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Matrix Effect of Human Reconstructed Epidermis on the Chemoselectivity of a Skin Sensitizing α-Methylene-γ-Butyrolactone: Consequences for the Development of *in Chemico* Alternative Methods

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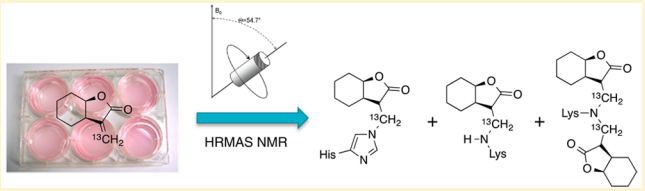
Supporting Information

ABSTRACT: Adoption of new legislations and social pressure are pushing toward the development of alternative methods to the use of animals for the assessment of most toxicological end-points including skin sensitization. To that respect, much efforts have been put in the first step of the adverse outcome pathway focusing on chemical interactions taking place between sensitizing chemicals or haptens and epidermal proteins. However, these *in chemico* approaches have been so far only based on the use of model nucleophiles, amino acids, peptides, or proteins in water/buffer solution and focused mainly on thiol reactivity. These studies even if bringing a valuable set of information are very far from reflecting chemical interactions that may happen between a xenobiotic and nucleophiles present in a complex heterogeneous tissue such as the epidermis. Recently, we have shown that using a high-resolution magic angle spinning (HRMAS) nuclear magnetic resonance (NMR) technique it was possible to characterize chemical interactions taking place between a skin sensitizer and nucleophilic amino acids present in a 3-D reconstructed human epidermis (RHE). We have now compared the chemical reactivity and chemoselectivity of a sensitizing α-methylene-γ-butyrolactone toward human serum albumin used as a model protein and RHE. Using this technique, we showed that amino acid modifications by this hapten was different according to the model used and that in RHE histidine residues seem to have an important role in the formation of adducts. Obviously, the role of histidine in the induction of skin sensitization has been so far neglected and should probably be taken into account for the refinement of *in chemico* approaches for the detection and potency classification of skin sensitizers.



INTRODUCTION

Adoption of the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) system[1] and also the seventh amendment to the EU Cosmetic Directive, banning from the European market cosmetic products containing ingredients tested on animals,[2] led to the development of alternative methods to predict toxicological end-points such as skin allergy. This need has been further reinforced by regulations in the European Union and soon within the Globally Harmonized System. The relevant EU legislation includes the Dangerous Substances Directive on dangerous chemicals[3] and the Dangerous Preparation Directive.[4] On the basis of the defined adverse outcome pathway (AOP) leading to skin sensitization, several key steps have been identified as the bases for the development of alternative methods. Indeed, the first key event of the AOP relies on the chemical modification of epidermal proteins by skin sensitizers, mainly through the formation of covalent bounds.[5,6] Thus, over the past decade, extensive knowledge on the chemistry of skin sensitizers has been acquired, and the elucidation of their reactivity toward peptides permitted researchers to propose the first mechanisms on the formation of hapten−protein adducts.[7−11] On the basis of this key step of the AOP and in the course of the development of alternative methods for the identification of chemical sensitizers, Gerberick et al. proposed a new approach based on the quantification of cysteine and lysine reactivity present on two synthetic peptides: the direct peptide reactivity assay (DPRA).[12,13] During the development of the DPRA, a large set of skin chemical sensitizers were assessed for their ability to react with peptides containing either a lysine or a cysteine residue. The DPRA was first endorsed by the European Center for Validation of Alternative Methods (ECVAM)[14] and was recently adopted as an OECD-guideline for the testing of chemicals under Section 4: Health Effects.[15] This *in chemico* approach is thus based on the assessment of the

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chemical reactivity of test chemicals toward cysteine and lysine reported as the most relevant nucleophiles present in proteins.

Already in the early 1970s, it was shown that natural products containing α-methylene-γ-butyrolactone moiety (Figure 1)

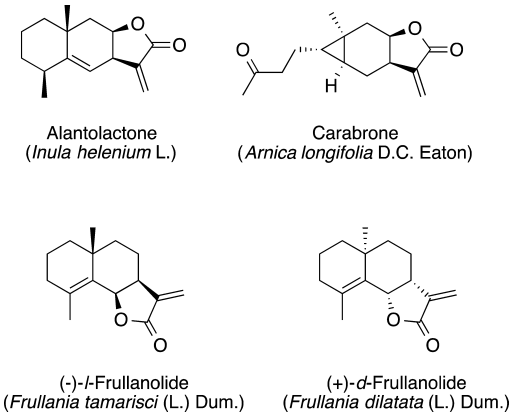
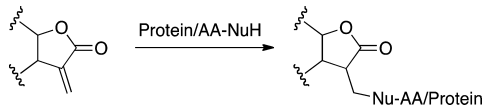


Figure 1. Structures of some α-methylene-γ-butyrolactones present in plants associated with skin allergies.

were associated with skin allergies and could react with nucleophiles such as cysteine or lysine to yield adducts (Scheme 1).[16,17] Such a reactivity was later confirmed by

Scheme 1. General 1,4-Michael Addition of Nucleophiles on α-Methylene-γ-butyrolactone Structures

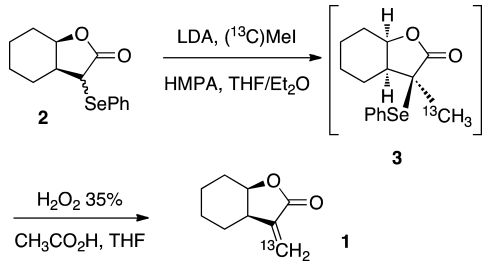


reaction studies between alantolactone (*Inula helenium* L.)[18] and both enantiomers of frullanolide (*Frullania dilatata* (L.) Dum. and *Frullania tamarisci* (L.) Dum.) toward the nucleophilic dipeptides *N*-Boc-Cys-Ala-OMe[19] and *N*-Boc-Lys-Ala-OMe.[20]

So far, reactivity studies of skin allergens toward nucleophilic residues have been performed exclusively in solution using either isolated amino acids or peptides/proteins. Indeed, these studies even though they bring a set of information are very far from modeling interactions that may happen between a xenobiotic and nucleophiles present in a complex heterogeneous tissue such as the epidermis. There is therefore a very strong need to develop more complex models in order to study and better understand the mechanisms of skin sensitization. Recently, we have shown that using a high-resolution magic angle spinning (HRMAS) nuclear magnetic resonance (NMR) technique, in association with a carbon 13 substituted methylating agent, it was possible to characterize chemical interactions taking place between this hapten and nucleophilic amino acids present in a 3-D reconstructed human epidermis (RHE).[21] RHE models have been developed as an alternative to animal experiments for the development of cosmetic, chemical, and pharmaceutical compounds. As keratinocytes represent the major cell type in the epidermis and play a key role in skin inflammatory reactions, keratinocyte cultures were chosen for the development of these models. Indeed, RHE models show high similarity with the *in vivo* human epidermis in terms of morphology and metabolic activity[22,23] even if some differences in terms of skin penetration[24] and enzymatic activation[25,26] should be pointed out.

In order to study the chemical response of RHE to the reactive α-methylene-γ-butyrolactone substructure, the α-(^{13}C)methylene hexahydrobenzofuranone **1** with a *cis* ring junction was chosen as an NMR probe (Scheme 2). This easily

Scheme 2. Synthesis of the α-(^{13}C)Methylene Hexahydrobenzofuranone 1 with a *cis* Ring Junction



accessible bicyclic α-methylene-γ-butyrolactone was previously reported to cause skin allergies in guinea pigs and to be a good model to study allergic reactions to sesquiterpene lactones of the alantolactone type.[27]

We now report the results of our investigations on α-(^{13}C)methylene hexahydrobenzo-furanone treated RHE using HRMAS NMR.

EXPERIMENTAL PROCEDURES

Caution: *Skin contact with α-methylene-γ-butyrolactones must be avoided. As potential sensitizing substances, these compounds must be handled with care.*

Chemicals and Reagents. (^{13}C)methyl iodide and deuterated solvents were purchased from Euriso-Top (Saint Aubin, France). All other chemicals were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France) unless otherwise noted and used without further purification. Aqueous solutions were prepared with deionized water. Human serum albumin (HSA) fraction V was purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). All air- or moisture-sensitive reactions were conducted in flame-dried glassware under an atmosphere of dry argon. All solvents were of reagent grade, and dried solvents were freshly distilled before use. Tetrahydrofuran and diethyl ether were dried over KOH and distilled from sodium/benzophenone. Chromatographic purifications were performed on silica gel columns according to the flash chromatography technique. All monodimensional nuclear magnetic resonance spectra (^{1}H and ^{13}C) were recorded on a Bruker AC300 [300 MHz (^{1}H) and 75 MHz (^{13}C)] spectrometer. Chemical shifts are reported in parts per million (ppm, δ) with respect to TMS. $CHCl_3$ was used as an internal standard (δ = 7.26 ppm). Multiplicities are indicated by s (singlet), d (doublet), t (triplet), and m (multiplet). The α-(^{13}C)-methylene hexahydrobenzofuranone **1** was synthesized according to a previously described procedure[25] using (^{13}C)MeI as the source of carbon 13.

(±)-*cis*-3-(^{13}*C*)-Methyl-3-(phenylselenyl)-hexahydrobenzofuran-2-one (3). To a solution of lithium diisopropylamine (LDA, 6.99 mmol, 1.5 equiv), prepared at −40 °C from diisopropylamine (0.986 mL, 6.99 mmol, 1.5 equiv) in dry tetrahydrofuran (10.0 mL) and a solution of *n*-butyllithium in hexanes (1.5M, 4.66 mL, 6.99 mmol, 1.5 equiv), was added dropwise at −78 °C a solution of **2** (1.38 g, 4.66 mmol, 1.0 equiv) in diethyl ether (8.7 mL). After stirring for 2 h at −78 °C, a solution of (^{13}C)methyl iodide (0.44 mL, 7.00 mmol, 1.5 equiv) and hexamethylphosphoramide (HMPA, 1.22 mL, 7.00 mmol, 1.5 equiv) in THF (8.7 mL) was added dropwise, and the mixture was allowed to warm up to 0 °C overnight. A solution of hydrochloric acid 10% (30 mL) was added. The aqueous phase was extracted with diethyl ether (3 × 80 mL), and the organic layer was washed with water (1 × 80 mL) and brine (1 × 80 mL), dried over

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Table 1. ^{1}H and ^{13}C Predicted and Experimental Resonance Assignments of Adducts Formed by the Reaction of Lactone 1 and N-Acetylated Nucleophilic Amino Acids as Well as by the Reaction of Lactone 1 with HSA

	predicted		experimental		HSA	
amino acid	$\delta^{1}H$ (ppm)	$\delta^{13}C$ (ppm)	$\delta^{1}H$ (ppm)	$\delta^{13}C$ (ppm)	$\delta^{1}H$ (ppm)	$\delta^{13}C$ (ppm)
Lys	2.63/2.68 (0.31)	48.3 (4.3)	3.05/3.14	43.31	3.20	43.50
			3.10[a]	46.73[a]	3.21[a]	46.25[a]
	2.13/2.32 (0.17)[a]	54.65 (6.7)[a]			3.12[a]	47.88[a]
His	3.71/4.09 (0.28)	49.8 (6.8)	4.13/4.26	43.7	4.28	45.23
Cys	2.74/2.93 (0.09)	31.88 (3.7)	2.72/2.92	35.80	2.90	39.9
Arg	3.53/3.57 (0.41)	43.06 (4.3)	ND	ND	ND	ND
Tyr	4.03/4.14 (0.42)	67.62 (4.3)	ND	ND	ND	ND
Ser	3.80/3.91 (0.42)	68.27 (4.3)	ND	ND	ND	ND

[a]Bis-adduct with lysine. ND = not detected.

$MgSO_4$, and concentrated. The selenyl derivative **3** (1.45 g) was obtained as a reddish oil and used without further purification.

(^{13}C)-(±)-3-Methylene-*cis*-hexahydrobenzofuran-2(3*H*)-one (1). To a solution of the crude selenyl derivative **3** (1.45 g, 4.67 mmol, 1.0 equiv) in dry tetrahydrofuran (25 mL) was added acetic acid (0.70 mL). The mixture was then cooled down to 0 °C, and 35% hydrogen peroxide (3.7 mL) was added. After 1 h of stirring at 0 °C, a saturated solution of aqueous $NaHCO_3$ (30 mL) was added. The aqueous phase was extracted with diethyl ether (3 × 60 mL), and the combined organic layers were washed with water (1 × 60 mL) and brine (1 × 60 mL), dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The crude product was purified on silica gel column chromatography (pentane/diethyl ether, 95:5) to give **1** (0.456 g, 2.98 mmol, 64% yield) as a yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.16−1.94 (m, 8H, 4× $-CH_2-$), 2.96−3.05 (m, 1H, H_{3a}), 4.52 (ddd, $^{3}J_1 = {}^{3}J_2 = {}^{3}J_1 = 6.2$ Hz, 1H, H_{7a}), 5.50 (dd, $^{2}J_{H-H} = 2.2$ Hz, $^{1}J_{H-C} = 160.4$ Hz, 1H, H_8), 6.13 (dd, $^{2}J_{H-H} = 2.6$ Hz, $^{1}J_{H-C} = 162.6$ Hz, 1H, H_8). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 20.5 ($-CH_2-$), 21.1 ($-CH_2-$), 26.3 ($-CH_2-$), 28.9 ($-CH_2-$), 39.6 (C_{3a}), 119.6 ($^{13}C_8$), 139.8 (d, $^{1}J_{C-C} = 74.8$ Hz, C_3), 171.0 (C_2).

Reaction of Lactone 1 with Model Amino Acids. (^{13}C)-(±)-3-Methylene-*cis*-hexahydrobenzofuran-2(3*H*)-one **1** was reacted with N-acetylated nucleophilic amino acids (His, Lys, Arg, Cys, Tyr, and Ser) according to the following general procedure. To a solution of *N*-Ac-amino acid (70 μmol, 10 equiv) in phosphate buffer (0.3 mL, 0.1 M, pH 7.4) was added a solution of **1** (1.07 mg, 7.0 μmol, 1.0 equiv) in CD_3CN (0.3 mL). The reaction mixture was filtered into an NMR tube for further analysis. A trace of acetonitrile was added as an internal reference (δ = 119.68) and reactions followed by ^{13}C NMR. Structures of adducts were assigned using g-HSQC experiments.

Reaction of Lactone 1 with HSA. To a solution of HSA (100 mg, 1.5 μmol, 1.0 equiv) in phosphate buffer (10 mL, 0.1 M, pH 7.4) degassed for 15 min was added a solution of **1** (46.1 mg, 300 μmol, 200 equiv) in a minimal amount of ethanol (1 mL). The reaction mixture was incubated at 37 °C for 10 days, and the solution was dialyzed against water (5 × 5 L), then lyophilized to give a yellowish solid (46 mg), which was dissolved in a mixture of H_2O (0.3 mL) and D_2O (0.1 mL). A drop of CH_3CN was added as internal reference and the mixture filtered into an NMR tube for further analysis. Structures of the different adducts were assigned using g-HSQC experiments carried out on a Bruker AC400 [400 MHz (^{1}H) and 100 MHz (^{13}C)].

Reconstructed Human Epidermis. The SkinEthic RHE model (SkinEthic, Lyon, France) was chosen as it could be obtained in a large 4 cm^2 format. SkinEthic RHE are normal human keratinocytes cultured for 17-days on an inert polycarbonate filter at the air−liquid interface. The 3-D reconstructed epidermis was 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 humidified atmosphere, according to SkinEthic's procedures.

Treatment of RHE with Lactone 1 and Control. SkinEthic RHE were topically treated with **1** in acetone (0.6M, 100 μL) and incubated for 24 h. The RHE negative control was not treated and incubated for 24 h. After incubation, the RHE were washed with fresh medium, separated from the polycarbonate support using a treatment with Dispase II (neutral protease, grade II, Roche, Mannheim) in Hepes buffer *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid), washed with water, and stored at −80 °C before NMR sample preparation.

Rotor Preparation and HRMAS Data Acquisition. Each sample was prepared at −20 °C by introducing 15 to 20 mg of frozen RHE into a disposable 30 μL KelF insert. To provide a lock frequency for the NMR spectrometer, 10 μL of D_2O containing 1% w/w TSP was also added to the insert. The insert was then sealed tightly with a conical plug and stored at −80 °C until the HRMAS analysis. Shortly before the HRMAS analysis, the insert was placed into a standard 4 mm ZrO_2 rotor and closed with a cap. The ensemble was then inserted into an HRMAS probe precooled at 3 °C. All HRMAS experiments were performed at 3 °C and were started immediately after the temperature inside the probe had reached the equilibrium condition (5 min).

HRMAS spectra were recorded on a Bruker Avance III 500 spectrometer (Hautepierre University Hospital, Strasbourg) operating at a proton frequency of 500.13 MHz. The spectrometer was equipped with a 4 mm double resonance (^{1}H, ^{13}C) gradient HRMAS probe, and a Bruker Cooling Unit was used to regulate the temperature at 3 °C. All NMR experiments were conducted on samples spinning at 3502 Hz in order to keep the rotation sidebands out of the spectral region of interest. Detailed acquisition procedures have been previously described.[21]

RESULTS AND DISCUSSION

Synthesis. In order to study the reactivity of the model bicyclic α-methylene-γ-butyrolactone in RHE, a carbon-13 substituted derivative was prepared. Thus, (^{13}C)-(±)-3-methylene-*cis*-hexahydrobenzofuran-2(3*H*)-one **1** was synthesized according to the methodology previously described by Fuchs et al.[28] but using (^{13}C)methyl iodide at the penultimate step (Scheme 2).

Reaction of Lactone 1 with Nucleophilic Amino Acids and HSA. In order to get ^{1}H and ^{13}C NMR chemical shift references of potential adducts formed by reaction of **1** with nucleophilic amino acids, predicted spectra were generated using ACD/CNMR and ACD/HNMR Predictor software (version 6.0, ACD/Laboratories, Toronto, Canada). Lactone **1** was also reacted with N-acetylated nucleophilic amino acids (Cys, Lys, His, Arg, Tyr, and Ser) that could be considered as potential hapten targets in proteins. Under the conditions used (10 equiv of nucleophile in phosphate buffer at pH 7.4) in order to favor a potential modification of amino acids, reactions were observed with cysteine, histidine, and lysine (Table 1 and Figure 2). Lactone **1** was also reacted with a model protein, HSA, and several adducts were observed. A tiny signal at

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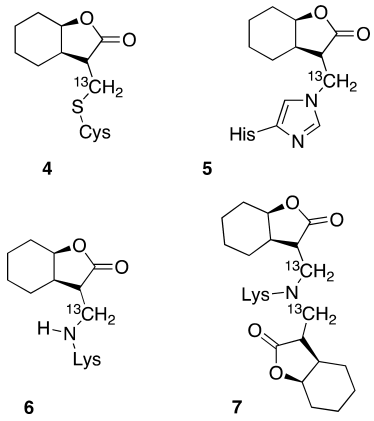

Figure 2. Structures of adducts formed between **1** and nucleophilic amino acids.

2.90(^{1}H)/39.90(^{13}C) (Figure S1), with respect to acetonitrile used as internal reference, was assigned to the formation of a cysteine adduct **4** (Figure 2). This correlation was in agreement with the theoretical/experimental values of a -CH_2-S- connection and was already observed by Franot et al. in previous studies using another model of lactone and dithiothreitol-reduced HSA.[29] The signal at 3.20(^{1}H)/43.50(^{13}C) was interpreted as arising from a lysine adduct **6**, while signals at 3.21(^{1}H)/46.25(^{13}C) and 3.12(^{1}H)/47.88(^{13}C) were assigned to a bis-adduct **7** formed by the reaction of the amino group with two equivalents of lactone **1**. Indeed, when reacting an excess of **1** with *N*-Ac-Lysine we first observed the formation of a monoadduct **6** with 3.05/3.14(^{1}H)/43.31(^{13}C) chemical shifts and then the formation of the bis-adduct **7** with 3.10(^{1}H)/46.73(^{13}C) chemical shifts. Finally, a very tiny signal at 4.28(^{1}H)/45.23(^{13}C) was assigned to the formation of histidine adduct **5** (Figure S1).

HRMAS NMR Analysis of RHE Treated with Lactone 1. To characterize the reactivity of **1**, the 2D ^{1}H−^{13}C g-HSQC edited sequence was used as previously described.[21] Representative HSQC edited spectra corresponding to RHE treated with lactone **1** compared with the HSQC edited spectrum of the control are presented in Figure 3 and Figure 4.

First, we can observe the residual signal of lactone **1** still present in the RHE at 5.74/6.19(^{1}H) and 124.10(^{13}C) ppm. The enlargement of the area characteristic of potential adducts is shown in Figure 4b. NMR data obtained on RHE treated by **1** compared to the control showed the presence of several groups of adducts. First, a large group of signals at 2.94(^{1}H)/32.29(^{13}C), 2.94(^{1}H)/28.82(^{13}C), and 2.68(^{1}H)/28.82(^{13}C) ppm was assigned to the haptenation of cysteine residues. The different chemical shifts observed in HRMAS NMR for cysteine adducts could reflect their different local microenvironment. Second, we observed the presence of a series of adducts at 3.17(^{1}H)/43.12(^{13}C), 3.17(^{1}H)/45.52(^{13}C), and 3.30(^{1}H)/45.54(^{13}C) ppm. These signals were assigned to the formation of a monoadduct with lysine **6** and to the formation of bis-adduct **7**. With the lactone being present in the RHE in excess, as confirmed by the residual signal of **1** present even after 24 h, the formation of a bis-adduct can be postulated. Finally, we observed much significant reactivity with histidine residues contained in RHE at correlations 4.26(^{1}H)/43.00(^{13}C), 4.32(^{1}H)/44.98(^{13}C), 4.18(^{1}H)/45.01(^{13}C), and 4.36(^{1}H)/47.08(^{13}C) ppm.

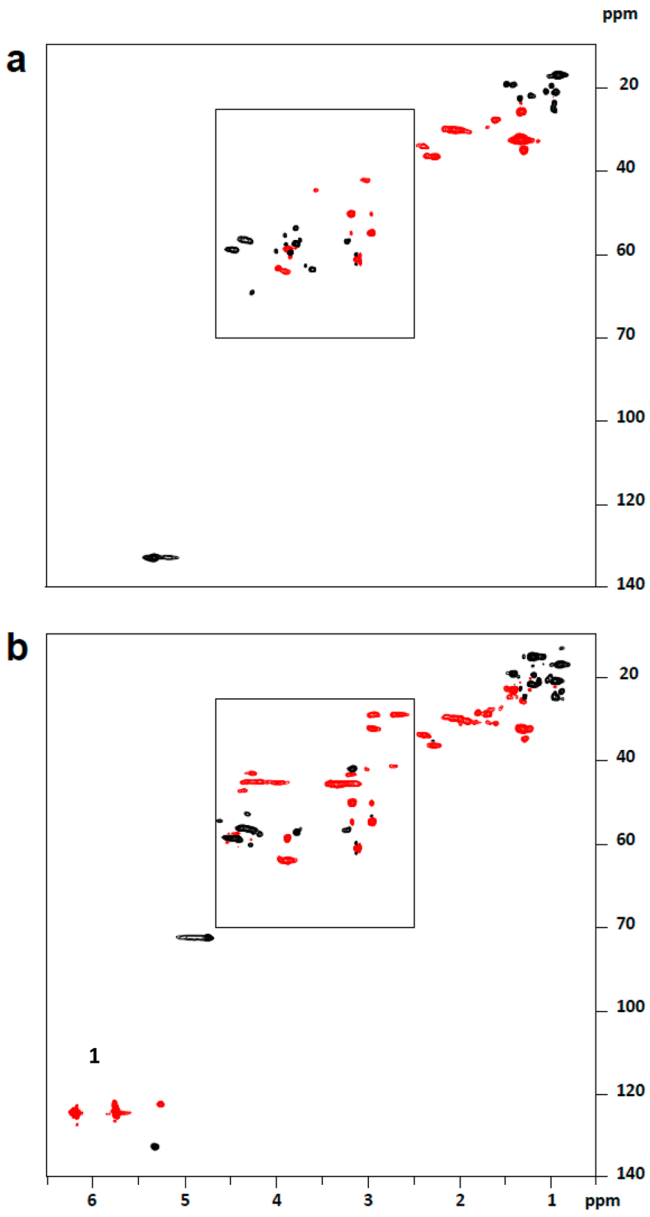

Figure 3. Full scale representative edited 2D HRMAS ^{1}H−^{13}C g-HSQC spectra of reconstructed human epidermis (a) without treatment and (b) with treatment with lactone **1**.

Chemoselectivity of *cis*-α-(^{13}*C*)Methylene Hexahydrobenzofuranone 1 in RHE. Reactivity studies carried out so far using model nucleophiles have shown chemoselectivity associated with most chemical sensitizers. Thus, even if lysine and cysteine have been often quoted as the main targets in proteins and therefore used to develop *in chemico* alternative methods for the detection of chemical skin allergens,[12,30] it has also been observed that other nucleophilic amino acids such as histidine,[31] tyrosine,[32] arginine,[33] and methionine[21] could be modified by chemical allergens. This chemoselectivity is expected to have a major impact on the activation and/or regulation of the immune response to chemical allergens. First, the modification of different amino acids will lead to the formation of different antigenic peptides during the processing of modified proteins by antigen presenting cells. Second, it has been shown that the chemoselectivity of haptens could play a major role in the activation of the Nrf2-ARE pathway[34] and

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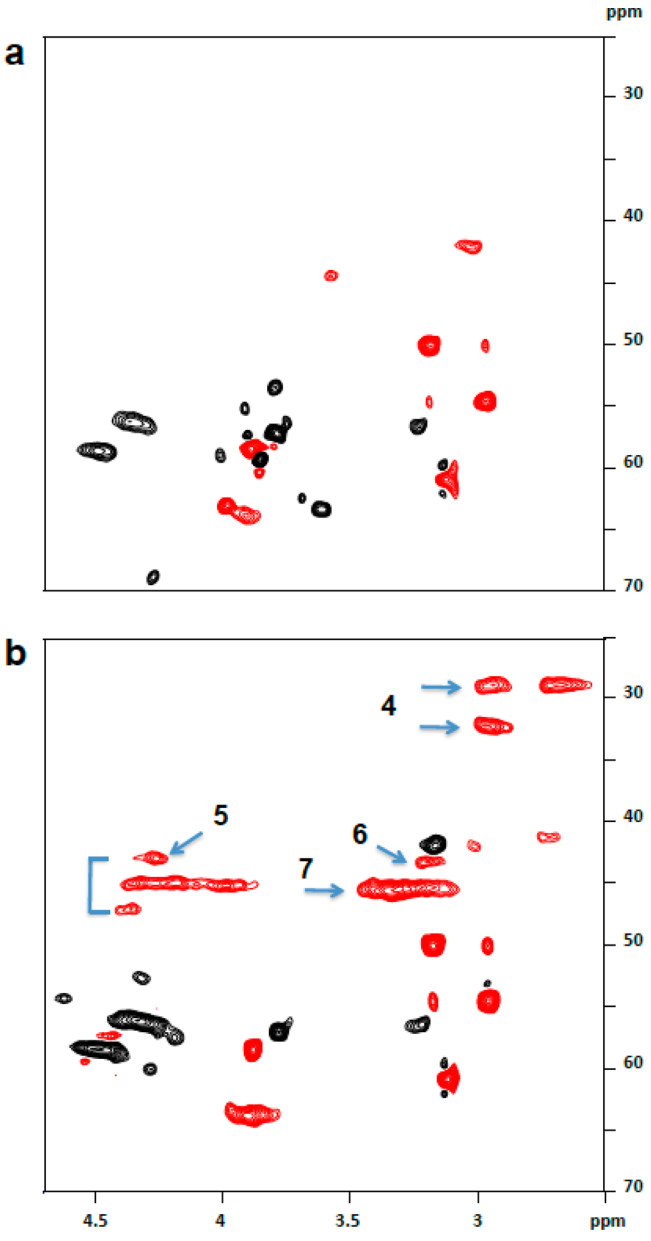

Figure 4. Enlarged representative edited 2D HRMAS $^{1}H-^{13}C$ g-HSQC spectra of reconstructed human epidermis (a) without treatment and (b) with treatment with lactone **1**. Arrows indicate new signals.

therefore contribute to the activation of detoxication pathways. Thus, chemicals able to react with thiol groups are also able to activate the Nrf2-ARE pathway and subsequently promote the expression of associated detoxication/protection systems such as heme oxygenase 1 (ho-1), NAD(P)H dehydrogenase, quinone 1 (nqo1), or interleukine 8 (Il-8), while sensitizing chemicals reacting only with lysine residues are not.[34] However, the chemoselectivity, as observed with model nucleophiles (amino acids, model peptides, and model proteins) in solution, could be somehow different from the one taking place in a 3-D tissue like the epidermis where diffusion and microenvironment parameters could be very different. Indeed, in RHE the chemoselectivity appeared to be different from the one observed when using a model protein such as HSA in solution with a different Lys/Cys balance and a much higher His reactivity in RHE (Table 2 and Figure 4b). If a single free

Table 2. ^{1}H and ^{13}C Resonance Assignments of Additional Signals Observed in RHE after Treatment by α-(^{13}C)Methylene Dehydrobenzofuranone 1

product(s)	$\delta^{1}H$ (ppm)	$\delta^{13}C$ (ppm)
1	5.74/6.19	124.10
4	2.94	32.29
	2.94	28.82
	2.68	28.82
6	3.17	43.12
7	3.17	45.52
	3.30	45.54
5	4.26	43.00
	4.32	44.98
	4.18	45.01
	4.36	47.08

cysteine residue present in the model protein could explain the observed greater reactivity toward cysteine residues in RHE compared to that of HSA, the difference in reactivity toward histidine residues is more striking. Indeed, this reactivity was very low in HSA despite the presence of 16 potentially reactive histidine residues, while 4 groups of signals corresponding to the reaction with histidine side chains were observed in RHE. It is interesting to observe that His, which is generally not considered as a major nucleophile in solution, seems to be a major target in 3-D RHE models. Similar observations on reactivity and chemoselectivity were observed with methyldodecanesulfonate, a methylating agent, with a different Lys/Cys balance and a much higher His reactivity in RHE.[21] Moreover, this is the first time that this type of reactivity is demonstrated for lactones in a living tissue. Indeed, to our knowledge, the only study, which describes the formation of protein-α-methylene-γ-butyrolactone adducts in cell medium, was performed on bacteria cultures.[35] In this study, the authors observed, by mass spectrometry, that only one cysteine residue was modified by the probe, while none of the 12 histidine residues reacted.

What Are the Consequences for the Development of *in Chemico* Alternative Methods? *In chemico* alternative methods are based on a series of approaches aimed at modeling the different chemical reactions taking place between potential skin sensitizers and nucleophilic amino acids present in epidermal proteins.[8] The selection of model nucleophiles has been based on existing knowledge, mainly derived from in solution chemistry using either model nucleophiles such as $BuNH_2$ or PhSH, amino acids, peptides, or model proteins. Under these conditions, the reactivity of histidine has never been reported as significant, and studies have been focusing mainly on cysteine and lysine residues. The fact that, in water solution, histidine adducts have not been considered could probably be associated with the fact that the imidazole moiety, in addition to being a potent nucleophile, is also a very good leaving group, leading in solution to equilibrated reactions. However, when performing such chemical reactions in a 3-D epidermis and using an *in situ* noninvasive observation, the microenvironment seems to stabilize adducts on histidine and prevent, in our case, a retro-Michael reaction from taking place. Such retro-Michael reactions on lactone derivatives bearing a good leaving group have been reported in the presence of HSA.[29]

The significance to the immune system of histidine interactions with haptens is poorly known with the only well

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documented example being the role of histidine residues on the human toll-like receptor 4 (TLR4) molecules explaining the skin sensitization to nickel.[36,37] Indeed, authors have been showing that histidine 456 and 458 are crucial for the binding of Ni^{2+} to the human TLR4 and that the absence of these residues in mouse TLR4 explains the impossibility to sensitize this animal species to nickel. Of course one may argue that Ni^{2+} is making coordination bonds with histidine and not covalent bonds as in the case of organic chemical allergens. Nevertheless, this illustrates that histidine residues can play a crucial role in skin sensitization to chemicals.

Another consequence of the formation of reversible histidine adducts could be to create a "reservoir" effect in the epidermis. Haptens could then be released over time to react with other amino acids leading to more thermodynamically stable adducts. This kind of reservoir/transport effect has been postulated for isocyanates derivatives, able to react first with cysteine to form reversible adducts that can be later transferred to an amino group making a more stable urea derivative.[38,39] However, this reservoir/transport effect reported with cystein still needs to be demonstrated for histidine.

Obviously, the role of histidine in the induction of skin sensitization has been so far neglected and should probably be taken into account for the refinement of *in chemico* approaches for the detection and potency classification of skin sensitizers.

CONCLUSIONS

This work demonstrated that the HRMAS NMR technique in association with RHE allowed one to investigate the reactivity of *cis*-α-(^{13}C)methylene hexahydrobenzofuranone in a 3-D-living tissue. Using this technique, we showed that amino acid modifications could be different according to the model studied and that histidine residues seem to have an important role in the formation of adducts in RHE.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.chemrestox.5b00363.

Enlarged representative edited 2D $^{1}H-^{13}C$ g-HSQC spectrum of HSA treated with lactone **1** (PDF)

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Notes

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ABBREVIATIONS

ACD, allergic contact dermatitis; AOP, adverse outcome pathway; DPRA, direct peptide reactivity assay; HRMAS, high-resolution magic angle spinning; HSA, human serum albumin; g-HSQC, gradient heteronuclear single quantum correlation; RHE, reconstructed human epidermis; TMS, tetramethylsilane; TSP, sodium tetramethylsilylpropionate

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