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Effect of Fluorination on Skin Sensitization Potential and Fragrant Properties of Cinnamyl Compounds

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A series of three α- and three β-fluorinated representatives of the family of cinnamate-derived odorants (cinnamaldehyde (**1**), cinnamyl alcohol (**2**), and ethyl cinnamate (**3**)) as used as fragrance ingredients is described. Olfactive evaluation shows that the fluorinated compounds exhibit a similar odor profile to their parent compounds, but the olfactive detection thresholds are clearly higher. *In vitro* evaluation of the skin sensitizing properties with three different assays indicates that α-fluorination of *Michael* acceptor systems **1** and **3** slightly improves the skin sensitization profile. α-Fluorocinnamyl alcohol **2b** is a weaker skin sensitizer than cinnamyl alcohol **2a** by *in vitro* tests and the fluorinated product drops below the sensitization threshold of the *KeratinoSens*® assay. On the other hand, β-fluorination of compounds **1** – **3** results in highly reactive products which display a worsened *in vitro* skin sensitization profile.

Keywords: cinnamyl derivatives, fluoroalkenes, fragrance ingredients, skin sensitization, *KeratinoSens*®.

Introduction

Cinnamaldehyde (**1a**; *Figure 1*) is the major constituent of cinnamon essential oil at around 90% and is responsible for the typical cinnamic taste and odor profiles.[1] It is commonly used to enhance the natural cinnamon note in fine fragrances, beauty or home care products, as well as in aroma compositions. For instance, North American consumers appreciate cinnamaldehyde as an apple cinnamon crumble scented candle, where the cinnamon is combined with a typical apple smell. Common cinnamaldehyde containing flavors include Chai spice and Speculoos. In fine fragrances, cinnamaldehyde is key for spicy complexes where the cinnamon character combines elegantly with the spicy clove character (mainly given by eugenol). This rich and sophisticated character is the basis for many perfumes of the spicy family. Indeed, Opium from Yves Saint Laurent is a classic example where the marriage of clove and cinnamon, along with the use of other spices is responsible for its spicy key character, which is its signature. Cinnamyl alcohol (**2a**; *Figure 1*) on the other hand is a key ingredient for balsamic notes in perfumery. It is reminiscent of styrax and its key ingredient phenyl propyl alcohol. It thus combines gracefully with rose notes for the formation of floral-oriental compositions, where it introduces the use of other balsamic notes such as Benzoin and Peru Balsam. It is further used in noble floral compositions such as lilac or hyacinth. Ethyl cinnamate (**3a**; *Figure 1*) is also a component of cinnamon essential oil, even though at much lower concentrations. In perfumery it is used at a lower extent, for instance to add a strawberry twist in compositions or to reveal the fruity side of a rose. However, a number of flavor creations rely on the sweet balsamic note of ethyl cinnamate where it is commonly used as a constituent of a fruity aroma, especially for strawberry compositions. The production of the three ingredients reaches several thousand tons *per annum*, with more than half of this amount being used in the flavor and fragrance industries. In most cases, the only stereoisomer of cinnamaldehyde and the related alcohol and ester from natural sources is the thermodynamically preferred (*E*)-isomer. This work is thus limited exclusively to this specific isomer.

In fine fragrance applications, the use of cinnamaldehyde **1a** is restricted to a level of 0.02 – 0.05% in final cosmetic products by IFRA

(International Fragrance Association) due to its skin sensitization properties (moderate – strong sensitization in the Local lymph node assay, LLNA).[2] The alcohol **2a** is classified as weak sensitizer, while ethyl cinnamate **3a** currently has no use restrictions. However, for flavor creations none of the three compounds has use restrictions. Both cinnamyl alcohol **2a** and cinnamaldehyde **1a** form part of the list of 26 allergens which need to be labeled on cosmetic products. Positive allergic reactions are regularly reported from dermatological clinics to both cinnamaldehyde and cinnamyl alcohol.[3][4]

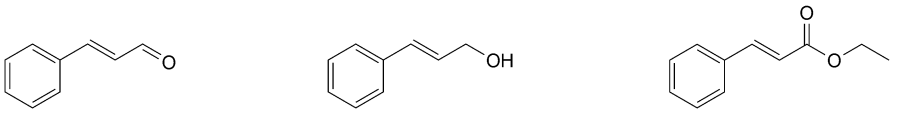
Results and Discussion

Synthesis and Olfactive Evaluation

In our endeavor to create safe to use and high impact fragrance ingredients, we embarked on a project to improve the toxicological and skin sensitization profiles of common fragrance ingredients. One such approach is to subtly alter the electronic properties of typical olfactive compounds without heavily influencing their overall shape – thus seeking to maintain their olfactive properties. Fluorine was identified as lead candidate for inclusion as a substituent, as its high electronegativity and small size make it a prevalent bioisostere of a hydrogen atom, while inducing an electronic impact on the neighboring functional groups without disturbing the steric conformation of the molecule. Surprisingly few fragrance ingredients bearing one or more fluorine atoms have been disclosed, especially when comparing with the large body of fluorinated pharmaceutical and agrochemical products which are introduced each year.[5] *Schlosser and co-workers* first introduced fluorine in terpenic odorants, and determined that the fluorine atom is small enough to only minimally influence the overall shape and thus the smell of the resulting fluorinated odorants.[6 – 8] Furthermore, *O'Hagan and co-workers* made use of geminal difluoro-substitution in macrocylic musk lactones and ketones to induce a conformational constraint by the fluorine *gauche* effect, and thus lock the molecule in the right conformation.[9][10] It is interesting to note that the electronic influence of the fluorine atom has never been used in fragrance ingredients in order to alter their toxicological footprint. We thus decided to synthesize fluorinated analogues of cinnamyl compounds **1** – **3** (*Figure 2*) bearing a fluorine atom either in the α- (compounds **1b**, **2b**, and **3b**) or in the β-position (compounds **1c**, **2c**, and **3c**) with respect to the oxygenated functional group (alcohol, aldehyde or ester moieties), in order to explore the influence of fluorine atom on the molecule's properties. Since we aimed to study the fluorine substitution of the (*E*)-configured double bond in the cinnamate series, the corresponding products all bear a (*Z*)-configured double bond, accounting for the higher priority of a fluorine *versus* a carbon atom.

Known compounds **1** – **3b** have already been synthesized in our laboratories by *Guérin et al.* using the following reaction sequence:[11] Ethyl (*Z*)-2-fluoro-3-phenylacrylate (**3b**) was obtained in 60% yield as a single double bond isomer by diethylzinc mediated olefination of benzaldehyde with ethyl dibromofluoroacetate (*Scheme 1*).[12] Reduction of ester **3b** by $LiBH_4$ in tetrahydrofuran (THF) afforded the alcohol **2b** in quantitative yield.[13] This latter compound was then oxidized by IBX (2-iodoxybenzoic acid) to give the desired 2-fluorocinnamaldehyde (**1b**) in 85% yield, as already published by *Wheeler et al.*[14]

Ethyl (*Z*)-3-fluoro-3-phenylacrylate (**3c**) was synthesized by the addition of silver fluoride to ethyl phenylpropiolate as reported by *Jiang and co-workers*, and is described in *Scheme 2*.[15] The fluorine addition proceeded in a regio- and stereoselective manner to afford exclusively the (*Z*)-configured product. DIBAL-H (diisobutylaluminium hydride) reduction described by *Konno et al.*[16] afforded the allylic alcohol **2c** in good yield, which was then further oxidized with MnO_2 in dichloromethane to give the desired β-fluorocinnamaldehyde (**1c**) in 65% yield.[17] The latter compound proved to be rather unstable and decomposed within several hours at room temperature or in the fridge. However, it was stable in neutral solution and when stored at −20 °C. The



(*E*)-Cinnamaldehyde (**1a**)
spicy, cinnamon-like, sweet, balsamic, cassia

(*E*)-Cinnamyl alcohol (**2a**)
balsamic, sweet

Ethyl (*E*)-cinnamate (**3a**)
sweet, balsamic, honey

Figure 1. The three major representatives of the cinnamyl family and their odor description.

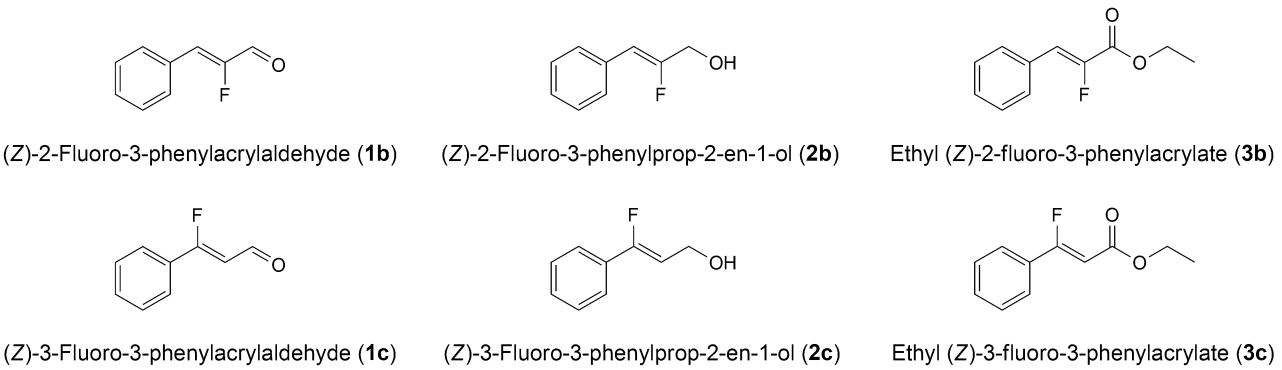
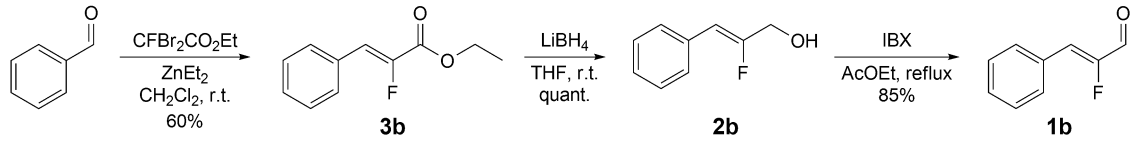
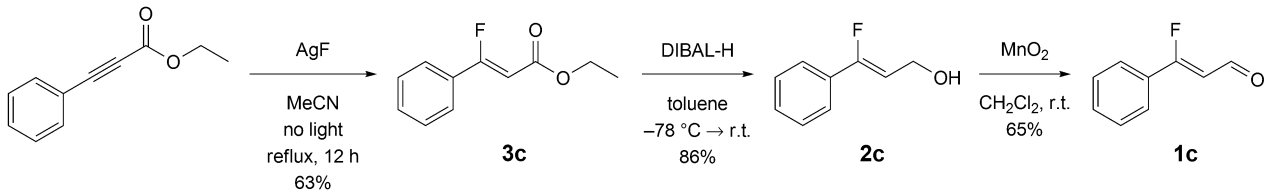


Figure 2. α- and β-fluorination of compounds **1** – **3**.



Scheme 1. Synthesis of α-fluorinated compounds **1b**, **2b**, and **3b**.



Scheme 2. Synthesis of β-fluorinated compound **1c**, **2c**, and **3c**.

authors assume that decomposition proceeds *via* elimination of hydrogen fluoride in the presence of trace amounts of water.

Olfactive evaluation of the six new compounds (as 10% solutions in dipropylene glycol (DPG), applied on a cellulose strip) was performed fresh and as dry-down, left to evaporate for several hours (*Table 1*). Simultaneously the fluorinated compounds were compared to their respective benchmarks; **1a** for aldehydes **1b** and **1c**, **2a** for alcohols **2b** and **2c**, and **3a** for ethyl esters **3b** and **3c**. The olfactive assessments indicate that the fluorinated compounds **b** and **c** displayed a very similar odor profile to their respective non-fluorinated compounds **a**. The evaluation by a trained perfumer indicated that compounds **1b**/**1c**, **2b**/**2c** and **3b** displayed a significantly lower intensity on smelling strip when compared to the respective benchmarks **1a** – **3a**, while ethyl (*E*)-3-fluoro-3-phenylacrylate (**3c**) surprisingly had a slightly stronger olfactive presence than benchmark **3a** fresh, as well as on dry-down.

To assess objectively the performance of compounds **1** – **3b** and **c**, their olfactive threshold concentrations were determined by GC-O (Gas Chromatography-Olfactometry),[18] also known as GC-Sniff measurements using 16 test persons (panelists) at discrete concentrations. For this technique, different dilutions of the tested compound are injected into a GC in decreasing order of concentration. The panelists smell the eluent at the sniffing port of the GC. The smelling is performed blind, and the panelists press a button upon perceiving an odor. The lowest concentration at which an odor is perceived at the correct retention time (as indicated by a flame ionization detector) is recorded as the individual odor threshold. The overall odor threshold is then obtained as the geometric mean of the measurements for all the panelists. For direct comparison, we also assessed the threshold concentration of cinnamates **a** with the same panelists. The odor threshold determines the minimal concentration of the compound per unit volume of air for its odor still to be perceived.

Table 1. Olfactive properties of cinnamic aldehydes, alcohols and esters **1** – **3**

Compound	Combined odor description (fresh and dry down)	Comparison (benchmark)[a]	GC-threshold (mean) [ng/l air]
1a	Spicy, cinnamon-like, sweet, balsamic, cassia		0.953
1b	Balsamic, cinnamic aldehyde-like	Intensity below benchmark	7.09
1c	Almondy, sweet, balsamic	Intensity below benchmark	4.92
2a	Balsamic, sweet		0.455
2b	Balsamic, cinnamyl alcohol like	Intensity below benchmark	3.36
2c	Floral balsamic, cinnamyl alcohol like	Intensity below benchmark	1.65
3a	Sweet, balsamic, honey		2.58
3b	Balsamic fruity, ethyl cinnamate like	Intensity below benchmark	6.41
3c	Floral balsamic, fruity strawberry	Slightly stronger than benchmark	15.31

[a] The comparative evaluation with the benchmark is a subjective evaluation by a trained perfumer.

As described in *Table 1*, the fluorinated cinnamyl derivatives have odor thresholds which are roughly one (or less) order of magnitude higher than their parent compounds, conveying that a larger amount of the respective compound is needed to be presented for the panelist to perceive the odor. Indeed, for the cinnamaldehydes **1** and alcohols **2**, roughly ten times more α-fluorinated substance is needed as for the parent compound. β-fluorination has a smaller effect, increasing the odor threshold only by a factor of five. In the case of ethyl cinnamate, the roles are reversed, as β-substitution has a larger effect on the olfactive threshold than α-substitution. This is indeed very surprising, since **3c** was the only compound which was perceived stronger on a paper smelling strip. In GC-sniff experiments, the respective compound is already in the gas phase, therefore the threshold relates mainly to the quality of receptor binding, and is only minimally influenced by physico-chemical properties like boiling point, diffusivity, and vapor pressure.[1] On the other hand, smelling the individual compounds on blotter might be heavily influenced by evaporation kinetics as well as interactions between the substance and the cellulose of the smelling strip.

Assessment of the Skin Sensitization Potency by in vitro *Methods*

In parallel to their olfactive assessment, all fluorinated analogues were tested by *in vitro* skin sensitization assays, namely *KeratinoSens*®[19]2 and by liquid chromatography-mass spectrometry (LC-MS) peptide binding assay. Furthermore, a specific assay was introduced to measure sulfotransferase-mediated activation of derivatives of **2a** to form peptide reactive sulfates.[20]

Skin sensitization is a T-cell mediated immune reaction. Small reactive molecules (haptens) are not large enough to be recognized by the immune system, but they can covalently modify skin proteins which then are recognized by the immune system as 'non-self' due to these novel modifications.[21] Some chemicals need to be activated by metabolic enzymes to form reactive metabolites which then act as haptens. These molecules are called prohaptens.[22]

The skin sensitization risk of new chemicals was classically assessed by animal tests. Over the last two decades, the local nymph node assay in mice was the main test used, as this test delivers a dose response and allows us to rate chemicals according to their sensitizer potency.[23] Recently, significant attempts have been made to replace animal testing,[24] and three new OECD (Organisation for Economic Co-operation and Development) guidelines for *in vitro* methods were published.[25 – 27] Herein we use three *in vitro* assays. In the *KeratinoSens*® assay (OECD guideline 442d), the Nrf2 (nuclear factor erythroid 2-related factor) dependent induction of a luciferase gene, which is under control of an antioxidant/electrophile response element (ARE/EpRE) is assessed. Nrf2 is a transcription factor, whose activity is controlled by another protein, Keap1 (Kelch-like ECH-associated protein 1). This latter protein is inactivated by covalent modifications of sulfanyl residues on its surface by electrophilic chemicals, which leads to activation of Nrf2. Therefore, this assay illustrates the response of skin cells to electrophilic chemicals, which nicely correlates with their skin sensitization potential.[28] Chemicals are added at increasing doses to the reporter cell-line, and the luciferase expression is measured as a fold-induction over solvent control treated cells. The EC1.5, EC2, and EC3 values (dose for inducing a 1.5-,

[1] The individual results of this odor threshold assesment can be found in the *Supplementary Information* (*SI*).

[2] *KeratinoSens*® is a registered trademark of *Givaudan SA*.

2-, or 3-fold increase in luciferase) are extrapolated from the dose-response curves and the maximal fold-gene induction is reported as the I_{max} value. In parallel, cytotoxicity to the cells is measured and expressed as IC_{50} value, *i.e.*, the concentration reducing cellular viability by 50%. Chemicals inducing the luciferase gene above 1.5 fold at a non-cytotoxic dose (> 70% viability) are rated as positive and potential sensitizers by this assay.[29] In the direct peptide reactivity assay (DPRA) (OECD guideline 442c), depletion of a Cysteine and a Lysine containing peptide after incubation for 24 h with an excess of the test chemical is monitored by LC-UV.[30][31] Here we use a modification of this assay with a peptide containing both a cysteine and two lysine-residues. In this peptide reactivity assay, depletion of the peptide and the formation of covalent adducts of test chemicals with the model peptide is assessed by LC-MS.[20] The third assay to test the cinnamyl alcohols (**2a** – **c**) incorporates a metabolic activation by sulfotransferase in S9 liver fractions. The assay is specific to test benzylic and allylic alcohols and has not been described before in the context of skin sensitization. It is described in more detail below.

The mechanism of skin sensitization of **1a** (and to a lesser extent **3a**) is attributed to its *Michael* acceptor properties, undergoing nucleophilic attack from cysteine residues of proteins (*Scheme 3,a*). The alcohol **2a** was long thought to be a prohapten and activated by skin enzymes to form **1a**, and then acting as a skin sensitizer by the same mode of action (*Scheme 3,b*, eq. 1). Indeed, around 50 – 70% of dermatological patients reacting to cinnamyl alcohol **2a** also react to cinnamic aldehyde **1a**,[3][4] which would indicate that they have been sensitized to the same chemical principle (*i.e.*, leading to the same structural modifications of skin proteins resulting in *Michael* adduct **4** and eventually **5**). However, this explanation does not explain the sensitization for those patients reacting to only cinnamyl alcohol **2a** and not to cinnamaldehyde **1a**. Evidence for two alternative modes of action was demonstrated recently. On the one hand, cinnamyl alcohol **2a** is also activated by skin enzymes to form a highly reactive epoxide **6**, which would then undergo attack by a skin protein, leading to a structurally distinct protein modification in compound **7** (*Scheme 3,b*, eq. 2).[32] Even more recently, human skin models were treated with ^{13}C labeled **2a**, and, using magical angle NMR analysis, evidence for protein adducts in the skin with the sulfur atom replacing the alcohol oxygen was presented. It was speculated that this could occur through activation of cinnamyl alcohol **2a** by a sulfotransferase (SULT) under participation of the cofactor 3′-Phosphoadenosine-5′-phosphosulfate (PAPS) to form a sulfate leaving group in compound **8**.[33] The resulting highly activated allylic methylene can then react with skin proteins (*Scheme 3,b*, eq. 3) in a nucleophilic substitution to yield adduct **9**. However, this enzymatic activation was so far not demonstrated in enzymatic assays.

2-Fluorocinnamaldehyde (**1b**) shows slightly weaker sensitization characteristics in direct comparison to cinnamic aldehyde **1a** in the *KeratinoSens*® assay, with an EC1.5 of 16.5 μM, compared to cinnamaldehyde **1a** at 10.9 μM (*Table 2*). However, cinnamaldehyde is regularly tested as a positive control in *KeratinoSens*®, and the historical overall average including more than 100 experiments for aldehyde **1a** is 15.1 μM,[3] therefore these values are not significantly different. Also the EC2 and EC3 values are almost identical for fluoroaldehyde **1b** and parent aldehyde **1a**. This indicates that both compounds should have an essentially similar reactivity and allergenic potential in *KeratinoSens*®. In the peptide reactivity test, the parent aldehyde **1a** formed 40.3% of the direct peptide adduct, while only 23% was observed for the fluoroaldehyde **1b**. In both cases, the mass of the formed adduct is consistent with *Michael* addition: For the adduct with **1a** $[M + H]^+ = 1041.7$ (= $[M + H]^+$ Peptide 909.6 + MW **1a**) and for **1b** $[M + H]^+ = 1060.7$ (=909.6 + MW **1b**). On the other hand, aldehyde **1c** shows a much stronger sensitization potential in *KeratinoSens*® with lower concentrations for induction of luciferase and much higher cytotoxicity. In the peptide reactivity assay, 98% peptide depletion is observed with concomitant formation of four different peptide adducts. The test peptide contains one cysteine and two lysine residues and was apparently modified by **1c** with one, two, or three equivalents under elimination of one, two, or three equivalents of HF ($[M + H]^+ = 1039.7 = 909.6 + MW$ **1c** – HF; $[M + H]^+ = 1169.7 = 909.6 + 2 \times MW$ **1c** – 2 HF; $[M + H]^+ = 1299.8 = 909.6 + 3 \times MW$ **1c** – 3 HF). This can be explained by reaction with the cysteine residue and the two lysine residues in the peptide where the *Michael* addition is immediately followed by elimination of hydrogen fluoride. The fourth adduct, with $[M + H]^+ = 1151.7 = 909.6 + 2 \times MW$ **1c** – 2 HF – 18 may be explained by addition of two equivalents of the test chemical and elimination of 2 × HF and one molecule of water. Most likely, this outcome can be attributed to a primary adduct formed by HF elimination and a second reaction of the free aldehyde functionality of the bound molecule by the intramolecular formation of a *Schiff*'s base with the second free

[3] Own unpublished data.

Table 2. Results from the *KeratinoSens*® assay and the peptide binding assay

	KeratinoSens® assay					Peptide reactivity
	I_{max} (maximal fold ind.)	Conc. for induction above threshold [μM]			Cytotoxicity IC_{50} [μM]	Estimated intensity of adducts in% of free peptide
		EC1.5	EC2	EC3		
1a	3.7	10.9	29.8	52.5	194.0	40%
1b	26.6	16.5	28.9	41.7	85.3	23%
1c	5.5	5.3	6.8	8.7	10.9	98%, double and triple adducts
2a	1.9	212.0	971.6	1371.9	1557.0	Trace, < 0.1%
2b	1.4	n.i.[a]	n.i.	n.i.	1190.2	No adduct observed
2c	5.1	10.2	28.2	59.5	363.9	22%
3a	3.6	18.6	37.4	109.6	405.6	9.3%
3b	2.8	44.0	109.3	n.i.	304.2	0.7%
3c	88.6	3.8	9.8	24.4	571.2	65%

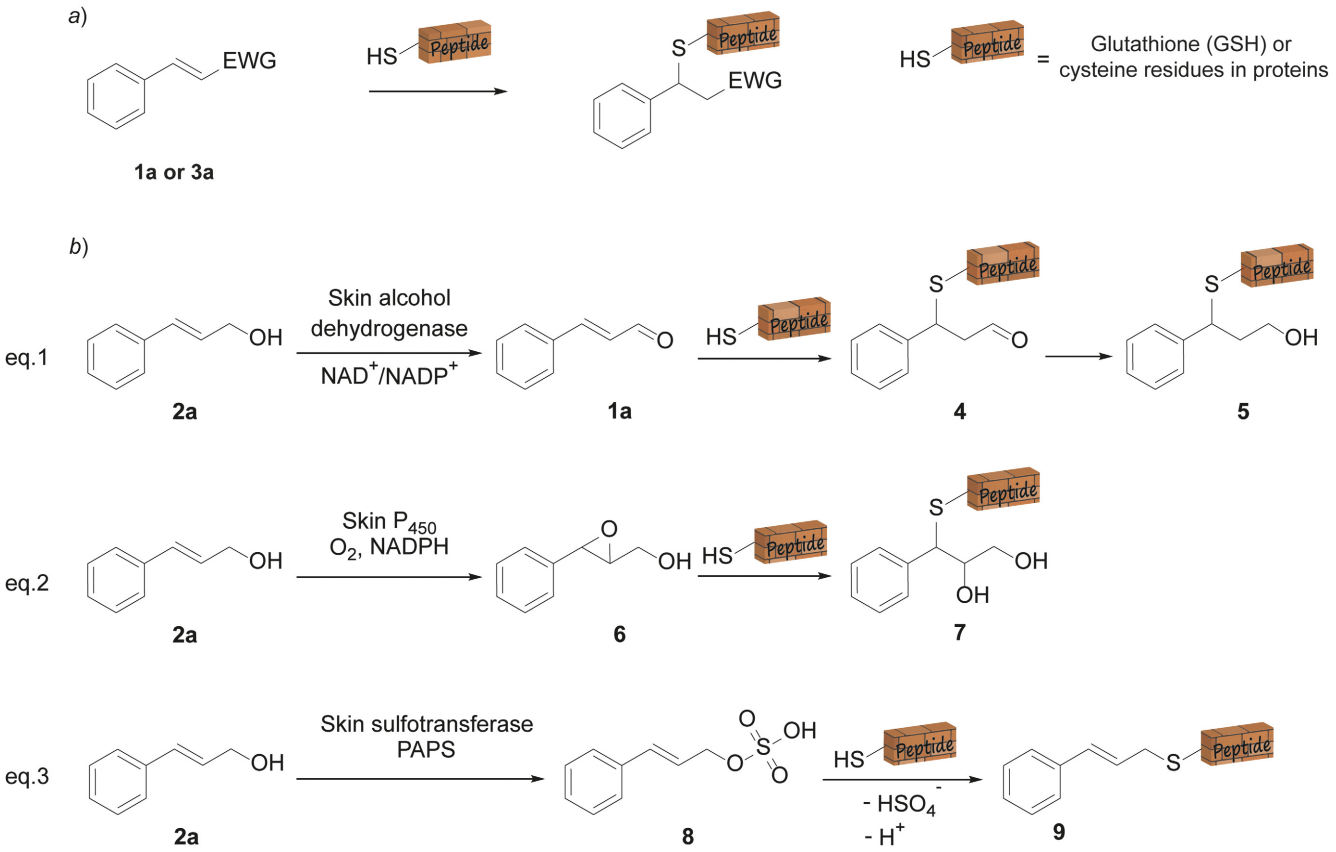
[a] n.i.: no induction.

amine functionality in the peptide, but there is a lack of experimental evidence to prove this. The very high reactivity of the β-fluorinated *Michael* acceptor system in **1c** is thus reflected by almost quantitative modification of all the nucleophilic cysteine and lysine residues in the test peptide.

α-Fluorocinnamyl alcohol (**2b**) shows no induction of the luciferase above 1.5-fold. This indicates that fluorine substitution of cinnamyl alcohol at the α-position lowers the skin sensitization activity in such a way that alcohol **2b** drops below the skin sensitization threshold of the *KeratinoSens*® assay. At the same time no adduct was observed in the peptide reactivity assay. The opposite is true when evaluating β-fluoro substitution in compound **2c** which triggers both gene induction and cytotoxicity in the *KeratinoSens*® assay at 20- and 5-fold lower concentrations, thus **2c** is expected to induce a significantly stronger sensitization response. Compound **2b** is also directly reactive in the peptide assay, again forming an adduct with a mass consistent with an addition-elimination reaction eliminating the hydrogen fluoride $[M + H]^+ = 1041.7 = 909.6 + MW$ **2c** $- HF$, and a second adduct with an additional loss of 18 mass units, thus formally loss of water. We suspect this mass to correspond to a product where an additional intramolecular substitution reaction occurred at the allylic alcohol. In the case of β-fluorinated alcohol **2c**, the fluorine substitution favours peptide addition and HF-elimination, even though at a lower rate compared to **1c**. While **2a** is a prohapten (requiring metabolic activation to sensitize), fluorination in β-position made **2c** a directly acting hapten, strongly modifying peptides without metabolic activation. It is thus a much stronger allergen and this mechanistic evidence from the peptide reactivity assay is also consistent with the *KeratinoSens*® result.

Ethyl 2-fluorocinnamate (**3b**) needs a 2.4-fold higher concentration than ethyl cinnamate (**3a**) to induce the luciferase 1.5-fold, and is thus a weaker sensitizer by the *KeratinoSens*® assay. Furthermore, the peptide reactivity assay yields *ca*. 10% direct *Michael* adduct formation for **3a**, while only traces (> 10 times less) are observed for the α-fluoroanalogue **3b**. Thus both assays indicate that in this case, the fluorinated ester **3b** appears to have a reduced reactivity thus decreasing the skin sensitization ability. Once again, the direct opposite is true for β-substitution with **3c** inducing luciferase at 5-times lower concentration compared to non-fluorinated **3a**. Peptide reactivity shows high values, with 65% peptide depletion and concomitant appearance of two adduct peaks, both again consistent with elimination of HF ($[M + H]^+ = 1083.7 = 909.6 + MW$ **3c** $- HF$). The two adducts are in a 1:2.5 ratio. The most straightforward explanation is that alcohol **3c** has added to either the cysteine or the lysine residue. Normally cysteine and lysine have strongly differing reactivity in DPRA, but this particular addition-elimination reaction with highly reactive compound **3c** appears to be possible with both nucleophiles as also seen in the addition of **1c** to multiple residues in the peptide.

As summarized above and in *Scheme 3*, cinnamyl alcohol **2a** was reported to act as prohapten with three different possible modes of action. We thus further tested the three alcohol derivatives **2a** – **2c** in an additional metabolism assay. To assess the potential to covalently modify sulfanyl groups in peptides and proteins, the alcohols were incubated with rat liver S9 fractions as the metabolic system, along with the sulfotransferase cofactor PAPS and glutathione (GSH) as trapping agent to indicate sulfanyl



Scheme 3. Reaction mechanism of cinnamyl ester and aldehyde directly acting as *Michael* acceptors (*a*) and three different modes showing how prohapten cinnamyl alcohol (**2a**) can be activated by skin enzymes to produce structurally different, potentially immunogenic, protein adducts (*b*).

Table 3. PAPS dependent formation of GSH-adducts with the general structure **9** in presence of a S9 mix as metabolic system (peak area, arbitrary unit)

	− S9 − PAPS		+ S9 − PAPS		+ S9 + PAPS	
	Sulfate	GSH-conjugate	Sulfate	GSH-conjugate	Sulfate	GSH-conjugate
2a	n.d.[a]	n.d.	n.d.	n.d.	2.3×10^6	1.4×10^8
2b	n.d.	n.d.	n.d.	n.d.	7.9×10^7	1.3×10^8
2c	n.d.	n.d.	n.d.	n.d.	2.8×10^6	2.2×10^8
3-phenylpropanol	n.d.	n.d.	n.d.	n.d.	1.9×10^7	n.d.

[a] n.d.: not detected.

group binding to form GSH adducts of type **9** (*Table 3*). As we have no synthetic reference standards, only peak areas are given assuming a similar response factor when comparing the different test compounds. In absence of the metabolic system and in absence of the cofactor PAPS, no GSH adduct was formed, while for all three cinnamyl alcohol derivatives **2a** – **c** the GSH adduct was formed in presence of the metabolic system and PAPS. The requirement for PAPS and the observation of the sulfated intermediate (determined by the exact mass by high resolution mass spectrometry, HR-MS) indicate that indeed the reaction proceeds *via* a sulfate intermediate. The activation by the metabolic system is in a similar range for the three compounds; hence this mode of action does not confer a major difference between them. On the other hand, the saturated analogue 3-

phenylpropanol was activated by PAPS to a sulfate but did not then form the GSH adduct, indicating that the allylic alcohol is a structural requirement for the sulfate intermediate to act as a peptide reactive entity.

Integrating all results suggests that β-substitution leads to a stronger reactivity and a different molecular mode of action with the possibility for addition-elimination reactions. Indeed, for **1c** and **3c**, the fluorine in the β position enhances the electrophilicity of the β-carbon, making it more prone to *Michael* addition generating an enolate which subsequently easily eliminates a fluoride. This addition-elimination reaction is even possible in the absence of a conjugated electron-withdrawing group for alcohol **2c**. This is translated for all three derivatives to a stronger direct peptide reactivity and stronger activity in *KeratinoSens*®. Substitution in the α-position reduces significantly the sensitization risk for the alcohol, only mildly for the ester, and has almost no effect on the aldehyde in the *KeratinoSens*® assay. This shows that the *Michael* acceptor properties of **1b** and **3b** are only mildly affected by the introduction of the highly electronegative fluorine atom in the α position in this test, even though a reduced amount of peptide adducts was observed. However, for the alcohol **2b**, α-fluorination leads to decreased reactivity. As shown in *Scheme 3*, all three mechanisms of sensitization of cinnamyl alcohol are at least two-step processes. If equation 1 is the major skin sensitization mechanism in the *KeratinoSens*® assay, α-fluorination of the alcohol would have the largest impact on the first oxidation step. Indeed, since fluorocinnamaldehyde **1b** has very similar sensitization reactivity to **1a**, the diminished reactivity of **2b** when compared to **2a** might be well explained by a decelerated $NAD^+/NADP^+$ dependent oxidation. Similarly, when regarding mechanistic possibilities 2 and 3, fluorine substitution can either alter the first, enzyme-catalyzed step, (P450 or PAPS). Alternatively, α-fluorination impacts the second step, more specifically the epoxide-opening or the substitution of the sulfate leaving group, which are spontaneous processes in both cases. Since the rate-determining step of both mechanistic alternatives is not identified, it would be futile to draw any further conclusions. However, the very similar reactivity of **2a** and **2b** in the PAPS-dependent formation of GSH adducts (*Table 3*) indicates that mechanistic proposal 3 is not the major skin sensitization mechanism in the *in vitro KeratinoSens*® assay.

Conclusions

Fluorine substitution of three members of the cinnamyl family (cinnamaldehyde (**1a**), cinnamyl alcohol (**2a**), and ethyl cinnamate (**3a**)) has been performed at both the α- and the β-positions of the olefins. Olfactive evaluation along with odor threshold determination indicates that the fluorine substitution alters only minimally the odor profile while conferring to the derivatives in most cases a lower odor intensity on blotter paralleled with a higher olfactive detection threshold. Both observations indicate that fluorine substitution at any olefin carbon diminishes the olfactive performance of the individual compound.

KeratinoSens® as well as peptide reactivity assays indicate that α-substitution for cinnamyl alcohol yields a less reactive fluorocinnamyl alcohol **2b**. However, this reduction is not seen for the SULT-mediated activation. Thus, only if this novel molecular mechanism is not a major driver for *in vivo* sensitization by cinnamyl alcohol, then **2b** has a reduced sensitization potential. Also, **2b** may also be activated to reactive and sensitizing **1b**. Thus, the reduction in the sensitization potential is probably less compared to the eight-fold loss of olfactive performance. Similarly, for **3b** a two-fold reduction in sensitization potential by *KeratinoSens*® and a more pronounced reduction in peptide reactivity is observed, along with a two-fold reduction in olfactive performance. No clear benefit in terms of sensitization (although a two-fold reduction in peptide reactivity is observed), but a clear loss of olfactive performance is found for **1b**.

The three β-fluorinated products **1** – **3c** however show drastically increased sensitization potential, which is due to increased reactivity towards skin proteins in a protein addition/HF-elimination sequence, hence β-fluorination led to a new molecular mode of action for all three derivatives, which could only be revealed by the mechanistic LC-MS based peptide reactivity assay. We thus confirm that the electronic properties of the double bond of known sensitizers plays a significant role in the sensitization profile.

While the results of this study are 'negative' in the sense that the new chemicals presented here have no improved risk/benefit ratio, this study illustrates the power of these new non-animal alternative tools in designing and early testing of new molecules and understanding their molecular mode of action. Combining these *in vitro* tools with olfactive assessment should thus allow the design and development of new fragrance ingredients with a safer use profile and improved risk/benefit ratio, which is currently a high priority of our laboratories. An example how such early *in vitro* testing (albeit for a different toxicological endpoint) led to the commercialization of a safer ingredient was published recently[34] and we will shortly report

examples with improved risk/benefit ratio regarding their skin sensitization potential.

Experimental Section

General Procedures

Commercially available reagents were used without further purification. Anhydrous solvents (acetonitrile and ethyl acetate) were purchased from *Sigma–Aldrich*. Anhydrous THF was distilled over sodium and benzophenone under nitrogen atmosphere and CH_2Cl_2 over CaH_2. Column Chromatography was performed on silica gel *60 Merck*, particle size 40 – 63 μm and aluminium oxide, basic, *Brockmann V*, particle size 50 – 200 μm, 60 Å, *Silicaflash P60* silica gel (40 – 60 μm) or a *Biotage Isolera* system *SNAP Ultra* prepacked cartridge. Mixtures of hexane and ethyl acetate (AcOEt) were used as eluent. Thin layer chromatography was performed on commercial 60-mesh silica gel plates, visualization was effected with short wavelength UV light (254 nm) and $KMnO_4$ or Ceric sulfate staining reagents. Standard GC analysis was performed on a *Agilent 5890* instrument with Chemstation software and a *ZB1* 15 m, 0.53 mm column. Carrier gas: H_2, 3.0 ml/min Sample amount: 10 μl. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a *Bruker AC300*, *Bruker ARX300*, *Bruker Avance-300* spectrometer (all three 300 MHz), or *Bruker Avance DPX-500* spectrometer; δ in ppm, J in Hz. Chemical shifts are given in ppm relative to internal TMS, while coupling constants are reported in Hertz (Hz). Deuterated chloroform and benzene were used as solvent. The multiplicity signals were indicated with the common abbreviations: *s* (*singlet*), *d* (*doublet*), *t* (*triplet*), *q* (*quartet*), *m* (*multiplet*), and br. (broad) and combinations thereof. In ^{13}C-NMR spectra the solvent itself served as the internal standard: $CDCl_3$ (δ(C) = 77.00 ppm, *t*, J_{CD} = 31.5). Signals corresponding to CH, CH_2, or Me groups were assigned from DEPT-135 and DEPT-90 spectra. The multiplicity is designated *q* (*quartet*) for Me, *t* (*triplet*) for CH_2, *d* (*doublet*) for CH, and *s* (*singlet*) for fully substituted carbon atoms. Mass spectra were measured in electron impact (EI) mode at 70 eV, with an ion source temperature of 230 °C. GC-MS was measured routinely on a *HP MSD 5975C* instrument with a 12m *BPX5* column from *SGE*. HPLC high resolution mass spectra (LC-HRMS) were recorded on a *Q-Exactive Orbitrap* (*Thermo Scientific*) instrument or on a *JEOL AccuTof 4G* spectrometer coupled to a *GC HP Agilent 7890* in chemical ionisation mode (CI).

The final products were purified by short path distillation using a *Büchi Kugelrohr B-585*. The vacuum was provided either by a rotary slide pump (0.05 mbar) or by a membrane vacuum pump (10 mbar).

GC-O experiments were performed on a *ThermoScientific*, *Trace 1300* equipped with an *AI1310* autosampler and a *Givaudan* in-house GC sniffing port with FID, splitting 1:1. The gas chromatographic system was set up with a *TSG-530660-D-10* 0.7 m – 0.53 mm precolumn and a *ZB-1* 15 m – 0.53 mm – 1.5 μm column, PTV on-column injection with a flow of 8 ml/min. The FID temperature was set to 250 °C.

Synthesis of Compounds **1b/1c, 2b/2c,** *and* **3b/3c**

Ethyl (2*Z*)-2-Fluoro-3-phenylprop-2-enoate (**3b**).[11][35] In an oven-dried three-neck flask equipped with a stir bar and flushed with argon, a solution of the benzaldehyde (1.1 g, 10.3 mmol.) and dibromofluoroethyl acetate (5.4 g, 20.6 mmol) in dry CH_2Cl_2 (100 ml) was prepared. A solution of diethylzinc (42.4 mmol, 1 M in hexanes) was then added slowly through cannula at 0 °C. The mixture was then allowed to warm to room temperature. The progress of the reaction was monitored by TLC and ^{19}F-NMR. When full consumption of starting material was observed, the reaction was stopped by adding a saturated solution of NH_4Cl very slowly because of the vigour of the reaction. The resulting mixture was filtered through a pad of *Celite*® to remove generated zinc salts and rinsed with CH_2Cl_2. The biphasic mixture was then concentrated under reduced pressure and the residue extracted three times with Et_2O. The organic layers were combined and dried over anhydrous $MgSO_4$. After removal of the $MgSO_4$ and evaporation of the volatile materials under reduced pressure, the crude was purified by flash chromatography on silica gel (cyclohexane/AcOEt 9:1) to afford the expected ester **3b** (1.2 g, 60% yield) as a colorless oil. ^{1}H-NMR (300.13 MHz, $CDCl_3$): 7.67 – 7.64 (*m*, 2 H); 7.38 – 7.41 (*m*, 3 H); 6.93 (*d*, $^3J_{H\text{-}F}$ = 35, 1 H); 4.36 (*q*, $^3J_{H\text{-}H}$ = 6, 2 H); 1.41 (*t*, $^3J_{H\text{-}H}$ = 6, 3 H). ^{13}C-NMR (75.47 MHz, $CDCl_3$): 161.1 (*d*, $^2J_{C\text{-}F}$ = 34); 146.8 (*d*, $^1J_{C\text{-}F}$ = 267); 130.9 (*d*, $^4J_{C\text{-}F}$ = 8); 130.0 (*d*, $^4J_{C\text{-}F}$ = 8); 129.4 (*d*, $^6J_{C\text{-}F}$ = 4); 128.6; 117.2 (*d*, $^2J_{C\text{-}F}$ = 5); 61.6; 13.9. ^{19}F-NMR (282.40 MHz, $CDCl_3$): −125.8 (*d*, $^3J_{H\text{-}F}$ = 35, 1 F). HR-MS (CI-TOF): 195.2080 ($C_{11}H_{12}FO_2^+$, $[M + H]^+$; calc. 195.2075).

(2*Z*)-2-Fluoro-3-phenylprop-2-en-1-ol (**2b**).[11][13] In an oven dried three-neck flask equipped with a stir bar and flushed with argon, a solution of ester **3b** (850 mg, 4.4 mmol) in dry THF (20 ml) was prepared. The mixture was cooled to 0 °C and lithium borohydride (670 mg, 30.8 mmol) was then added slowly. The mixture was allowed to warm to room temperature. The progress of the reaction was monitored by

TLC and ^{19}F-NMR. When full consumption of the starting material was observed, the reaction was stopped by adding a saturated solution of NH_4Cl very slowly. The resulting mixture was then extracted with AcOEt, the organic layers were combined, washed with brine and dried over $MgSO_4$. After removal of the $MgSO_4$ and evaporation of the volatile materials under reduced pressure, the crude was purified by flash chromatography on silica gel (cyclohexane/AcOEt 9:1) to afford the expected 2-fluorocinnamyl alcohol **2b** in quantitative yield (669 mg, 99% yield) as a white solid. ^{1}H-NMR (300.13 MHz, $CDCl_3$): 7.55 (*d*, $^3J_{H-H}$ = 8, 2 H); 7.34 – 7.26 (*m*, 3 H); 5.79 (*d*, $^3J_{H-F}$ = 39, 1 H); 4.26 (*d*, $^3J_{H-F}$ = 15, 2 H); 4.01 (br., 1 H, OH). ^{13}C-NMR (75.47 MHz, $CDCl_3$): 158.0 (*d*, $^1J_{C-F}$ = 268); 132.6 (*d*, $^3J_{C-F}$ = 3); 128.5 (*d*, $^5J_{C-F}$ = 7); 128.3; 127.3 (*d*, $^6J_{C-F}$ = 3); 107.3 (*d*, $^2J_{C-F}$ = 7); 61.3 (*d*, $^2J_{C-F}$ = 32). ^{19}F-NMR (282.40 MHz, $CDCl_3$): −113.3 (*dt*, $^3J_{H-F}$ = 14, 39, 1 F). HR-MS (CI-TOF): 153.0698 ($C_9H_{10}FO^+$, $[M + H]^+$; calc. 153.0710).

(2*Z*)-2-Fluoro-3-phenylprop-2-enal (**1b**).[11][14] To a solution of alcohol **2b** (360 mg, 3.36 mmol) in AcOEt (Volume: 15 ml) was added 2-iodoxybenzoic acid, IBX (940 mg, 10.1 mmol). The mixture was heated to reflux for 4.5 h, filtered through a pad of *Celite*®, and then concentrated under reduced pressure affording **1b** (826 mg, 98% yield) as an off-white solid. ^{1}H-NMR (400 MHz, $CDCl_3$): 9.35 (*d*, $^3J_{H-F}$ = 17.0, 1 H); 7.74 – 7.71 (*m*, 2 H); 7.46 – 7.44 (*m*, 3 H); 6.63 (*d*, $^3J_{H-F}$ = 34.3, 1 H). ^{13}C-NMR (100 MHz, $CDCl_3$): 184.0 (*d*, $^2J_{C-F}$ = 24.9); 154.8 (*d*, $^1J_{C-F}$ = 272); 130.9 (*d*, $^3J_{C-F}$ = 2.9); 130.7; 130.6; 129.0; 126.8. ^{19}F-NMR (282 MHz, $CDCl_3$): −128.5 (*dd*, $^3J_{H-F}$ = 34.3, 17.0, 1 F). HR-MS (CI-TOF): 151.0554 ($C_9H_8FO^+$, $[M + H]^+$; calc. 151.0553).

Ethyl (2*Z*)-3-Fluoro-3-phenylprop-2-enoate (**3c**). The title compound was prepared according to a literature procedure.[15] Silver(I) fluoride (14.57 g, 115 mmol) was added to a solution of ethyl 3-phenylpropiolate (10 g, 57.4 mmol) in acetonitrile (50 ml). The mixture was wrapped in aluminum foil before being heated to reflux and stirred overnight. The mixture was filtered a total of three times through a pad of silica gel (to get rid of the large amount of silver salts), washed with MTBE, hexane, and AcOEt. The volatiles were evaporated. The product was purified by flash chromatography using a 50 g cartridge with an eluent from 0 to 10% AcOEt in hexane over ten column volumes, then 100% AcOEt over two column volumes. After evaporation of the volatiles, ethyl (2*Z*)-3-fluoro-3-phenylprop-2-enoate (**3c**; 6.97 g, 62.5% yield) was obtained as a yellowish oil. A sample of olfactive quality was obtained by Kugelrohr distillation (0.05 mbar, 125 – 180 °C). ^{1}H-NMR (400 MHz, $CDCl_3$): 7.67 (*dd*, *J* = 1.5, 8.3, 2 H); 7.52 – 7.42 (*m*, 3 H); 5.92 (*d*, *J* = 33.3, 1 H); 4.28 (*q*, *J* = 7.3, 2 H); 1.35 (*t*, *J* = 7.2, 3 H). ^{13}C-NMR (101 MHz, $CDCl_3$): 166.3 (*d*, *J* = 277.0); 164.0 (*d*, *J* = 2.5); 131.5; 130.7 (*d*, *J* = 26.5); 128.8 (*d*, *J* = 1.7); 125.6 (*d*, *J* = 8.3); 97.2 (*d*, *J* = 7.5); 60.4; 14.3. ^{19}F-NMR (376 MHz, $CDCl_3$): −96.13 (s, 1 F). HR-MS (APCI): 195.0815 ($C_{11}H_{12}FO_2^+$, $[M + H]^+$; calc. 195.0816).

(2*Z*)-3-Fluoro-3-phenylprop-2-en-1-ol (**2c**). The compound was prepared according to a literature procedure.[16] DIBAL-H (25% in toluene, 10 ml, 14.87 mmol) was added to a solution of ester **3c** (2.5 g, 12.87 mmol) in CH_2Cl_2 (20 ml) at −78 °C. The mixture was stirred for 2 h at −78 °C. After this, a second portion of DIBAL-H (25% in toluene, 10 ml, 14.87 mmol) was added and the mixture was allowed to stir overnight while warming to room temperature. The mixture was then diluted with ether and cooled to 0 °C. Then, 1.4 ml of H_2O, 1.4 ml of NaOH (3 M) and further 3.5 ml H_2O were added. The cooling bath was removed, and the mixture stirred 15 min until the appearance of a white precipitate. The suspension was dried with $MgSO_4$, filtered over cotton, and the volatiles were evaporated. The product was then purified by column chromatography using a 25 g cartridge with a gradient from 10 – 50% AcOEt in hexane over 15 column volumes to yield 1.69 g (86% yield) of the title compound **2c** as a colorless oil. A sample of olfactive quality was obtained by Kugelrohr distillation (0.06 – 0.08 mbar, 120 – 145 °C). ^{1}H-NMR (400 MHz, $CDCl_3$): 7.58 – 7.49 (*m*, 2 H); 7.41 – 7.33 (*m*, 3 H); 5.66 (*td*, *J* = 7.1, 36.6, 1 H); 4.45 (*dd*, *J* = 2.1, 7.1, 2 H); 1.87 (*s*, 1 H). ^{13}C-NMR (101 MHz, $CDCl_3$): 158.1 (*d*, *J* = 250.4); 129.3; 128.6; 128.5; 124.4; 104.8 (*d*, *J* = 15.8); 56.1 (*d*, *J* = 8.3). ^{19}F-NMR (376 MHz, $CDCl_3$): −117.21 (*s*, 1 F). HR-MS (APCI): 135.0605 ($C_9H_{11}FO_2^+$, $[M - H_2O + H]^+$; calc. 135.0605).

(2*Z*)-3-Fluoro-3-phenylprop-2-enal (**1c**). According to a literature procedure,[17] manganese dioxide (9.60 g, 110 mmol) was added to a solution of (2*Z*)-3-fluoro-3-phenylprop-2-enal (**2c**; 2.1 g, 13.80 mmol) in CH_2Cl_2 (Volume: 75 ml). The mixture was stirred at room temperature for 8 h. The mixture was filtered through a pad of silica, washed with CH_2Cl_2, and the volatiles evaporated. The crude product was purified by column chromatography on a 25 g cartridge using an eluent from 1 – 10% AcOEt in hexane over 15 column volumes, then 50% AcOEt in hexane over two column volumes to yield (2*Z*)-3-fluoro-3-phenylprop-2-enal (**1c**; 1.41 g, 65% yield, 95% purity) as a bright yellow oil. A sample of olfactive quality was obtained by Kugelrohr distillation (0.06 – 0.08 mbar, 95 – 110 °C) as colorless oil. Aldehyde **1c** is very unstable under ambient

conditions, and should be stored diluted or below −20 °C. ^{1}H-NMR (400 MHz, CD_2Cl_2) 10.17 (*d*, *J* = 7.6, 1 H); 7.76 – 7.69 (*m*, 2 H); 7.59 – 7.53 (*m*, 1 H); 7.53 – 7.46 (*m*, 2 H); 6.10 (*dd*, *J* = 7.6, 34.0, 1 H). ^{13}C-NMR (101 MHz, CD_2Cl_2) 188.4 (*d*, *J* = 12.4); 171.4 (*d*, *J* = 274.5); 132.4, 129.0 (*d*, *J* = 1.7); 129.5 (*d*, *J* = 25.7); 125.9 (*d*, *J* = 9.1); 107.3 (*d*, *J* = 5.0). ^{19}F-NMR (376 MHz, CD_2Cl_2): −106.55 (*s*, 1 F). HR-MS (APCI): 151.0554 ($C_9H_8FO^+$, $[M + H]^+$; calc. 151.0554).

KeratinoSens® Assay

The standard operating procedure described before[18] was used in compliance with OECD guideline 442 days. Briefly, cells were exposed in triplicate to the test chemicals for 48 h at twelve binary dilutions in the range from 0.98 to 2000 μM, and then luciferase activity and cytotoxicity were determined. Three replicates were performed for each sample analysed. For each chemical in each repetition and at each concentration, the gene induction compared to DMSO controls was determined. Furthermore the maximal fold-induction (I_{max}) and the EC1.5, EC2, and EC3 values (concentration in μM for induction above the given threshold, based on linear extrapolation) along with IC_{50} values (concentration yielding 50% reduction in cellular viability) were calculated.

Peptide Reactivity Assessment

The LC-MS based peptide reactivity assay reported before[11] was applied. In this approach, depletion of the test peptide Cor1-C420 (Ac-NKKCDLF; 0.1 mM start concentration) after 24 h by test chemical (1 mM) is determined. Due to presence of both cysteine and lysine in the peptide, both cysteine and lysine-reactive chemicals react with and deplete this peptide and formation of adducts at multiple sites is possible. At the same time adduct formation between peptides and test chemicals and chemical-induced peptide oxidation are measured by LC/MS. LC/MS analysis was performed on a *VELOS PRO* Mass spectrometer (*Thermo SCIENTIFIC*, San Jose, CA, U.S.A.) operated in the ESI(+) mode. Mass spectra were recorded from 200 – 2000 amu. A *ZORBAX Eclipse XDB-C18* column, 2.1 mm ID, 150 mm, *5-Micron* (*Agilent Technologies*) was used. The mobile phase consisted of H_2O (*A*) and methanol (*B*) each containing 0.1% formic acid (v/v). The solvent flow was 250 μl/min and the following gradient (ratio *A*:*B*) was used: 0 min, 95:5; 2 min, 40:60; 10 min, 2:98; 12 min, 2:98. The integration was performed with *Xcalibur Quan Browser*™.

Sulfotransferase Activation Assay

Activation of benzyl alcohols and conjugated alcohols by sulfotransferase (SULT) under participation of the cofactor 3′-Phosphoadenosine-5′-phosphosulfate (PAPS) to form a metabolite with a sulfate leaving is a new mode of action probably relevant for skin sensitization. To assess the potential to covalently modify sulfanyl groups in peptides, test chemicals (0.2 mM) were incubated with rat liver S9 fractions (1 mg protein/ml) in 0.1 mM *Tris* buffer, pH 7.4 as metabolic system, along with the sulfotransferase cofactor PAPS (2 mM) and glutathione (GSH) (1 mM) as trapping agent to indicate sulfanyl group binding to form GSH adducts specifically in presence of PAPS. Samples were analyzed on a *Dionex UltiMate XRS 3000* HPLC system coupled to a *Q-Exactive* orbitrap mass spectrometer (*Thermo Scientific*) with electrospray ionization (ESI) in positive mode. As no synthetic reference standards were available, only peak areas are given assuming a similar response factor when comparing the different test compounds.

Supplementary Material

Supporting information for this article is available on the WWW under https://doi.org/10.1002/cbdv.201800013.

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Author Contribution Statement

J. C. conducted the syntheses, coordinated the study, and co-wrote the manuscript. *R. E.* performed the biological assays. *H. K.* performed the odor threshold experiments. *D. L.* did the olfactive evaluation. *X. P.* supervised and designed the synthetic experiments. *S. C.-B.* supervised and designed the synthetic experiments and contributed to the manuscript. *A. N.* designed the biological assays and co-wrote the manuscript. *A. B.* coordinated the study.

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