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Is isoeugenol a pre-hapten? Characterization of a thiol-reactive oxidative byproduct of isoeugenol and potential implications for skin sensitization.

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Keywords: Skin sensitization, isoeugenol, chemical reactivity, *in chemico* methods, stability studies

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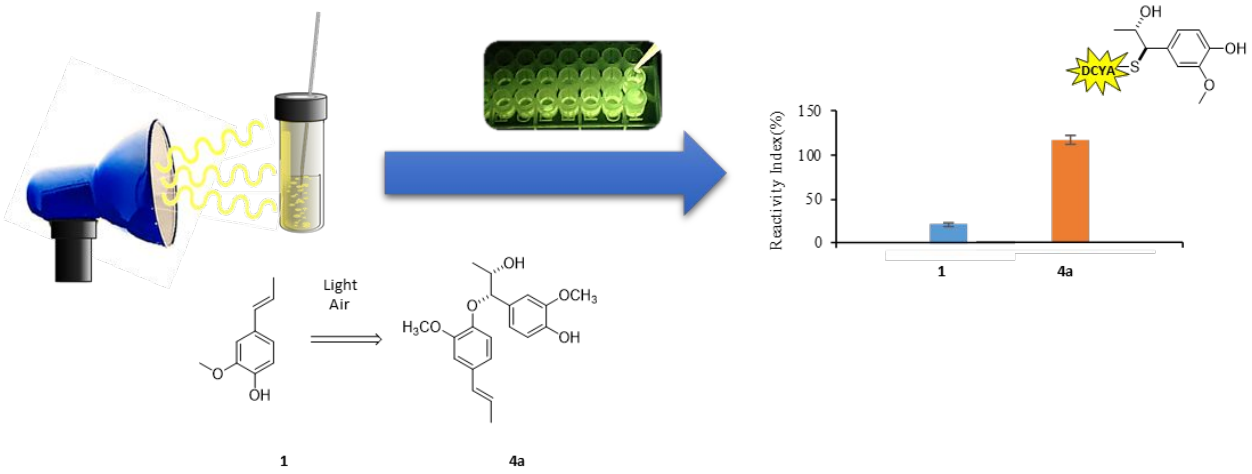

Abstract

Isoeugenol is widely used by the cosmetic and fragrance industries, but it also represents a known cause of skin sensitization adverse effects. Although devoid of a structural alert, isoeugenol has been classified as pre-hapten in virtue of the presence of a pre-Michael acceptor domain. Isoeugenol oxidation could theoretically lead to the generation of reactive toxic quinones, and photo-induced oxidative degradation of isoeugenol was reported to generate strongly thiol reactive byproducts. Nonetheless, the isoeugenol degradation product responsible for increased reactivity was found to be elusive. In the present study, an aged isoeugenol sample was subjected to reactivity-guided experiments to trap elusive thiol reactive species with a fluorescent nucleophile, *viz*. dansyl cysteamine (DCYA). The results herein presented demonstrate that photo-oxidation of isoeugenol led to the formation of a dimeric 7,4'-oxyneolignan with strong chemical reactivity, capable of nucleophilic substitution with thiols. The results were confirmed by isolation, structural characterization, and further NMR reactivity studies. Isoeugenol is already well-known as moderately reactive in thiol depletion assays and was herein demonstrated to be capable of converting to more potent electrophilic species upon degradation, thus acting as a pre-hapten. The application of the reactivity-guided strategy described herein was shown to serve as an effective tool to investigate elusive skin sensitizers.

Introduction

Contact dermatitis is a chronic pathology that cannot be cured, but only mitigated by avoidance of physical contact with the allergen. The timely identification of potential skin sensitizers is, therefore, an important aspect in the development of safer formulations, for cosmetics and other topical products.

Fragrance ingredients are commonly found in everyday preparations, such as personal care, foods, household, and industrial products, including insecticides, cleaners, and detergents. Some fragrance ingredients are known to cause Allergic Contact Dermatitis (ACD) by acting as chemical sensitizers and resulting in type IV delayed allergic reactions.[1]

Isoeugenol (IE), one of the major constituents of clove essential oil, is broadly used by the cosmetic and fragrance industries primarily due to its desirable sweet, spicy, woody, and carnation olfactory notes, but it also represents a known cause of adverse effects, *viz*. skin sensitization. The occurrence of allergic reactions to IE is so widespread that it has been included among the eight constituents of the fragrance mix I (FM I) used in routine patch tests.[2–4] Along with 23 other pure compounds and two natural extracts, IE has been included among the 26 cosmetic fragrance ingredients which must be declared on the ingredients list in commercial products regulated by the EU Cosmetics Directive.[5]

Skin sensitizers are predominantly small, lipophilic electrophiles that can trigger delayed allergic reactions following recurring skin contact.[6] Chemical sensitizers typically initiate the immune-eliciting pathways leading to ACD by covalent binding to reactive nucleophilic amino acids in skin proteins. This formation of covalent adducts is at the core of the haptenation process, which is considered the Molecular Initiating Event (MIE) according to the Adverse Outcome Pathway (AOP) described for skin sensitization.[7] At a first glance, the structural feature of isoeugenol lacks of a mechanistic electrophilic domain capable of triggering the MIE. Indeed, although sensitization to isoeugenol has been mainly attributed to biotic activation by skin enzymes,[8] evidence has also been reported about isoeugenol being susceptible to abiotic activation through rapid oxidation.[9] The chemical reactivity of isoeugenol, observed versus both thio- and amino-peptides, has been explained by Patlewicz *et al*. based on the presence of a pre-Michael acceptor domain, which could generate reactive quinone derivatives upon oxidation.[10] Electrochemical oxidation of

IE resulted in the generation of multiple *O*-demethylated and hydroxylated species capable of adduct formation with proteins.[11] During a stability-thioreactivity correlation study, IE was identified as moderately unstable under exposure to photo-induced oxidative conditions, resulting in about 40% degradation within 100 days of exposure to air and light at room temperature.[12] The crude aged isoeugenol (AIE) was shown to change over time, with the photo-oxidized material being more thiol reactive, but until now candidates responsible for the observed reactivity remained elusive.[12,13]

The first *in chemico* method adopted by the OECD was the Direct Peptide Reactivity Assay (DPRA), which aims to characterize the MIE by measuring the covalent binding of chemicals to skin proteins.[14] The DPRA relies on the classification of chemicals based on the quantification of unreacted peptides by HPLC-UV. In June 2019, a second chemical method, the Amino acid Derivative Reactivity Assay (ADRA), has been added to the list of validated non-animal alternatives. Both methods rely on low-throughput, indirect quantification of reactivity and have not been validated for testing multi-component articles.[14]

A fluorescent high throughput method using a dansyl thiol (HTS-DCYA™) was developed as a convenient strategy to improve the sensitivity and throughput of *in chemico* methods.[15] The HTS-DCYA™ relies on the same rationale as DPRA, but it enables the direct quantification of the hapten-thiol adducts by fluorescence detection. The optimized workflow is particularly convenient for rapid, simultaneous screening of a large number of test articles in a short time frame. Also, integration of the fluorescence assay with analytical techniques, such as LC-MS, can facilitate the rapid identification of reactive compounds by trapping them as stable fluorescent adducts. Indeed, the DPRA method has been modified to Spectro-DPRA invigorated from HTS-DCYA™ rationale and utility of such method was applied to cosmetic ingredients.[16]

In the present study, a DCYA-reactivity guided strategy was applied to identify elusive compounds responsible for the increased chemical reactivity of photo-oxidized IE. The aged IE mixture was subjected to chemical reactivity experiments and reactive compounds were trapped by reaction with the fluorescent DCYA. Resulting adducts were isolated, characterized, and potential reaction mechanisms associated with the observed reactivity were further explained by experiments with the NMR-DCYA™ method.

Material and Methods

1. Materials

Isoeugenol **1** (CAS. 97-54-1, mixture of *cis* and *trans*) with 98% purity and reagents for synthesis were purchased from Sigma-Aldrich (St. Louis, MO, USA). The fluorescent compounds, DCYA and DCYA disulfide, were synthesized as described previously.[15] Maleimide polymer-supported (SiliaBond®, Maleimide, ≥0.75 mmol/g) was purchased from SiliCycle (Quebec City, Quebec, Canada). Standardized buffer solution pH 10, microcentrifuge tubes and polypropylene solvent-resistant 96 well microplates were purchased from Fisher Scientific (Suwanee, GA, USA). Silica gel flash cartridges (SNAP Ultra 100 g and SNAP HP-Sil 10 g) were purchased from Biotage (Charlotte, NC, USA). Flash chromatography purifications were performed using an Isolera Four system (Biotage). Isoeugenol was irradiated for 100 days[12,13] by bubbling air at a constant flow rate and simultaneously exposing the samples to cool light irradiation conditions (6 h day/night cycles) using a compact fluorescent light bulb (860 lm, 6500 K, 60 W equivalent, Philips® Andover, MA, USA). The samples were constantly stirred at room temperature for the entire duration of the study, then stored at -20 ˚C. Chemical analysis of the resulting aged isoeugenol was performed by GC-MS.[12]

2. HTS-DCYA™ method

The chemical reactivity of test articles was investigated using the *in chemico* HTS-DCYA™ method.[15] Briefly, a 2.5 mM solution of DCYA was mixed with an equal volume of 5.0 mM of test samples (821 μg/ml for AIE) in acetonitrile. The resulting solution was then transferred into two microcentrifuge tubes (150 μL in each tube). Aqueous pH 10 buffer (30 μL) was added to the reaction (R) tubes; acetonitrile (30 μL) was added to the second tube (blank, Bl) instead of buffer. DCYA controls were prepared by mixing the 2.5 mM solution of DCYA to an equal volume of acetonitrile. The obtained DCYA solution (150 μL in each tube) was then incubated with aqueous pH 10 buffer (30 μL) for negative control (NC), or with acetonitrile (30 μL) for positive control (PC). The samples were incubated for 23 min with vigorous shaking at room temperature. Afterward, the reaction solutions were added to the vial containing 5 mg of maleimide resin and incubated for 45 min with vigorous shaking. Sixty microliters of the supernatant were diluted to

a final volume of 500 µL with ACN. Forty microliters (in triplicate) of the diluted solutions and 120 µL of ACN were put into a 96 well black with clear bottom microplate. Each plate was read in triplicate using fluorescence end-point readings (excitation 350 nm, emission wavelength 520 nm, cutoff 420 nm). The degree of reactivity is shown as the reactivity index (RI), corresponding to the estimated formation of covalent DCYA adducts with the test article. The RI was calculated based on the average of nine data (three readings/well in triplicate) as follows:

$$\mathrm{RI} = 100 \times \left(1 - \frac{\mathrm{Bl} - \mathrm{R}}{\mathrm{PC} - \mathrm{NC}} - \frac{\mathrm{PC} - \mathrm{Bl}}{\mathrm{PC}}\right)$$

3. Isolation and characterization of DCYA adducts

Five mL of 215 mM DCYA solution in ACN was mixed with the same volume of 585 mM AIE solution. Aqueous pH 10 buffer (0.5 mL) was added dropwise and the reaction was stirred for one hour at room temperature. The reaction mixture was concentrated under vacuum, brine solution was added and the solution was extracted with ethyl acetate. The organic phase was treated over anhydrous Na_2SO_4, filtered, and the filtrate was evaporated to yield a crude residue which was purified by flash chromatography on silica gel. The sample was loaded on a SNAP Ultra 100 g silica cartridge (Biotage) and eluted with hexane: ethyl acetate (5%-100%, stepwise gradient). The resulting fractions were dried and analyzed by NMR and HPLC-DAD-MS.

4. *Isolation and characterization of 7,4'-oxyneolignan*

AIE (200 mg) was separated using a SNAP HP-Sil 10 g silica cartridge (Biotage) and eluted with 25% ethyl acetate in hexane. The 7,4'-oxyneolignan **4a** (70.4 mg, 32.3 %) was isolated as a yellow oil and the structure was elucidated by 1D- and 2D- NMR experiments and mass spectroscopy.

5. HPLC-DAD-MS analysis

High performance liquid chromatographic analyses were performed using an Agilent 1290 Infinity HPLC system equipped with a quaternary pump, autosampler, column compartment, photodiode array detector, and Agilent Open Lab ChemStation data acquisition software (Agilent Technologies, Santa Clara, CA, USA). An SB-C18 RRHD, 2.1x100 mm, 1.8 µm column was used (Agilent Technologies). Isoeugenol

samples were characterized using the following method: the solvent system was composed of solvent A (water) and B (ACN) both containing 0.2% acetic acid; the flow rate was set at 0.2 mL/min. The gradient used for characterization of isoeugenol samples was 20% to 45% B in 3 min then to 85% B in 15 min, to 90% B in 1 min hold for 3 min. UV was monitored at λ_{254nm}. Positive and negative ESI spectra were acquired from 100 to 1000 amu. Chemical analysis of DCYA experiments was performed by HPLC-MS using the same conditions as above except for the following: 45% to 60% B in 3 min, then to 70% B in 17 minutes and to 90% B in 3 minutes, hold for 4 min. UV absorption profile was monitored at λ_{330nm}.

6. NMR-DCYA™

The NMR-DCYA™ assay was performed as described previously.[17] Compound **4a** was dissolved to 100 mM in $CDCl_3$ (125 µL) and mixed with a solution 100 mM DCYA in $CDCl_3$ (125 µL, containing 10 mM of 2,5-dimethylfuran as the internal standard). A set of control ^{1}H NMR spectra was recorded every 5 min for approximately 70 min before addition of 1,5-diazabicyclo[4.3.0]non-5-ene (DBN). A 10 mM solution of DBN in $CDCl_3$ (25 µL) was then added, mixed and the reaction was analyzed by recording one spectrum every 5 min for approximately 300 min. The spectra were processed using MNOVA 9.0 software. Depletion of electrophilic signal (*doEs*) was calculated as follows:

$$doEs = 100\% \frac{\int C}{\int IS}$$

With C = area of the signal of interest of the compound(s) and IS = area of the internal standard peak at 5.8 ppm.

7. m-Chloroperoxybenzoic acid (m-CPBA) oxidation

A solution of 2.56 mmol of 80% *m*-CPBA was added dropwise to a solution of isoeugenol (1.28 mmol) in $CHCl_3$ at 0 ^{o}C. The mixture was stirred at for 30 min and then brought to room temperature and stirred for additional 1 h. Then the mixture was quenched with saturated $NaHCO_3$ solution, diluted with distilled water and extracted with $CHCl_3$. The organic layer was separated, dried over anhydrous Na_2SO_4, evaporated under reduced pressure and the resulting residue was purified by flash chromatography.

8. tert-Butyl hydroperoxide (t-BHP) oxidation

A Solution of *t*-BHP (2.44 mmol, 70% in water or 5.0-6.0 M decane) was added dropwise to a stirred solution of isoeugenol (1.22 mmol) in CH_2Cl_2 at 0 ^{o}C. The mixture initially was stirred at 0 ^{o}C for 30 min and then at room temperature. After 5h of stirring, the mixture was quenched with saturated $NaHCO_3$ solution, diluted with water, extracted with DCM, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to yield crude mixture.

9. Epoxidation with N-Bromosuccinimide (NBS)

N-Bromosuccinimide (270 mg) in DME (4 mL) was added to a stirred solution of isoeugenol (250 mg) in 70% aqueous DME at 0 ^{o}C portion wise. After 3 h of stirring, diluted with water (15 mL) and the mixture was extracted twice with CH_2Cl_2. The combined organic layer was washed with saturated $NaHCO_3$ solution, dried over anhydrous Na_2SO_4, filtered and the filtrate was concentrated. The resulting bromohydrin residue was subjected to the next reaction without further purification. The crude residue was dissolved in methanol (10 mL) and added to a stirred suspension of anhydrous potassium carbonate (630 mg) in methanol (10 mL). After stirring for 1 h, the solution was neutralized by addition of 2N HCl, extracted twice with EtOAc. The combined organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The resulting crude product was purified by flash chromatography.

Results

Isoeugenol thiol reactivity increases upon photo-oxidation.

In order to understand the plausible chemical transformation of pre-haptens during the forced photo-oxidative conditions tested, IE and AIE were probed for their thiol reactivity using the HTS-DCYA™ method. The AIE was obtained upon photo-induced oxidative degradation, to accelerate the degradation process in the absence of solvent. Pure isoeugenol was classified as sensitizer with moderate thiol reactivity toward DCYA (Table 1), despite the absence of direct reactivity alerts. After degradation, the resulting AIE showed about three times increase in fluorescence response compared to IE, although it was not clear

whether fluorescence changes were related to increased concentration of IE-derived activated species or to the generation of different, more reactive byproducts.

Table 1. HTS-DCYA™ reactivity of isoeugenol samples after 23 min incubation.

Samples	Reactivity Index (RI)
Isoeugenol (**1**)	21.6 ± 2
Aged isoeugenol mixture (AIE)	62.9 ± 4
Compound **4a** (*cis*/*trans*)	116.6 ± 5

Fluorescence-guided isolation of reactive AIE components.

To identify the elusive reactive compounds generated upon IE degradation, additional trapping experiments with DCYA were carried out. The AIE was treated with DCYA in pH 10 buffer. The thin-layer chromatography profile of the reaction mixture showed one major fluorescent spot in addition to the ones corresponding to unreacted DCYA and its autoxidation byproduct $(DCYA)_2$ (Figure S1). The reaction mixture was purified by flash chromatography and the structures of the major adduct **2** and a minor adduct **3** (Figure 1) were determined using 1D- and 2D-NMR and HPLC-MS experiments (Table S1, Figures S2-S13). The regioselectivity of adduct **2** was confirmed by HMBC correlation (Figure S8) between the ^{1}H (3.36 ppm) of 7″, belonging to the IE moiety, and the ^{13}C signal of the CH_2S of cysteamine (31.4 ppm). The yield of isolated major adduct **2** was approximately 15%, and those of unreacted DCYA, isoeugenol and $(DCYA)_2$ were 17.8, 26.5 and 12.2%, respectively. Based on thorough structural characterization by NMR, adduct **2** could have resulted from nucleophilic attack of DCYA on 7,4'-oxyneolignan **4a**, a dimeric oxidation product of isoeugenol.[18,19] The thioacetal derivative **3** could have resulted from vanillin as a result of thioacetalization with two molecules of DCYA.

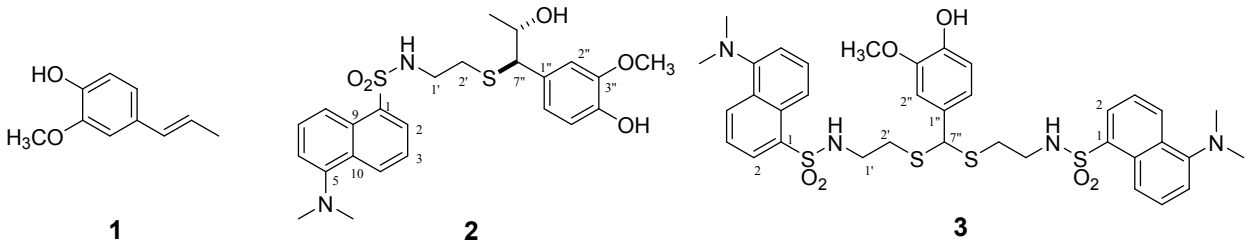

Figure 1. The structure of isoeugenol (**1**), and isolated DCYA adducts **2** and **3**.

Based on the structures of these adducts, it was initially postulated that degradation of **1** may occur with the generation of reactive endoperoxide or epoxide species. Oxidative degradation byproducts have been often associated with skin sensitization adverse effects due to their reactive, electrophilic nature.[20] It has been previously observed that some terpenoids, such as geranial and linalool, and aromatic compounds such as cinnamyl alcohol, produce epoxide or peroxide *via* radical mechanisms. In several instances, such autoxidation byproducts have been linked to evidence of skin sensitization in both *in vivo* and *in vitro* models.[21–25] It has also been reported that isoeugenol spontaneously autoxidized to give dimers and vanillin through hypothetical formation and cleavage of a dioxetane intermediate(s).[26–28] Formation of aldehyde derivatives of structurally similar compounds, such as anethole and eugenol, as a result of photochemical oxidation, has also been reported.[29]

Attempts to prepare isoeugenol epoxide.

Based on known oxidation mechanisms for similar volatile organic components (VOC), and the isolated DCYA adducts, a plausible degradation pathway was proposed as shown in Figure 2. To validate this hypothesis, attempts to prepare isoeugenol epoxide were carried out using reported methodologies. To our surprise, isolation of IE epoxide derivatives was found to be quite elusive. Classic epoxidation conditions[29–31] using *m*-chloroperoxybenzoic acid (*m*-CPBA) and *t*-butyl hydroperoxide (*t*BHP), as well as two-step process with *N*-bromosuccinimide (NBS) and alkali on isoeugenol and acetylated isoeugenol failed to provide the expected epoxides. Only a major adduct of isoeugenol with *m*-CPBA was produced, while

reaction with NBS led to the formation of methoxylated phenylpropanoid and benzofuranoid as sole byproducts (Figure S14). Epoxidation using the bulky peroxide, *t*BHP, as well as attempts of epoxidation of the acetylated IE derivative did not yield the anticipated isoeugenol epoxide.

The elusive nature of the epoxide, and its tendency to ring-opening in the presence of oxidizing agents, led to the supposition that such oxygenated intermediate may be too unstable and can easily undergo nucleophilic attack in the presence of nucleophiles, such as *m*CPBA or isoeugenol. Such unexpected results led to the premise that the epoxide may be too reactive to accumulate as a stable byproduct in AIE. It was nonetheless clear that some reactive, non-epoxy degradation byproduct was present in the AIE sample.

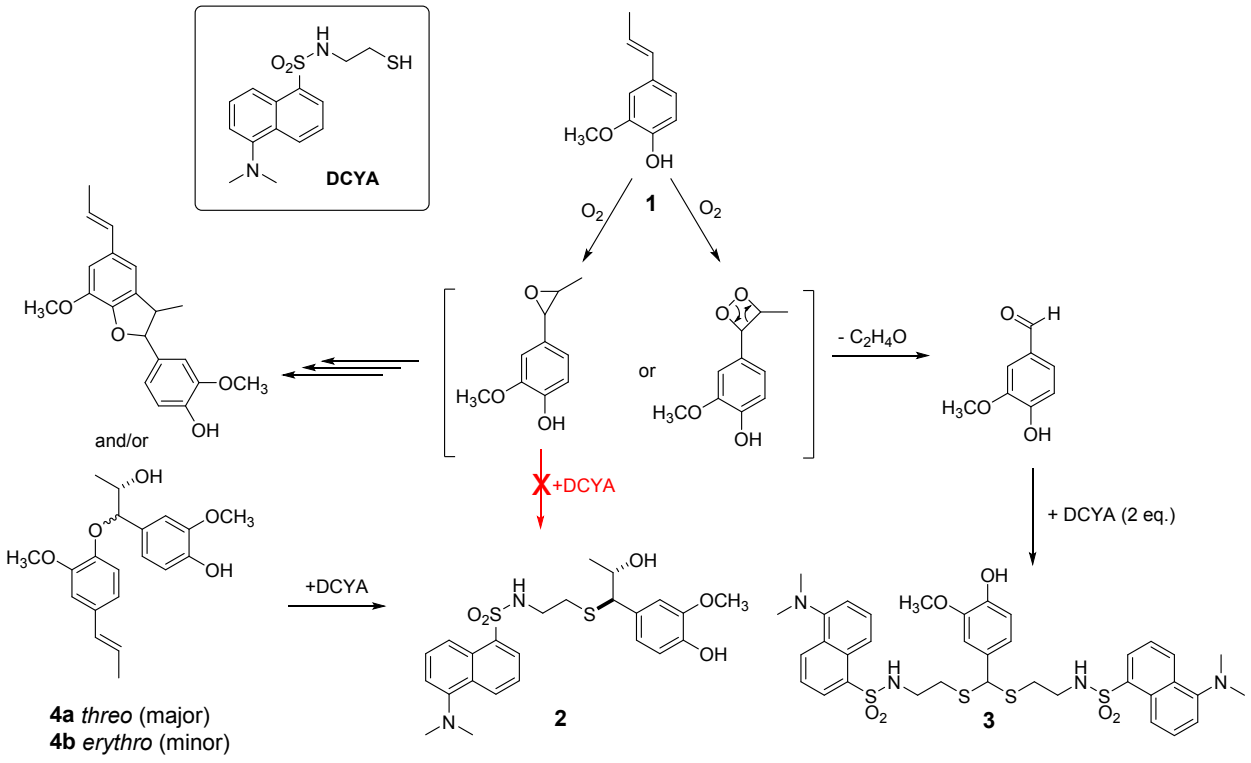

Figure 2. Proposed degradation pathway of isoeugenol and formation of DCYA adducts

Isolation and characterization of IE photo-oxidative byproducts.

As observed in the ^{1}H, ^{13}C and 2D-NMR spectra of crude AIE mixture, at least two additional compounds were identified along with isoeugenol (Figures S15, S16) as major component. This was

expected as we have previously shown that isoeugenol underwent only partial degradation when subjected to forced degradation conditions.[13] AIE was therefore subjected to flash chromatography, and the 7,4'-oxyneolignan **4a** was isolated along with IE. From the NMR analysis (Table S2, Figure S17-S18), compound **4a** was identified as diastereoisomeric mixture (*trans*:*cis* = approximately 5:1).[18,19,32] The OH group at C8 was confirmed by COSY and HMBC correlations between the doublet at 0.99 ppm and the multiplet at 4.05 ppm (Figure S19, S21). In the ^{1}H NMR spectrum of AIE, the integral ratio of δ_H 6.12-6.02 (H-8 of **1** and H-8' of **4a**) and δ_H 4.49-4.41 (H-7 of **4a**) was shown as 3:1 (Figure S15), indicating that there were 2:1 mixture of isoeugenol (**1**) and its oxidized dimer **4a** along with several minor compounds including **4b**.

Although **4a** did not contain structural alerts, testing using the HTS-DCYA™ method showed maximal reactivity in the fluorescence assay (Table 1).

Detailed NMR-DCYA™ experiments (Tables S3-S4 and Figures 3, S22, S23) were then performed in order to gain a mechanistic understanding of the reaction of **4a** in the presence of the nucleophilic thiol.[33] Incubation of **4a** in the presence of DCYA alone did not cause any reactivity in the control reaction, thus ruling out a possible pro-oxidant effect of **4a** on DCYA dimerization. The addition of DBN as a catalyst resulted in rapid depletion of **4a** with the generation of adduct **2** and isoeugenol. The stoichiometric equivalent of this reaction appears to be 1:1:1:1 (depletion of **4a**: depletion of DCYA: generation of DCYA adduct **2**: generation of isoeugenol (**1**), Figure 3B), although further validation with kinetic NMR experiments will be required. In any case, the data obtained by NMR experiments, therefore, confirmed that **4a**, a major component generated presumably from isoeugenol under oxidative conditions, is indeed very reactive with thiols *via* nucleophilic substitution. It is worth noticing that substitution of **4a** with DCYA occurs through the re-generation of one mole equivalent of isoeugenol as a byproduct and warrants further experimentation to establish the possible reaction mechanism.

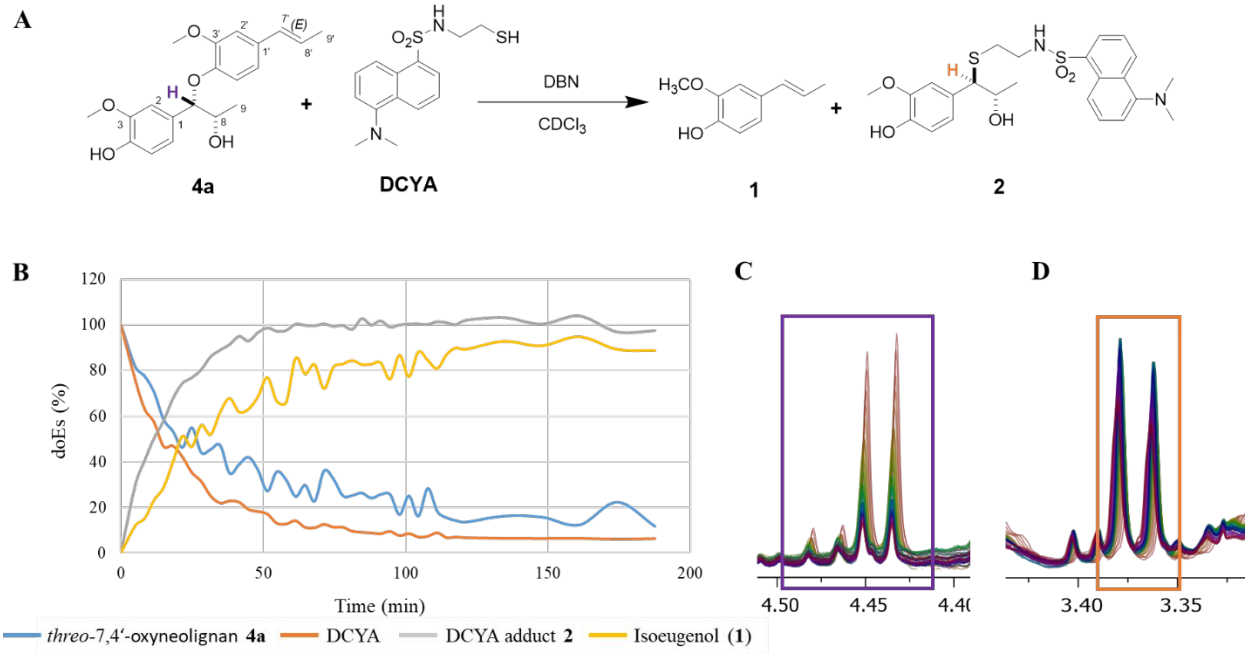

Figure 3. Observed NMR-DCYA™ experimental data with **4a** and DCYA. A) Nucleophilic substitution of DCYA on **4a**. B) Relative ratios of depletion of DCYA and **4a** as well as formation of adduct **2** after addition of DBN over a period of 200 min. C) Arrayed ^{1}H NMR spectra indicating depletion of signal corresponding to **4a** (in purple box, H-7, δ_H 4.49-4.42 ppm) after addition of DBN. D) Arrayed ^{1}H NMR spectra indicating increase in intensity of signal corresponding to **2** (in orange box, H-2, δ_H 3.41-3.35 ppm) after addition of DBN.

Discussion

Autoxidation of VOCs is a well-studied phenomenon, which may occur with the generation of potentially harmful byproducts. Attention to the chemical instability of fragrances and the change in their reactivity is therefore an important aspect to establish the safety of formulations with respect to proper storage and handling conditions.

The present investigation demonstrated that the photo-induced oxidation of isoeugenol predominantly generates a 7,4'-oxyneolignan **4a** with strong chemical reactivity. Isoeugenol is already well-known as moderately reactive in thiol depletion assays[12], and it can be activated to more potent electrophilic species by oxidation, thus acting as a pre-hapten.[20]

Aliphatic terpenoids are especially prone to photo-oxidation *via* radical mechanisms. Oxidative by-products such as conjugated aldehydes, peroxides, peroxyradicals, endoperoxides, and epoxides have been linked to increased skin sensitization adverse effects of aged geraniol, linalool, and cinnamyl alcohol among others.[21,22,24,25] Interestingly, in the case of isoeugenol, typical epoxidation procedures failed to provide stable products *via* chemical synthesis. Focused isolation of major degradation byproducts of IE resulted in the identification of a strongly reactive 7,4'-oxyneolignan with no structural alert for skin sensitization. Regardless of the lack of mechanistic domains, the compound was clearly reactive toward DCYA as model nucleophile. Based on the data herein presented, it is therefore plausible that IE oxidative byproduct **4a** could trigger the initial key events leading to skin sensitization.

It is worth notice that several authors reported the dimerization of IE to 8,4'-oxyneolignan(s) along or in lieu of the 7,4'-oxyneolignan derivative herein described.[18,27] Based on experimental evidence, it seems that reaction conditions play a pivotal role in the dimerization process. The presence and protic nature of the solvent, along with the choice of radical initiators, may constitute experimental factors critical to the fate of IE photo-oxidative reactions.

It is also important to mention *in vivo* data reported previously[4] on skin sensitization potential of the 8-*O*-4' regioisomer. Takeyoshi *et al*. reported that the 8,4'-oxyneolignan was only weakly reactive in guinea pig maximization tests and caused no lymph node cell proliferation in LLNA experiments. The 8-*O*-4' regioisomer was not isolated in aged isoeugenol samples, nor any thiol adduct of the same was identified. Based solely on thiol trapping experiments on IE photo-oxidized in the absence of solvents, it seems that the 7,4'-oxyneolignan may be of higher toxicological concern than its regiomeric analog.

Haptenation events are a fundamental KE in skin sensitization pathways; however, elicitation of inflammatory pathways in keratinocytes and dendritic cells should follow in order to lead to clinical outcomes. Further *in vitro* experiments will be required to determine whether such cellular cascades are also activated upon exposure to **4a**. More kinetic experiments will also be performed to further characterize the mechanism of isoeugenol sensitization.

Trace amounts of **4a** were also found in commercial lots of pure isoeugenol, which were stored in dark glass bottles at 4 ˚C for an extended period of time. These observations confirmed findings reported previously by Oka *et al*.[19] The authors also observed that small traces of **4a** are sufficient to antagonize the response to isoeugenol *in vitro* by inhibiting the murine olfactory receptor mOR-EG. Prolonged storage in the presence of an oxygen source could thus be sufficient to generate compounds of toxicological and organoleptic concern. This is especially relevant in the cosmetic and fragrances industry, where high volumes of pure chemicals are likely stored during the manufacturing process. Therefore, the formation of reactive byproducts such as **4a** is important for quality as well as for safety control of bulk materials under good manufacturing practices.

Acknowledgments

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Abbreviation

ACD, Allergic Contact Dermatitis; ADRA, Amino acid Derivative Reactivity Assay; AIE, Aged Isoeugenol; AOP, Adverse Outcome Pathway; DBN, 1,5-diazabicyclo[4.3.0]non-5-ene; DME, Dimethoxy-ethane; DPRA, Direct Peptide Reactivity Assay; GC-MS, Gas Chromatography coupled with Mass Spectrometry; HPLC-MS, High-Performance Liquid Chromatography coupled with Mass Spectrometry; HTS-DCYA™, High Throughput Screening using Dansyl Cysteamine; IE, Isoeugenol; KE, Key Event; MIE, Molecular Initiating Event; NMR, Nuclear Magnetic Resonance; OECD, Organisation for Economic Co-operation and Development; RI, Reactivity Index; VOC, Volatile Organic Compounds.

Supporting Information

The NMR data of adducts, isolated IE byproducts and aged isoeugenol mixture, epoxidation attempts and detailed data from NMR-DCYA™ experiments were supplied in the supporting information.

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