

Bioactivation of Cinnamic Alcohol Forms Several Strong Skin Sensitizers

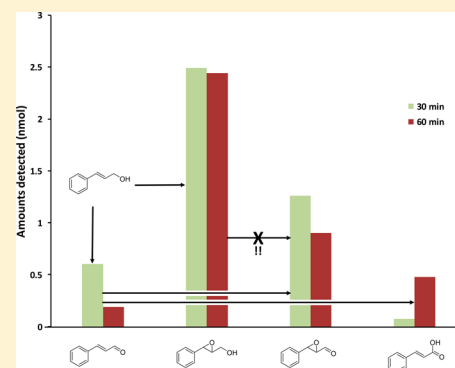
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S Supporting Information

ABSTRACT: Cinnamic alcohol is a frequent contact allergen, causing allergic contact dermatitis (ACD) in a substantial number of individuals sensitized from contacts with fragrances. Hence, cinnamic alcohol is one of the constituents in fragrance mix I (FM I) used for screening contact allergy in dermatitis patients. Cinnamic alcohol lacks structural alerts for protein reactivity and must therefore be activated by either air oxidation or bioactivation to be able to act as a sensitizer. In the present study, we explored the bioactivation of cinnamic alcohol using human liver microsomes (HLM), and the potential pathways for these reactions were modeled by *in silico* (DFT) techniques. Subsequently, the reactivity of cinnamic alcohol and its metabolites toward a model hexapeptide were investigated. In addition to cinnamic aldehyde and cinnamic acid, two highly sensitizing epoxides previously unobserved in studies of bioactivation were detected in the incubations with HLMs. Formation of epoxy cinnamic aldehyde was shown (both by the liver microsomal experiments, in which no depletion of epoxy cinnamic alcohol was observed after initial formation, and by the very high activation energy found for the oxidation thereof by calculations) to proceed via cinnamic aldehyde and not epoxy cinnamic alcohol.



INTRODUCTION

Cinnamic alcohol and cinnamic aldehyde (Figure 1) are frequent contact allergens, causing allergic contact dermatitis

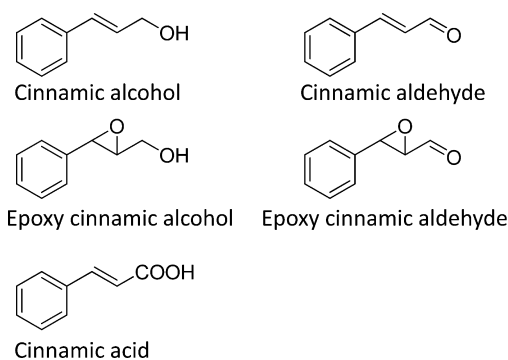


Figure 1. Structures of compounds studied.

(ACD) in a substantial number of individuals sensitized from contacts with flavors and fragrances. Therefore, they are two of eight constituents of fragrance mix I (FM I) used in the baseline series for screening contact allergy in dermatitis patients. Contact allergy is a T-cell-mediated delayed-type hypersensitivity reaction caused by the formation of immunogenic complexes that is considered to take place between electrophilic compounds (haptens) and nucleophilic functional groups on

proteins in the skin. Some compounds that are not directly reactive can nevertheless cause contact allergy; these need to be activated first, either abiotically (e.g., via autooxidation; prohaptens) or by bioactivation (prohaptens). The toxicological and dermatological properties of cinnamic alcohol have been extensively reviewed.¹ Cinnamic alcohol lacks structural alerts for protein reactivity in its own right but has been shown to act as a prohaptens by forming the hapten cinnamic aldehyde via metabolic oxidation in the skin.^{2,3} Cinnamic alcohol and cinnamic aldehyde are generally considered to be the most prominent example of a prohaptens–hapten pair. Therefore, cinnamic alcohol has been the preferred prohaptens in numerous experimental studies on skin metabolism over the years.^{4–6} In a recent study, we have shown that cinnamic alcohol can be readily activated outside the skin by autooxidation, thus acting not only as a prohaptens but also as a prehaptens.⁷ The identified sensitizers were cinnamic aldehyde and epoxy cinnamic alcohol.

Epoxides and aldehydes can be formed via metabolic oxidation^{8–11} and/or autooxidation.^{7,12} In these processes, the oxidation products could be identical even though they are formed via totally different pathways. A well-studied example is the fragrance terpene geraniol. Geraniol is an unsaturated alcohol structurally related to cinnamic alcohol that has been shown to be activated both via autooxidation, acting as a

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prehapten, and via bioactivation, acting as a prohapten. Epoxides were formed via both activation routes.^{13,14} However, to the best of our knowledge, to date, the only reported metabolites formed from cinnamic alcohol are cinnamic aldehyde and cinnamic acid.

The aim of the present study was to investigate whether other skin sensitizers besides cinnamic aldehyde (i.e., epoxides) can be formed via bioactivation as has been shown for geraniol. The formation of oxidation products was investigated using human liver microsomes, and potential pathways for these reactions were modeled by *in silico* (DFT) techniques. Furthermore, the chemical reactivity of cinnamic alcohol and the metabolites toward a hexapeptide was investigated, as chemical reactivity toward peptide nucleophiles can be correlated to sensitizing potency.¹⁵

■ EXPERIMENTAL PROCEDURES

Caution: These chemicals are dangerous. This study involves skin-sensitizing compounds that must be handled with particular care.

Chemicals. Epoxy cinnamic alcohol and epoxy cinnamic aldehyde (Figure 1) were synthesized as described previously.⁷ Cinnamic alcohol and cinnamic aldehyde (Figure 1) were purchased from Aldrich Chemicals (Stockholm, Sweden) and purified before use with preparative HPLC and column chromatography, respectively. The AcPHCKRM peptide was purchased from Peptide 2.0 Inc. (Chantilly, VA, USA). Acetone was purchased from Merck (Darmstadt, Germany), and olive oil, from Apoteket AB (Gothenburg, Sweden). Unless otherwise indicated, reagents were obtained from commercial suppliers and used without further purification.

Instrumentation and Mode of Analysis. *Gas Chromatography/Mass Spectrometry (GC/MS).* Electron-ionization mass spectral analysis (70 eV) was performed on a Hewlett-Packard 5973 mass spectrometer connected to a gas chromatograph (Hewlett-Packard 6890) equipped with a splitless capillary inlet and an HP-5MSi fused silica capillary column (30 m × 0.25 mm, 0.25 μm, Agilent Technologies, Palo Alto, CA, USA). Helium was used as the carrier gas, and the flow rate was 1.3 mL/min. The temperature program started at 50 °C for 1 min, increased by 10 °C/min, and ended at 250 °C for 5 min. For mass spectral analysis, the mass spectrometer was used in the SIM mode, detecting ions with *m/z* values 119 and 148 for epoxy cinnamic aldehyde, 103 and 132 for cinnamic aldehyde, 91 and 134 for cinnamic alcohol, 132 and 150 for epoxy cinnamic alcohol, and 131 and 150 for cinnamic acid.

Preparative High-Performance Liquid Chromatography (HPLC). HPLC was performed using a Gilson pump model 305, a Gilson UV/vis detector model 119, and a Zorbax semipreparative column (250 mm × 9.4 mm, 5 μm particles, Agilent Technologies, Palo Alto, CA, USA); the flow rate was 6.0 mL/min, and the compounds were monitored at 230 nm. Aliquots of 100 μL were injected onto the column and eluted with acetonitrile (35%) in Milli-Q water.

LC/MS/MS System. LC/MS/MS analysis (1200 series, Agilent Technologies, Palo Alto, CA, USA) consisted of a high-pressure pump, an autosampler, and a mass spectrometer (Triple Quad LC-MS 6410) equipped with an electrospray interface and a triple-quadrupole mass analyzer. The electrospray triple quadrupole mass spectrometer was operated in the positive ionization mode. The positive ions of the analytes were detected by following transitions in multiple reaction monitoring mode (MRM).

LC/MS Analyses. LC/MS was performed using electrospray ionization (ESI) on a Hewlett-Packard 1100 HPLC/MS. The system included a vacuum degasser, a binary pump, an autoinjector, a column thermostat, a diode array detector, and a single quadrupole mass spectrometer. The HPLC was equipped with a HyPURITY C18 column (150 × 3 mm i.d., particle size 3 μm, Thermo Hypersil-Keystone, Thermo Electron Corp., Bellafonte, PA, USA). The mobile phases consisted of 0.005% pentafluoropropanoic acid, 0.1% acetic acid, and 5% acetonitrile in water (solvent A) and 0.005% pentafluoropropanoic acid, 0.1% acetic acid, and 50% water in

acetonitrile (solvent B). A linear gradient from 0 to 100% B in 30 min followed by 10 min of isocratic elution was used. The flow rate was 0.40 mL/min, and the column temperature was set to 40 °C. The electrospray interface was used with the following spray chamber settings: nebulizer pressure, 40 psig; capillary voltage, 3500 V; drying gas temperature, 350 °C; and drying gas flow rate, 10 L/min. Fragmentor voltage was set to 120 V to produce molecular fragments. The mass spectrometer was used in scan mode, detecting molecular ions with *m/z* values ranging from 50 to 1600.

Liver Microsomal Incubations. The microsomal incubations were performed using human liver microsomes (HLM) (0.5 mg of protein pooled from 29 male and female donors, BD Biosciences), substrate (10 μM), potassium phosphate buffer (100 mM, pH 7.4) and a nicotinamide adenine dinucleotide phosphate (NADPH)-regenerating system (1.3 mM NADP⁺, 3.3 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase, and 3.3 mM magnesium chloride, BD Biosciences) in a total volume of 500 μL. All incubations were performed in triplicate, and control samples were run in the absence of substrate or in the absence of the NADPH-regenerating system. The incubations were initialized by the addition of the substrate after 5 min of preincubation at 37 °C and were terminated after 30 or 60 min by addition of ethyl acetate (500 μL) containing internal standard (2 μM). The extracts were collected after centrifugation at 3000 rpm and dried over molecular sieves before analysis was performed using GC/MS and LC/MS/MS.

Reactions of Metabolites from Cinnamic Alcohol with the Model Peptide Ac-Pro-His-Cys-Lys-Arg-Met-OH (AcPHCKRM).

All solvents were degassed with argon prior to use. Solutions of cinnamic alcohol in dimethyl sulfoxide (DMSO) (40 mM, 100 μL) together with potassium phosphate buffer (100 mM, pH 7.4) (200 μL) were added to a vial purged with argon containing AcPHCKRM in DMSO (4 mM, 100 μL). Accordingly, final concentrations of cinnamic alcohol and the model peptide in the reaction mixture were 10 and 1 mM, respectively. The reaction was kept under argon at room temperature and monitored with HPLC/ESI-MS for 20 h. Samples were collected from two sets of reactions to be able to analyze every 20 min. All metabolites were treated identically, and their reactions were investigated using the method described earlier.

Stability Experiments. To study the stability of the metabolites formed from cinnamic alcohol under the microsomal conditions, we prepared 10 μM solutions of each metabolite in potassium phosphate buffer (100 mM, pH 7.4). The metabolite solutions (0.5 mL) were either immediately extracted or incubated in a water bath at 37 °C, where they were extracted after 30 or 60 min. All extracts were analyzed with GC/MS, and the incubated samples were compared with the immediately extracted samples to enable the calculation of recovery. The stability of the epoxides under the reactivity experiment conditions was studied using 10 mM solutions of each epoxide in potassium phosphate buffer (100 mM, pH 7.4) and DMSO (1:1). Samples were withdrawn every hour for 10 h and analyzed with GC/MS.

Computational Methods. The P450 heme active site was modeled using a methoxy radical, as described in the literature.^{16–22} All calculations were carried out at the B3LYP-D3/6-31+G**^{23,24} level of theory in Jaguar, part of the Schrödinger suite of programs. Structures were initially minimized in MacroModel before a LST search was undertaken in Jaguar for the transition state. If no transition state was initially forthcoming, then alternative attempts were tried, including variation of the initial guess and finding the initial *in vacuo* transition states, which were subsequently solvated. The solvent used for all calculations was implicit water, using the PBF model.^{25,26} Results were analyzed with the quick reaction coordinate (QRC) method of Goodman et al.²⁷ Calculations were performed on the SNIC facilities of C3SE, located at Chalmers University of Technology, Gothenburg.

■ RESULTS AND DISCUSSION

In this study, we demonstrated the formation, reactivity, and interconversion of previously undetected bioactivation products of cinnamic alcohol. We found that bioactivation of cinnamic

alcohol forms two highly sensitizing epoxides in addition to the previously reported strong sensitizer cinnamic aldehyde and the nonsensitizer cinnamic acid (Figures 1 and 2).

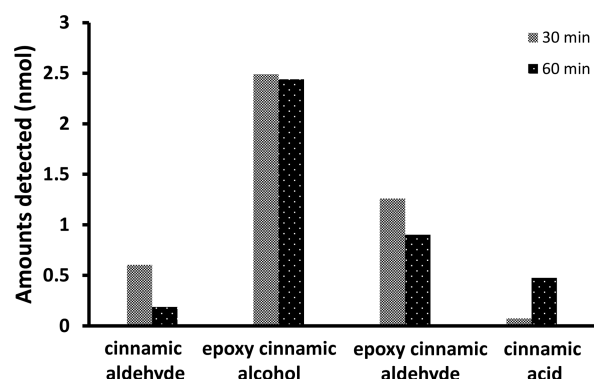


Figure 2. Metabolic activation of cinnamic alcohol in human liver microsomal incubations. The total amounts of the metabolites formed in the incubation mixtures are presented as the mean of three individual incubations. The microsomal incubations were performed using human liver microsomes (0.5 mg of protein), substrate (10 μ M), potassium phosphate buffer (100 mM, pH 7.4), and a NADPH-regenerating system in a total volume of 500 μ L. The incubations were terminated after 30 or 60 min and analyzed with GC/MS or LC/MS/MS (cinnamic acid).

Liver Microsomal Incubations. Human liver microsomes were used to study the bioactivation of cinnamic alcohol, a method that has previously been used within our group to study bioactivation of prohapten.²⁸ Liver microsomes were used as a model system because no comprehensive skin metabolism model exists today; however, similar metabolic processes are considered to occur in the liver as in the skin.^{29,30} Three major metabolites (retention time t_R = 6.2, 6.4, and 7.3 min) were detected in the human liver microsomal incubations of cinnamic alcohol when analyzing with GC/MS. The identity of the peaks was confirmed on the basis of their corresponding mass spectra using synthesized or purified purchased reference compounds for comparison. The peaks correspond to epoxy cinnamic aldehyde, cinnamic aldehyde, and epoxy cinnamic alcohol, respectively. LC/MS/MS analysis was used to detect formation of cinnamic acid in the microsomal incubations because of difficulties with the limit of detection of this compound in the GC/MS analysis.

The quantification of the formed metabolites (Figure 2) was based on the response from calibration curves of pure reference compounds and the use of an internal standard. Reported values are averages of triplicate experiments for each time point (30 and 60 min). Incubation of cinnamic alcohol (5 nmol) for 30 and 60 min with HLM resulted in epoxy cinnamic aldehyde (1.26 and 0.90 nmol), cinnamic aldehyde (0.60 and 0.19 nmol), epoxy cinnamic alcohol (2.49 and 2.44 nmol), and cinnamic acid (0.075 and 0.48 nmol). Only 12% of the total applied dose of cinnamic alcohol was detected as cinnamic aldehyde after 30 min. The concentration of cinnamic aldehyde decreased to 4% of the total applied dose of cinnamic alcohol after 60 min, possibly because of further oxidation to cinnamic acid. This is supported by a corresponding increase of the concentration of cinnamic acid (1.4 to 9.5%) during the same time period. The two epoxides were detected at higher concentrations (epoxy cinnamic aldehyde 25%, epoxy cinnamic alcohol 50%) than the cinnamic aldehyde after 30 min, but although the level of epoxy

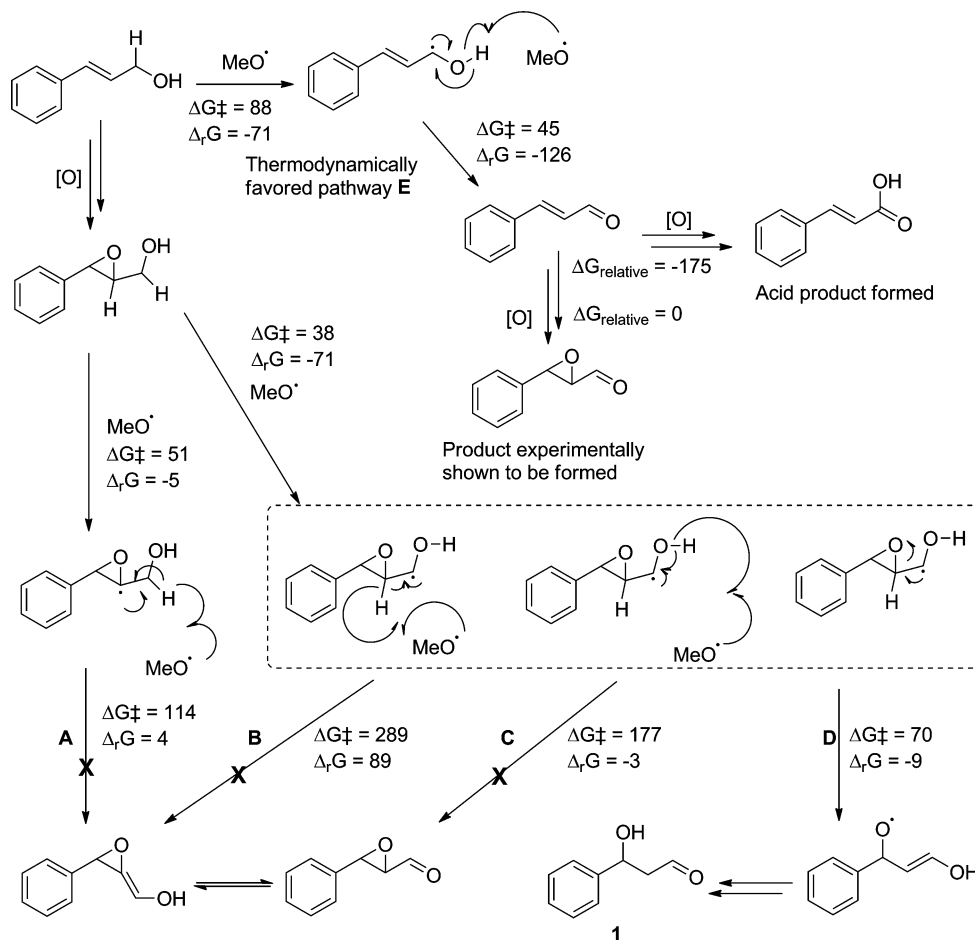
cinnamic alcohol was stable over time, the level of epoxy cinnamic aldehyde decreased with time. These epoxides have been undetected in previous studies^{3,31} of cinnamic alcohol metabolism. However, in those earlier studies^{3,31} that reported a recovery of 65–82%, HPLC with a methanolic mobile phase was used as a compound identification method. Therefore, according to our experience, detection of the epoxides would be unlikely, as both compounds are unstable under such conditions (data not shown).

The enzyme that is believed to be mainly responsible for the oxidation of haptens similar to that discussed in this article has been shown, with *in vitro* enzyme mixtures, to be CYP2B6.^{13,32,33} This is, as are other P450 enzymes,³⁴ a rather flexible enzyme.^{35,36} The flexibility makes attempts to model reactivity using the whole protein by mixed QM/MM methods impossible with current levels of computational power because of the time scales needed to handle the flexible protein. A model system was therefore chosen in which the methoxy radical was used as a substitute for the single-electron processes at the heme active site, as has earlier been described in the literature.^{16–22}

As shown in Figure 2, the amount of epoxy cinnamic alcohol, once formed, does not decrease over time. The possible pathways for further metabolism were modeled using the methoxy-radical model, as shown in Scheme 1. Key results from this study include the following. First, the oxidation of epoxy cinnamic alcohol to epoxy cinnamic aldehyde is a surprisingly high-energy process, with the possible pathways (Scheme 1, pathways A–C) all requiring a significantly higher activation energy (at least 26 kJ mol⁻¹) and being significantly less favored than the oxidation of cinnamic alcohol to cinnamic aldehyde (pathway E, with a total $\Delta_r G$ of -197 kJ mol⁻¹) in the same model system. Additionally, the obvious side reaction (opening of the epoxide to yield 3-hydroxy aldehyde 1 (pathway D in Scheme 1)) was also found to be of high activation energy and low thermodynamic driving force. However, the activation energy was lower compared to that of pathways B and C. The possible cause of the high activation energy of these pathways is a “pinning-back” of the alcohol proton by hydrogen bonding, rendering it less likely to take part in any form of oxidation processes, as can be seen in the minimum-energy conformer (Figure 3). Thus, epoxy cinnamic alcohol becomes a stable point in the overall pathway, trapping large amounts of the initial reactant epoxy at this state and resulting in a lower yield of epoxy cinnamic aldehyde than might otherwise be expected. Second, as has been discussed, the decrease of cinnamic aldehyde is almost identical to the increase of cinnamic acid, suggesting that the principal end point for cinnamic aldehyde is as cinnamic acid rather than epoxy cinnamic aldehyde. This is supported by calculations, with the pathway for oxidation to cinnamic acid being 175 kJ mol⁻¹ lower than that for oxidation to epoxy cinnamic aldehyde. In conclusion, the principal end points for oxidative processes in this model system and the liver microsomal assay are the strongly sensitizing epoxy cinnamic alcohol and the nonsensitizing cinnamic acid, as opposed to large quantities of the traditional culprit for allergy to cinnamic alcohol, cinnamic aldehyde.

Reactivity of Cinnamic Alcohol and Its Metabolites toward AcPHCKRM. A key event in both sensitization and elicitation of contact allergy is the formation of immunogenic hapten–protein complexes. In this study, the acetylated hexapeptide AcPHCKRM was used as a model to investigate the ability of cinnamic alcohol and its metabolites to modify either of the nucleophilic amino acids within this peptide

Scheme 1. Potential Intermediates and the Activation Energies ($\Delta G^\ddagger$) and ΔG Values (in kJ mol^{-1}) for Their Formation for the Interconversions between the Oxidation Products Cinnamic Aldehyde, Epoxy Cinnamic Alcohol, Epoxy Cinnamic Aldehyde, and Cinnamic Acid and Unobserved Side Product 1 Discussed Herein^a



^aAll energies are derived from transition states calculated in Jaguar at the B3LYP/6-31+G** level using a methoxy radical as model for the heme active site of CYP P450 enzymes. $\Delta_r G$ values are calculated for a single reaction; $\Delta G_{\text{relative}}$ are the relative free energies of conversion for the two possible isomeric oxidation products of cinnamic aldehyde.

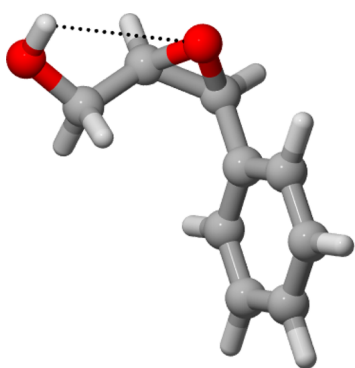


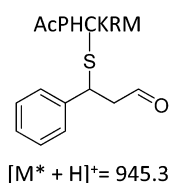
Figure 3. Minimum-energy structure (generated at B3LYP-D3/6-31+G**) of epoxy cinnamic alcohol, including the proposed hydrogen bond that is pinning back the alcohol proton.

covalently. The peptide has previously been used to assess the reactivity of contact allergenic haptens and prohaptens.^{37,38}

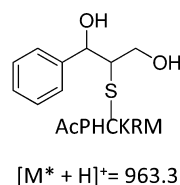
Cysteine adducts (Figure 4) were rapidly formed in reactivity experiments of the peptide and cinnamic aldehyde, epoxy cinnamic alcohol, and epoxy cinnamic aldehyde, as indicated by the appearance of $[M^* + H]^+$ and $[M^* + 2H]^{2+}$ ions (* denotes

one haptentation) in the mass spectra (Table 1). In addition, signals corresponding to the y_5^* , y_4^* , y_3 , y_2 , b_2 , and b_1 fragments were found in the mass spectra of all conjugates, indicating that the cysteine residue of the peptide were modified. The results are in good agreement with the well-known high reactivity of cysteine.³⁹ For cinnamic aldehyde, the adduct contained the base ions m/z 945.3 and 473.6, corresponding to $[M^* + H]^+$ and $[M^* + 2H]^{2+}$, respectively, which is in agreement with a Michael addition of peptide to the 2,3-unsaturated aldehyde (Figure 4A). For epoxy cinnamic alcohol, the adduct contained the base ions m/z 963.3 and 482.2, corresponding to $[M^* + H]^+$ and $[M^* + 2H]^{2+}$; the conjugate (shown in Figure 4B and Scheme 2) was formed via an opening of the epoxide, mainly at the 2-position, by the cysteine (the 2-position is slightly preferred (1 kJ mol^{-1}) to the 3-position in computational models using a methanethiolate model of the cysteine nucleophile) (Scheme 2). This (very weak) selectivity is due to the competition between electronic stabilization of the transition state because greater stabilization is available from the phenyl ring than from the alcohol and because of the better steric availability of the 2-position. Because this is an S_N2 process, the steric availability of the 2-position (free of the bulk

A. Reaction with cinnamic aldehyde



B. Reaction with epoxy cinnamic alcohol



C. Reaction with epoxy cinnamic aldehyde

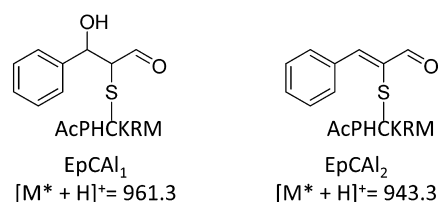


Figure 4. Suggested structures of conjugates formed in the reactivity experiments with the model peptide AcPHCKRM and (A) cinnamic aldehyde, (B) epoxy cinnamic alcohol, and (C) epoxy cinnamic aldehyde.

of the phenyl group) is dominant in determination of the regioselectivity.

In the reactivity experiment with epoxy cinnamic aldehyde, two cysteine adducts (EpCAL₁ and EpCAL₂) were observed immediately, as revealed by the presence of the y_5^* , y_4^* , y_3 , y_2 , b_2 , and b_1 fragments in the mass spectra (Table 1 and Figure 4C). EpCAL₁ contained the base ions m/z 961.3 and 481.2, corresponding to $[(M_{\text{peptide}} + M_{\text{epoxy cinnamic aldehyde}}) + H]^+$ and $[(M_{\text{peptide}} + M_{\text{epoxy cinnamic aldehyde}}) + 2H]^{2+}$, respectively. EpCAL₁ is the result of an epoxide opening via a nucleophilic substitution at the 2-position (35 kJ mol⁻¹ preference for the 2-position as opposed to the 3-position when modeled with a methanethiolate nucleophile) with the cysteine residue (Figure 4C and Scheme 2B). This strong preference is the result of combination of the steric preference for the more exposed site and electronic preference because of the strong conjugation of the transition state with the aldehyde. It should be noted that epoxidation of the Michael acceptor has therefore reversed the regioselectivity toward soft nucleophiles from the 3-position to the 2-position. This is of potential future interest to synthetic

chemists, as it leads to substitution on the otherwise harder-to-access 2-position. It derives from the aldehyde C=O interacting with the epoxide and activating the 2-site, which is conventionally considered to be unreactive to nucleophilic attack in a Michael acceptor.

The ions observed for EpCAL₂ were m/z 943.3 and 477.2, with the interpretation of $[(M_{\text{peptide}} + M_{\text{epoxy cinnamic aldehyde}} - 18) + H]^+$ and $[(M_{\text{peptide}} + M_{\text{epoxy cinnamic aldehyde}} - 18) + 2H]^{2+}$. We hypothesize that EpCAL₁ loses water to form the 2,3-unsaturated aldehyde EpCAL₂ (Figure 4C). Also, a third cysteine adduct (EpCAL₃) was present in the reaction mixture (identified by the presence of the y_5^* , y_4^* , y_3 , y_2 , b_2 , and b_1 fragments (Table 1)). EpCAL₃ gives the ions m/z 985.3 and 493.3 for $[(M_{\text{peptide}} + M_{\text{epoxy cinnamic aldehyde}} + 24) + H]^+$ and $[(M_{\text{peptide}} + M_{\text{epoxy cinnamic aldehyde}} + 24) + 2H]^{2+}$ or $[(M_{\text{peptide}} + M_{\text{EpCAL}_2} + 42) + H]^+$ and $[(M_{\text{peptide}} + M_{\text{EpCAL}_2} + 42) + 2H]^{2+}$. At present, no structure has been assigned to this adduct, but a similar pattern has previously been reported for another epoxyaldehyde and this peptide.³⁸

The conjugates formed from cinnamic alcohol of commercial quality in reactions with the peptide were probably due to reactive impurities because no adducts were observed in the reactivity experiment with purified cinnamic alcohol. This is congruent with our previous study of the autoxidation of cinnamic alcohol.⁷ The conjugates formed could not be separated with the LC/ESI-MS method used ($t_R = 11.4$ – 13.3 min). The ions and fragments found in the mass spectra are congruent with the ions and fragments of adducts of cinnamic aldehyde, epoxy cinnamic alcohol, and EpCAL₁ (Table 1). Individual extraction of these ions resulted in chromatograms with peaks at $t_{Ra} = 11.5$ min, $t_{Rb} = 11.6$ min, and $t_{Rc} = 12.6$ min, which corresponds to $t_{REpCAL_1} = 11.5$ min, $t_{REpoxy cinnamic alcohol} = 11.6$ min, and $t_{Rcinnamic aldehyde} = 12.6$ min, indicating that the impurities present are similar to metabolites epoxy cinnamic aldehyde, epoxy cinnamic alcohol, and cinnamic aldehyde, thus confirming the findings in the study of autoxidation where both cinnamic aldehyde and epoxy cinnamic alcohol were identified as impurities in cinnamic alcohol of commercial quality.

Depletion of unreacted peptide is, according to literature, a method that could be used for reactivity assessments.^{40–42} Figure 5 summarizes the depletion data for AcPHCKRM. As expected, epoxy cinnamic aldehyde and cinnamic aldehyde were the most reactive metabolites, causing 88 and 77% depletion, respectively, already after 15 min when used in 10-fold excess. Interestingly, epoxy cinnamic alcohol did not react as fast as the aldehydes even though almost the same depletion was reached after 360 min. For cinnamic alcohol (purified by HPLC), no depletion was observed throughout the whole experiment (20 h). However, for cinnamic alcohol of commercial quality, the depletion after 200 min was 20%, which most probably was due to reactions with impurities present in commercial cinnamic

Table 1. Ions and Fragments Observed for Monoadducts Formed in the Reactivity Experiments with AcPHCKRM^a

compound	adduct	$[M^* + H]^+ m/z$	$[M^* + 2H]^{2+} m/z$	$y_5^* m/z$	$y_4^* m/z$	$y_3 m/z$	$y_2 m/z$	$b_2 m/z$	$b_1 m/z$
cinnamic aldehyde		945.3	473.6	806.3	669.3	434.2	306.2	277.1	140.1
epoxy cinnamic aldehyde	EpCAL ₁	961.3	481.2	822.3	685.3	434.1	306.2	277.1	140.1
	EpCAL ₂	943.3	477.2	804.3	667.3	434.1	306.1	277.1	140.1
	EpCAL ₃	985.3	493.3	846.3	709.3	434.1	306.1	277.1	140.1
epoxy cinnamic alcohol		963.3	482.2	824.3	687.3	434.1	306.1	277.1	140.1

^aThe reactivity experiments of the metabolites of cinnamic alcohol were performed as described in the Experimental Procedures. The reactions were monitored with HPLC-ESI-MS. M^* and y^* indicate haptenated peptide and haptenated fragments, respectively.

Scheme 2. Activation Energies ($\Delta G^\ddagger$) and ΔG Values (in kJ mol^{-1}) Calculated in Jaguar at the B3LYP/6-31+G** Level for the Opening of the Epoxide Ring in Epoxy Cinnamic Alcohol (A) and Epoxy Cinnamic Aldehyde (B) by a Methanethiolate Nucleophile

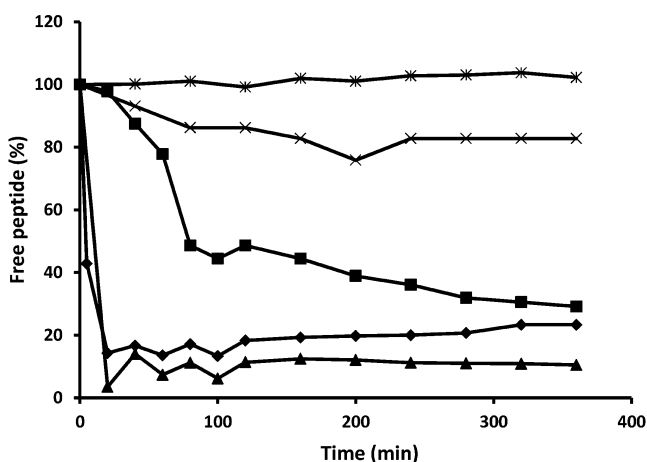
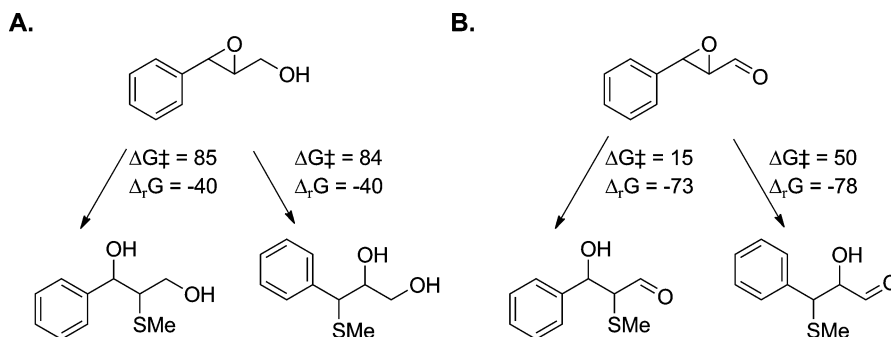


Figure 5. Depletion (consumption rate) of model peptide Ac-Pro-His-Cys-Lys-Arg-Met-OH (AcPHCKRM) obtained with cinnamic alcohol (purified with HPLC) (*), cinnamic alcohol of commercial quality (x), epoxy cinnamic alcohol (■), cinnamic aldehyde (◆), and epoxy cinnamic aldehyde (▲) in the reactivity experiments, normalized to 100%.

alcohol without purification, in accordance with results of our previous work.⁷

Autoxidation has also been shown to increase the sensitizing potency of cinnamic alcohol in the LLNA,⁷ reinforcing the idea that autoxidation can be important in contact allergy. As cinnamic alcohol and cinnamic aldehyde are generally considered to be the most prominent example of a prohaptent–haptent pair, cinnamic alcohol is the preferred prohaptent in numerous experimental studies on skin metabolism;^{4–6} therefore, cinnamic alcohol is also commonly used for evaluation of new *in vitro* assays. In method development, the increased sensitizing potency derived from the autoxidation products can lead to misinterpretations of the results; thus, evaluation of *in vitro* assays should be undertaken with pure cinnamic alcohol. In such studies, the metabolic formation to epoxides from cinnamic alcohol should also be investigated. In addition, consideration of the possibility of formation of a reactive 2,3-epoxide among the structural alerts related to 2,3-unsaturated alcohols should be included.

Because the oxidation products are already present, the dosage of sensitizers received under air exposure could be higher than the concentrations formed solely via metabolism. In spite of this, metabolism must not be ignored because it can further increase concentrations of the oxidation products.

Moreover, once formed, the metabolites do not need to penetrate the skin before reaction with proteins, and sensitization can most likely occur at lower concentrations than those required if penetration had to be considered. The amounts of metabolites formed are probably sufficient for an elicitation reaction in a sensitized individual when pure cinnamic alcohol is applied on the skin. Cinnamic alcohol is considered to be an almost as frequent cause of ACD as cinnamic aldehyde.⁴³ Patch-test results from the U.S. have shown fewer reactions to cinnamic aldehyde than to cinnamic alcohol when tested at the same concentrations.⁴⁴ More recent studies^{45,46} have demonstrated that between 21 and 38% of the patients who patch tested positive to cinnamic alcohol did not react to cinnamic aldehyde, which has been previously assumed to be the only culprit. The high frequency of allergic reactions to cinnamic alcohol might be explained by a higher degree of exposure to cinnamic alcohol than to cinnamic aldehyde.^{46,47} The discrepancy in reactions observed for cinnamic alcohol and cinnamic aldehyde can also be due to metabolic activation of cinnamic alcohol, forming allergenic epoxides in addition to cinnamic aldehyde. Moreover, cinnamic alcohol of commercial quality is used for testing dermatitis patients in the clinic. Thus, the presence of both cinnamic aldehyde and epoxy cinnamic alcohol in the patch-test material could affect the obtained test results. Such an effect seen on clinical test results is not necessarily negative because it has been previously shown in studies of geraniol that testing with oxidized material or with the sensitizing oxidation products (the haptens) can detect more cases of contact allergy compared to using the pre- or prohaptent solely.^{48,49}

CONCLUSIONS

In the present study, we have investigated the bioactivation of cinnamic alcohol, and the reactivity and interconversion of the metabolites formed thereof were explored. For the first time, we could show that, besides the already known sensitizer cinnamic aldehyde, two highly sensitizing epoxides were formed when cinnamic alcohol was incubated with human liver microsomes. Moreover, cinnamic alcohol of commercial quality showed peptide reactivity because of the presence of reactive impurities. The formation of epoxy cinnamic aldehyde was shown to proceed via cinnamic aldehyde and not via epoxy cinnamic alcohol, which was confirmed by computational methods. The low reactivity of the epoxy cinnamic alcohol may be due to the pinning back of the alcohol to the epoxide, resulting in a stable point on the pathway from which further oxidation is impossible. The results from the reactivity experiments were

congruent with the previously reported sensitizing potencies based on LLNA data (Table 2). Additionally, a reversal of

Table 2. LLNA Data and Depletion of AcPHCKRM with Cinnamic Alcohol and Its Metabolites

compound	chemical reactivity ^a (%) ^b	LLNA EC3 value		classification ^c based on LLNA data
		w/v (%)	M	
cinnamic alcohol (commercial quality)	20 ^d	21 ^e	1.7 ^f	weak
cinnamic alcohol (purified with HPLC)	0	nt ^g	nt	nt
cinnamic aldehyde	77	0.75 ^h	0.057	strong
epoxy cinnamic alcohol	71	0.58 ^h	0.039	strong
epoxy cinnamic aldehyde	91	2.22 ^h	0.015	strong
cinnamic acid	nt	nt	nt	nonsensitizer ⁱ

^aChemical reactivity toward the nucleophilic hexapeptide Ac-PHCKRM in DMSO/phosphate buffer (pH 7.4). ^bPercent depletion of the free peptide after 360 min. ^cThe sensitizing potency is classified as follows: EC3 value <0.1%, extreme; ≥0.1 to <1%, strong; ≥1 to <10%, moderate; and ≥10%, weak.⁵⁰ ^dCinnamic alcohol of commercial quality was used. ^eLiterature value, no additional purification stated.^{1,51} ^fMolarity is calculated with the molar mass of cinnamic alcohol. ^gnt, not tested. ^hLLNA data from Niklasson et al.⁷ The EC3 values (the estimated concentration required to induce an SI of 3) are calculated using linear interpolation. ⁱClassified as a nonsensitizer in guinea pig maximization test.⁵²

selectivity of the attack of soft nucleophiles on Michael acceptors has been demonstrated, moving the site of attack to the (normally unreactive) 2-position instead of either the 3-position or direct attack.

■ ASSOCIATED CONTENT

Supporting Information

Structure files for all transition states, reactants, and products. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

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■ ABBREVIATIONS

ACD, allergic contact dermatitis; AcPHCKRM, Ac-Pro-His-Cys-Lys-Arg-Met-OH; DFT, density functional theory; DMSO, dimethyl sulfoxide; ESI, electrospray ionization; HLM, human

liver microsomes; LLNA, local lymph node assay; LST, linear synchronous transit; NADPH, nicotinamide adenine dinucleotide phosphate

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Supporting Information:

Bioactivation of Cinnamic Alcohol Forms Several Strong Skin
Sensitizers

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Index of Structures contained within supporting information file:

