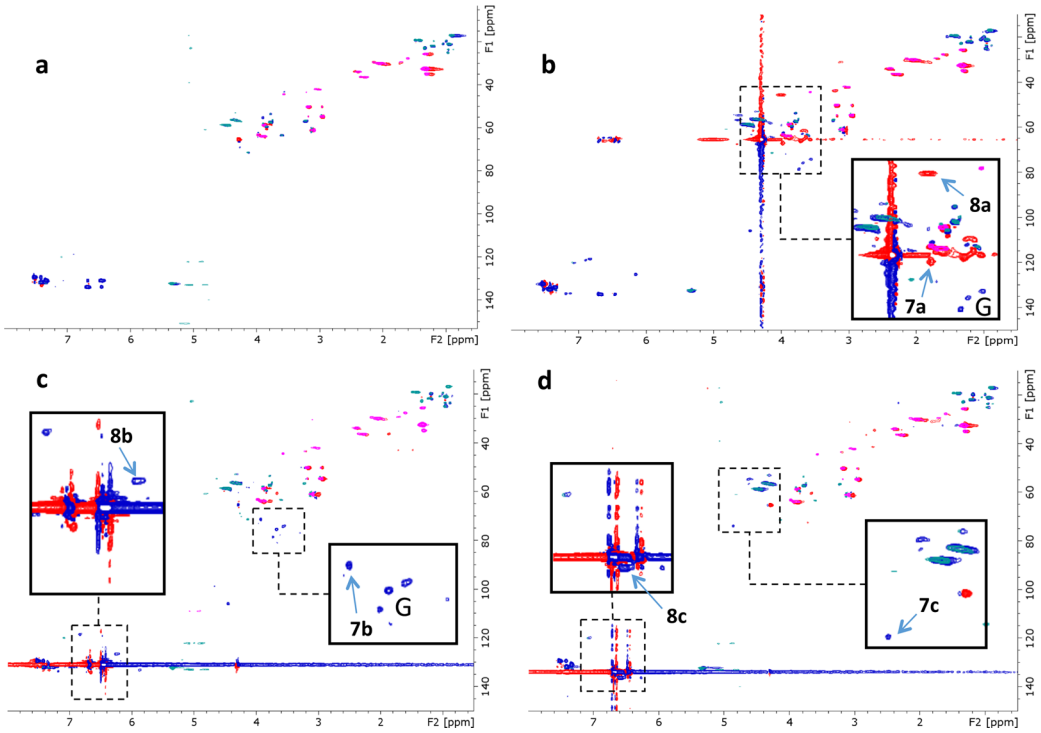
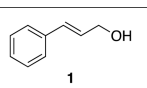
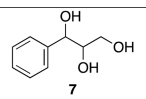
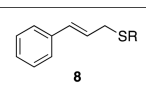


Figure 2. 2D $^{1}H-^{13}C$ g-HSQC HRMAS NMR spectra of a reconstructed human epidermis treated with (a) cinnamyl alcohol **1**, (b) 1-(^{13}C)cinnamyl alcohol **1a**, (c) 2-(^{13}C)cinnamyl alcohol **1b**, and 3-(^{13}C)cinnamyl alcohol **1c** after 8 h of incubation. **G** corresponds to signals associated with the increased concentration of glucose.

Combining these data, 2 structures in agreement with ^{1}H and ^{13}C chemical shifts can be suggested. Thus, combining NMR data from the 2 nonvinylic −CH- positions with the $-CH_2-$ signal at 3.97/67.0 ppm a triol **7** (Table 1) can be proposed,

Table 1. $^{1}H/^{13}C$ NMR Predicted[a] Values in Solution vs Experimental[b] HRMAS Values in RHE for Structures 1, 7, and 8

	 1		 7		 8	
Position	Predicted	Experimental	Predicted	Experimental	Predicted	Experimental
1	4.28/62.7	4.65/67.6	3.50/63.9	3.97/67.0	3.43/41.3	3.98/45.6
2	6.33/130.7	6.47/130.7	3.62/74.5	3.93/71.6	6.19/127.1	6.24/126.4
3	6.60/133.9	6.65/133.9	4.42/75.7	4.85/73.9	6.45/136.4	6.56/136.1

[a]ACD/CNMR and ACD/HNMR Predictor software (version 6.0, ACD/Laboratories, Toronto, Canada). [b]2D $^{1}H-^{13}C$ g-HSQC was obtained on a Bruker Avance III 500 spectrometer equipped with a 4 mm double resonance (^{1}H, ^{13}C) gradient HRMAS probe.

while combining NMR data from the 2 vinylic −CH- positions with the $-CH_2-$ signal at 3.98/45.6 ppm an allylic sulfide **8** can be suggested. Indeed NMR chemical shifts of the $-CH_2-$ signal at position 1 are characteristic of the heteroatom attached to it. A ^{13}C upfield signal at 45.6 ppm can indeed only be associated with the presence of a sulfur atom, while a ^{13}C downfield signal at 67.0 ppm can only be associated with the presence of an oxygen atom at position 1.

DISCUSSION

It has been widely reported in the literature that cinnamyl alcohol **1**, not chemically reactive, was activated (oxidation) by an alcohol dehydrogenase into the protein reactive cinnamaldehyde **2**. This hypothesis was mainly supported by the high frequency of concomitant reactions to **1** and **2** as well as the demonstrated presence in the epidermis of functional ADHs. However, when looking into more details to clinical and experimental data several observations are not in full agreement with this proposed activation mechanism. Thus, patch test studies performed in the U.S. have shown less positive reactions to cinnamaldehyde **2** than to cinnamyl alcohol **1** when the two compounds were tested at the same concentration.[31] This greater occurrence of ACD to cinnamyl alcohol **1** could be explained by a higher degree of exposure of the population, but concomitant positive patch tests to cinnamaldehyde should be expected. Indeed several clinical studies have demonstrated that a significant number of patients (about one-third) sensitized to cinnamyl alcohol **1** do not react when patch tested to cinnamaldehyde **2**.[20,21] In addition to these clinical observations, *ex vivo* studies have investigated the absorption and behavior of **1** and **2** in full-thickness human skins.[29,30] If the reduction of cinnamaldehyde **2** into cinnamyl alcohol **1** and its oxidation into cinnamic acid **3** were clearly observed (Scheme 1), no conversion of cinnamyl alcohol **1** into the protein-reactive cinnamaldehyde **2** was clearly proven. Indeed, it is on the base of the formation of cinnamic acid **3** that oxidation of cinnamyl alcohol **1** into cinnamaldehyde **2** was suggested.

The difficulty when investigating the formation of reactive intermediates in the skin is that classical methods usually rely on the diffusion of metabolites outside the epidermis for subsequent analysis. This is of course a major drawback when investigating intermediates that are not likely to diffuse outside

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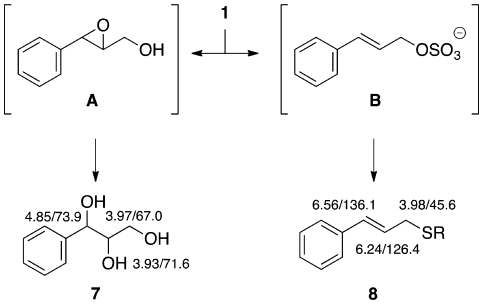
of the epidermis and that would react *in situ* with nucleophilic residues. The use of HRMAS in association with carbon-13 substituted chemicals could therefore be a very useful tool to observe the metabolism and subsequent reactivity of xenobiotics in a living tissue with out fastidious and time-consuming extraction processes.

On the basis of our experiments, the traditional hypothesis of cinnamaldehyde **2** being the source of sensitization to cinnamyl alcohol **1** was not supported. Indeed, if the reduction of **2** into **1** was evidenced, the reverse was not observed. In addition, metabolites formed are rather suggesting either the formation of an epoxy-alcohol [**A**] or the activation of the allylic hydroxyl function potentially through a sulfate [**B**] even if neither [**A**] nor [**B**] was detected in these experiments (Scheme 3).

Scheme 3. Potential Metabolic Activation of Cinnamyl Alcohol 1 in Reconstructed Human Epidermis Leading to the Formation of Products 7 and 8



On the one hand, epoxide [**A**] was already reported to form when cinnamyl alcohol **1** was incubated with human liver microsomes[32] probably through a CYP enzymatic system.[33] Such an epoxide [**A**] has also been reported to form by autoxidation of cinnamyl alcohol **1** when exposed to air.[19] The subsequent hydrolysis of [**A**] mediated by an epoxide hydrolase would give triol **7**. On the other hand, there are reports in the literature suggesting that primary hydroxyl groups can be activated in the skin to form reactive intermediates. This pathway has been proposed to explain the skin toxicity associated with the 12-hydroxy nevirapine (12-OH-NVP), one of the liver metabolites of nevirapine.[34,35] It was postulated that 12-OH-NVP could be sulfated in the epidermis and that this product would subsequently react with nucleophilic residues on proteins. It should be noted that epidermal sulfotransferases are highly expressed and active in RHE models.[27,28] This mechanism that would explain skin sensitization is also supported by the clinical case of a worker who developed severe allergic contact dermatitis after accidental exposure to cinnamyl chloride.[36] At patch-testing, he was found positive to cinnamyl chloride but also to cinnamyl alcohol. Cinnamyl chloride would be expected to form the same protein adducts as the sulfate ester of cinnamyl alcohol, thus explaining the concomitant reaction to both cinnamyl derivatives.

One should therefore consider that the sensitization route of cinnamyl alcohol and cinnamaldehyde are most likely mechanistically independent and that the high frequency of concomitant reactions to **1** and **2** could be explained by a concomitant exposure to these chemicals frequently associated in consumer products.[37]

CONCLUSIONS

For the first time, we demonstrated that HRMAS NMR in association with carbon-13 substituted chemicals allows for the *in situ* observation of the metabolism of chemicals in a reconstructed human epidermis. Our data do not support the hypothesis that cinnamyl alcohol, a fragrance prohapten, is activated by an enzymatic oxidation into the reactive cinnamaldehyde. Furthermore, the metabolites identified suggest the formation of an epoxy-alcohol [**A**] and/or an allylic sulfate [**B**] as potential electrophiles.

Therefore, our data suggest that cinnamyl alcohol is inducing skin sensitization through a route independent of the one involving cinnamaldehyde. Cinnamyl alcohol should therefore be considered as a skin sensitizer on its own even if alternative pathways cannot be excluded as RHE models have no cross-talks with the dermis and antigen presenting cells or the endothelium is not present.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

ACD, allergic contact dermatitis; ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; FM I, fragrance mix I; FM II, fragrance mix II; HRMAS, high-resolution at magic angle spinning; g-HSQC, gradient heteronuclear single quantum coherence; RHE, reconstructed human epidermis

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