

## Review

### Chemical aspects of allergic contact dermatitis: a 21<sup>st</sup> century perspective

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## Keywords

Allergic contact dermatitis, skin sensitization, chemical aspects, nucleophile-electrophile mechanisms, radical mechanisms

## Declarations

Elena Giménez-Arnau, Bertrand Vilenó and David W Roberts declare no conflict of interest.

## Human and animal rights

This article does not contain any studies with human or animal subjects performed by any of the authors.

## Abstract

**Purpose of review:** Although the biological mechanism of skin sensitization is a quite complex multi-stage process, the sensitization potency (or absence of potency) of a chemical is dependent on its chemical properties. Here we present an overview of the advances that have been made in our understanding of the chemical properties that determine sensitization potency, and how this understanding can be applied to rationalize existing data and to make predictions for new chemicals.

**Recent findings:** It has been known for many decades that for a chemical to be a sensitizer it must be able to form a covalent reaction product with skin protein. There are several reaction mechanisms by which these covalent products can be formed. Five major mechanisms in which the sensitizing chemical is electrophilic are recognized. For each of these mechanisms we summarise how these are related to chemical structure and what is now understood about the relationship between chemical properties and sensitization potency. A further mechanistic domain recently recognized, covering chemicals reacting as nucleophiles, is also discussed. Recent developments have made it possible to investigate free radical chemistry *in cutaneo*, and we discuss how these can be applied to investigate free radical sensitization mechanisms.

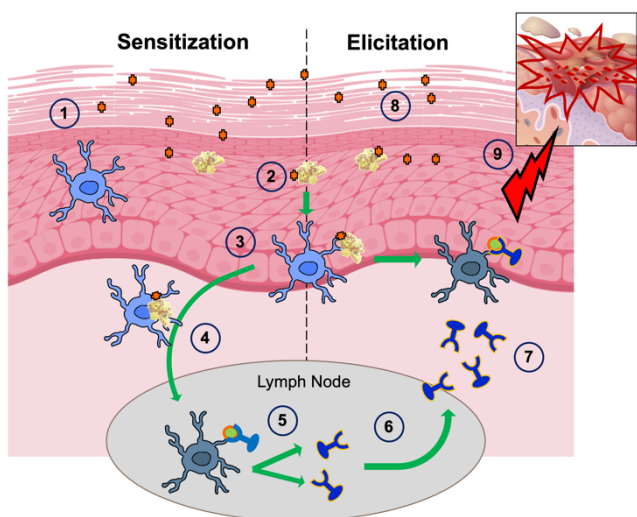
**Summary:** An update on the chemistry related to skin sensitization is presented.

## Abbreviations

ACD, allergic contact dermatitis; AOP, adverse outcome pathway; BP, benzophenone; C, carbon; DC, dendritic cell; Citr-OOHs, citronellol hydroperoxides; DEPMPO, 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide; DST, dermal sensitization threshold; DFT, computational density functional theory; DPRA, direct peptide reactivity assay; E, electrophile; EPR, electron paramagnetic resonance; EU, European Union; HRMAS-NMR, high-resolution magic angle spinning nuclear magnetic resonance; KE, key event; LG, leaving group; Lim-OOHs, limonene hydroperoxides; Lin-OOHs, linalool hydroperoxides; NMR, nuclear magnetic resonance; Nu, nucleophile; O, oxygen; PACD, photoallergic contact dermatitis; PhO<sup>•</sup>, phenoxyl radical; PhOH, phenol; REACH, European registration, evaluation, authorization and restriction of chemicals; RHE, reconstructed human epidermis; R<sup>•</sup>, carbon radical; RO<sup>•</sup>, alkoxyl radical; ROO<sup>•</sup>, peroxy radical; ROOHs, hydroperoxides; ROS, reactive oxygen species; SB, Schiff base; SC, spin scavenging; ST, spin trapping

## Introduction

Allergic contact dermatitis (ACD) is an important occupational and environmental health issue. It is an immunotoxicity reaction caused by activation of antigen-specific T-cells resulting from skin sensitization to a xenobiotic after repeated contact [1]. ACD occurs through a two-phase process: (i) the sensitization phase, *i.e.*, sensitization of the individual acquired after first contact with the allergen, followed by (ii) the elicitation clinical phase triggered after re-exposure of the sensitized individual to the allergen [Fig. 1].



**Fig. 1** ACD mechanism: (1) penetration of the allergen, (2) binding to skin proteins in the epidermis, (3) dendritic cells (DC) incorporation of modified proteins (4) DC maturation and migration to lymph nodes, (5) presentation of allergen-protein antigenic modification to naïve T-lymphocytes, (6) proliferation of antigen-specific memory T-cells, leaving the lymph nodes (7) and entering the circulation, (8) re-exposure to the allergen, re-incorporation of another modified protein by DC and maturation, (9) presentation of the antigen to memory T-lymphocytes, release of inflammatory agents and ACD clinical response (eczema, oedema, vesicles, scaling, fissures).

The incidence and prevalence of ACD in the general population is not readily determinable as most available information is derived from specialized patch testing data collected at dermatological centres. Yet, according to Scheinman *et al.* 20 to 25% of the adult population in the European Union (EU) are thought to suffer from ACD to one or more skin allergens present in everyday life [2]. There is currently no available treatment for ACD other than symptomatic. Prevention of the disease therefore relies mainly on early identification of the sensitizing potential of chemicals prior to their introduction to the market and on upstream studies to investigate their mechanism(s) of action for risk assessment purposes.

For at least the past 25 years and following (i) the 3Rs (replacement, reduction and refinement) principle developed over 50 years ago for the ethical use of animals for scientific purposes [3,4], (ii) the EU 7<sup>th</sup> Amendment to the Cosmetics Directive that placed a complete ban on animal testing in 2013 for cosmetic ingredients [5] and (iii) the European Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) regulation that compels the use of *in vitro* and *in silico* approaches as first choice to assess the skin sensitization potential of chemicals [6,7], developing *in silico*, *in chemico* and *in vitro* approaches to assess the skin sensitization potential of chemicals is a major concern of both involved industry and academia. The publication of the Adverse Outcome

Pathway (AOP) for skin sensitization in 2012 [8] accelerated the development of predictive tools for risk assessment aligned to the four defined key events (KE) associated with skin sensitization : (KE1) binding of chemicals to skin proteins, (KE2) keratinocytes activation, (KE3) dendritic cell (DC) activation and maturation, and (KE4) proliferation of antigen-specific T cells [9] . KE1 is the chemical event related to the ability of small chemicals to modify epidermal proteins and form antigenic structures that trigger the immune system. KE1 is clearly considered the molecular initiating event in the AOP for the sensitization process.

The earliest investigation into why some chemicals are skin sensitizers and why some are not was reported by Landsteiner and Jacobs in 1935 [10] , at a time when the field of mechanistic organic chemistry was still largely unexplored. They examined 20 compounds with the basic structure of a benzene ring substituted with nitro groups and/or halogen atoms. Each compound was heated with aniline in methanol for 2 h, and each was tested for sensitization in a guinea pig assay. They found that 10 of the compounds reacted with aniline and were sensitizers, while the other 10 did not react and were negative in the sensitization test. They concluded that ability to react with protein nucleophiles, modelled by aniline in their experiments, was necessary for a chemical to be a sensitizer. Landsteiner and Jacobs also considered the poison ivy allergens, not directly reactive but that can be oxidised to *ortho* quinones which are highly reactive towards nucleophiles. By the early 1980s mechanistic organic chemistry had become a more developed field and several investigations into relationships between chemistry and skin sensitization had been reported. In 1982 Dupuis and Benezra published a book describing how the skin sensitizing properties of many well-known allergens could be rationalised in terms of their reaction chemistry [11]. Among the chemicals discussed were the sesquiterpene lactones, found in plants of the *Compositae* family. Sesquiterpene lactones are cyclic compounds with a total of 15 carbon atoms, including a cyclic ester (*i.e.* lactone) group and those with a double bond adjacent to the ester group (now recognised as a Michael acceptor reactivity alert) were found to be sensitizers. From the 1980s onwards the field of chemistry-sensitization relationships has grown substantially, including advances in quantitative structure-potency models. In-depth knowledge gained over all these years by chemists specializing in mechanistic organic chemistry and the reactive chemical functions present in the molecular structure of skin sensitizers has led to the development of reactivity assays which are now routinely used in the non-animal risk assessment of chemicals for skin sensitization potential [9].

Skin sensitizers react with proteins in the skin through the formation of stable covalent bonds. In some cases, the formation of the initial covalent bond with protein is significantly reversible and, in such cases, the thermodynamically most stable reaction product rather than the initial reaction product may be the entity that gives rise, via the later stages in the AOP, to sensitization. Reactivity of a sensitizer can be direct (hapten) if the molecule already contains the reactive chemical function, or

can be acquired *via* metabolic activation (pro-hapten) or by air-, heat- or light-mediated chemical conversions (pre-hapten) [12]. The formation of a covalent bond requires two electrons that can be provided either by (i) one of the atoms especially rich in electrons, the nucleophile, sharing them with another atom poor in electrons, the electrophile, or (ii) contribution of both species involved, each one sharing an electron in a radical based reaction. The aim of this publication is to review the chemical mechanisms and reactive chemical functions (*a.k.a.* structural alerts) recognised for skin sensitizers, including those reacting by nucleophile-electrophile mechanisms and those reacting through radical mechanisms.

### **Skin sensitizers reacting through nucleophile-electrophile mechanisms**

In the majority of cases the skin sensitizer is the electrophile and the protein is the nucleophile. Indeed, many sensitizers contain electrophilic chemical functions. The side chains of several amino acids in proteins contain electron-rich or nucleophilic groups that can react with electrophiles. Cysteine (the nucleophile is a ionised SH group) and lysine units (the nucleophile is the NH<sub>2</sub> group of the -(CH<sub>2</sub>)NH<sub>2</sub> side chain) are the most usually considered in ACD, but amino acids such as histidine, methionine, tyrosine and arginine can also act as nucleophiles [13], though they are less reactive. The N-terminal units of proteins (general structure NH<sub>2</sub>-CHX-CONH-) are also reactive, possibly more reactive than lysine [14]. A terminal cysteine unit (NH<sub>2</sub>-CH(CH<sub>2</sub>SH)-CONH-) may in some cases be particularly important since it has two nucleophilic centres (NH<sub>2</sub> and ionised SH) and can give rise to thermodynamically stable cyclic adducts (e.g. with aldehydes [15]). Another amino acid rarely if ever considered in this context is selenocysteine (-NH-CH(CH<sub>2</sub>SeH)-CONH-). The -SeH group, even when not ionised, is a substantially more reactive nucleophile than ionised -SH groups [16,17]. Selenocysteine-containing proteins are known to be present in the skin at levels of about 1% based on total skin protein and it is plausible that one of their functions is to react with exogenous electrophiles.

### **Reaction mechanistic domains**

When chemicals are grouped according to their reaction mechanisms, it is usually found that those in the same reaction mechanistic domain fit a quantitative relationship between sensitization potency and chemistry parameters (in some cases a reactivity parameter alone, in some cases together with a hydrophobicity parameter). The identity of reactive skin protein(s) involved in skin sensitization is not known, but it is well established in organic chemistry that for chemicals reacting via a common mechanism, reactivity towards one reagent is correlated with reactivity to others. Consequently, within a reaction mechanistic domain sensitization potency of electrophilic chemicals can be correlated with reactivity towards a model nucleophile (such as a cysteine-based peptide or butylamine) or computational reactivity parameters.

There are five major reaction mechanistic domains for electrophilic sensitizers [18,19] and a sixth updated mechanistic domain covering nucleophilic sensitizers [20], which can react with electrophilic groups in proteins. They are all shown in Fig. 2 and described below.

**The S<sub>N</sub>2 domain.** A nucleophile attacks a saturated carbon atom and a leaving group (LG) is expelled, in a concerted single step. Examples of sensitizers in this domain are sulphonate and sulphate esters such as dimethyl sulphate, *p*-nitrobenzyl bromide, epoxides and many others. Relationships between structure and chemical reactivity for this mechanistic domain have been extensively studied going back to the mid 20<sup>th</sup> century [21]. Some of the key points most relevant to skin sensitization are described below.

Reactivity depends on the reaction centre and on the LG. For a given LG, reactivity at a methyl centre is greater than reactivity at a higher primary alkyl centre, and reactivity at a secondary alkyl centre is usually much lower, although this is not always the case, particularly when the reaction centre and the LG are part of a cyclic system (e.g. the sultone shown in Figure 2). Allylic ( $\text{-C=C-CH}_2\text{LG}$ ), benzylic ( $\text{Ar-CH}_2\text{LG}$ ) and styrylic ( $\text{Ar-C=C-CH}_2\text{LG}$ ) centres are activated by conjugation in the transition state and reactivity at these centres is greater than at an alkyl centre.

For a given reaction centre, LG can vary quite widely in their effectiveness. Usually, the LG is expelled as its anion, and reactivity is partly correlated with the ability of LG to carry a negative charge. This can be quantified by the acidity ( $\text{pK}_a$ ) of the conjugate acid LGH. Halide ( $\text{-Cl}$ ,  $\text{-Br}$  or  $\text{-I}$ ),  $\text{-OSO}_2\text{R}$  (alkane sulphonate) and  $\text{-OSO}_2\text{OR}$  (alkyl sulphate) are all good LG, being expelled as anions of very strong acids ( $\text{pK}_a < 0$ ). Carboxylate ( $\text{-OCOR}$ ) and sulphate ( $\text{-OSO}_3^-$ ) are relatively poor LG, being expelled as anions of weaker acids (carboxylic acids,  $\text{pK}_a$  typically in the range 2.5 to 5; bisulphate ion,  $\text{pK}_a = 2$ ). Unless they are bonded to activated reaction centres, carboxylate esters are not reactive enough to sensitize. Thus, benzyl benzoate (benzylic reaction centre) and allyl octanoate (allylic reaction centre) are both sensitizers. Allyl acetate, although similarly reactive to allyl octanoate, is less hydrophobic and is a non-sensitizer in the local lymph node assay. Ethers, in which the LG (alkoxide ion) is the anion of an alcohol ( $\text{pK}_a$  values usually above 14) usually have very low reactivity, although epoxides, in which the ether is part of a 3-membered ring, are in many cases reactive enough to be sensitizers. Alcohols ( $\text{R-OH}$ ), in which the LG is the hydroxide ion, are not significantly reactive as S<sub>N</sub>2 electrophiles but there is evidence that in some cases they can be metabolically converted in the skin to the corresponding sulphates ( $\text{R-OSO}_3^-$ ). These are not reactive enough to sensitize when R is alkyl [22], but are more reactive when the reaction centre is activated. Based on NMR studies in reconstructed human epidermis (RHE) it has been proposed that cinnamyl sulphate ( $\text{PhCH=CHCH}_2\text{OSO}_3^-$ ) may be the reactive metabolite involved in skin sensitization by cinnamyl alcohol [23] and this is supported by kinetics calculations indicating that it is sufficiently

reactive to sensitize [22]. Other alcohols whose allergenicity may be rationalised by this *in cutaneo* sulphation mechanism include benzyl alcohol and the allylic alcohols geraniol and farnesol [22].

Conjugated dienes are not directly reactive but, in some cases, particularly when one or both of the double bonds is part of a cyclic system, they can be activated by conversion in the skin to  $S_N2$ -electrophilic epoxides [24]. Styrene, in which the double bond is conjugated with the aromatic ring, is thought to sensitize by this mechanism [25].

Potency of  $S_N2$  sensitizers is dependent on both reactivity and hydrophobicity [18,19,25].

**The  $S_NAr$  domain.** A nucleophile attacks an aromatic carbon atom and a LG is expelled. 2,4-Dinitrochlorobenzene and 2,4-dinitrobenzenesulphonic acid, both widely used as “standard allergens” in dermatological research, are  $S_NAr$  electrophiles, the LG being the chloride and the sulphite ion, respectively. The  $S_NAr$  reaction is much less concerted than the  $S_N2$ , with the new bond being formed to a large extent before the bond to the LG starts to weaken significantly [26,27]. Consequently, the structure-reactivity relationships for this domain are very different from those for the  $S_N2$  domain. The pioneering work of Landsteiner and Jacobs in the 1930s [10], demonstrating the connection between the ability to react with a nucleophile (in their studies aniline) and the ability to sensitize, was based on  $S_NAr$  electrophiles, although the term had not been coined at that time and the field of organic reaction mechanistic chemistry was still largely undeveloped. For this domain, structure-reactivity relationships (reviewed in [27]) and relationships between reactivity and skin sensitization potency have been quite extensively investigated, indicating that potency depends on reactivity but not hydrophobicity [28,29].

**The Michael acceptor domain.** A nucleophile attacks one of the carbon atoms of a double or triple carbon-carbon bond that is activated by conjugation with at least one unsaturated electronegative unsaturated group (e.g. carbonyl groups  $C=O$  in aldehydes ( $-CHO$ ), ketones ( $COR$ ) and esters ( $CO_2R$ )) [18,19,30]. Some chemicals not directly reactive are thought to sensitize via activation to Michael acceptors. Examples include hydroquinones and catechols being oxidized to highly reactive quinones [25]. A pro-Michael mechanism has been widely considered to apply for cinnamyl alcohol (oxidation to cinnamaldehyde) but more recent evidence suggested that a pro- $S_N2$  mechanism may be more likely [22,23].

Potency of Michael acceptor sensitizers is dependent on reactivity, although to a lesser extent than the  $S_N2$  and  $S_NAr$  domains, and only marginally, if at all, dependent on hydrophobicity [29,30].

**The Schiff base (SB) electrophile domain [31,32].** Chemicals in this domain are aldehydes or ketones. As mentioned above, when the aldehyde or ketone group is bonded to a double or triple carbon-carbon bond the Michael acceptor mechanism is also possible. Ketones that are not activated by neighbouring electronegative groups are less reactive than aldehydes. Examples of Schiff base domain sensitizers include lilial (aldehyde), butane-2,3-dione (diketone in which one keto group

makes the other more reactive) and methyl pyruvate (an ester group makes the keto group more reactive). A nucleophile attacks the carbon atom of a carbonyl C=O group and the oxygen atom becomes protonated. The initial adduct may then react further, as shown in Fig. 2. When the nucleophile is a primary amine RNH<sub>2</sub> the reaction product has the general structure RN=C known as a Schiff base (SB). This is why chemicals in this mechanistic domain are referred to as SB electrophiles. However, it is not known whether sensitization actually involves SB formation (e.g. via reaction with lysine units) or not. SB formation is readily reversible under aqueous conditions, as also is hemithioacetal formation, and it is possible that equilibrium thermodynamics contribute to potency [14]. Figure 2 shows what we consider to be the most likely possibilities for protein binding of SB electrophiles. A pro-SB mechanism whereby an aliphatic amino group -CH<sub>2</sub>NH<sub>2</sub> is oxidized to CH=O is considered to apply for sensitization by ethylenediamine and may be more widely applicable.

Potency of SB electrophile sensitizers is dependent on a combination of reactivity and hydrophobicity [31,32].

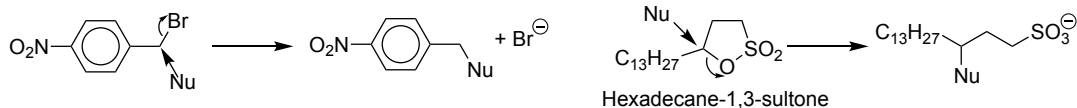
**The acyl transfer domain [18,19].** Chemicals in this domain are carboxylic acid derivatives. They have a carbonyl group bonded to a LG and can be represented by the general structure RCOX. The nucleophile attacks the carbonyl group and the intermediate expels the LG X as its anion. Acyl chlorides RCOCl, acid anhydrides and some phenolic esters can sensitize by this mechanism. Simple alkyl esters are unable to react in this way, since the alkoxide ion is too strongly basic to act as a good LG. If the acid HX has a pK<sub>a</sub> significantly above about 10, RCOX is unlikely to sensitize by the acyl transfer mechanism. Not enough acyl transfer sensitizers have been studied to develop firmly established structure-potency relationships.

**Nucleophilic sensitizers [20].** It has recently been found that a substantial number of sensitizers, with no electrophilic or pro-electrophilic alerts, can be simply explained in terms of the sensitizer acting as a nucleophile towards electrophilic centres in protein. Disulphide linkages S-S of cystine units are the most prevalent electrophilic centres in proteins and it is also possible that selenium analogues S-Se and/or Se-Se could be involved. In some cases, the nucleophilic reaction centre of the sensitizer is a sulphur or phosphorus atom, and in other cases it is an aromatic carbon atom activated by  $\pi$ -electron-donating groups. Examples are sodium metabisulphite (inorganic sensitizer), 1-dodecanethiol and 1-thioglycerol (an ionized SH group being the reaction centre), triphenyl phosphite (with a trivalent phosphorus atom as the reaction centre), 1,3-diaminobenzene (aromatic carbon activated by two NH<sub>2</sub> groups being the reaction centre) and cardol (aromatic carbon activated by two OH groups being the reaction centre).

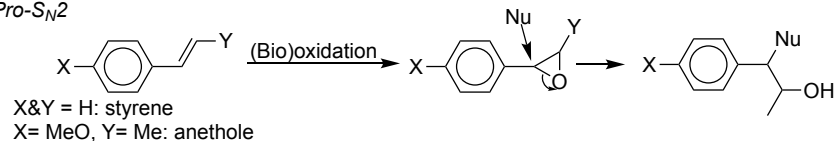
Preliminary indications are that potency of nucleophilic sensitizers depends on a combination of nucleophilic reactivity and hydrophobicity [20].

## S<sub>N</sub>2 electrophiles

### Examples

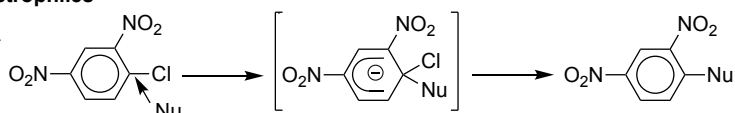


### Pro-S<sub>N</sub>2



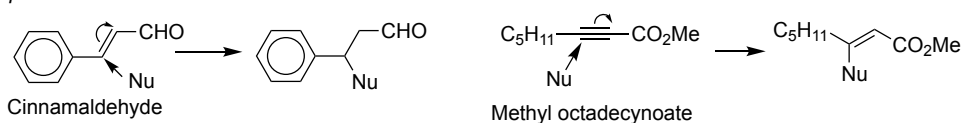
## S<sub>N</sub>Ar electrophiles

### Examples

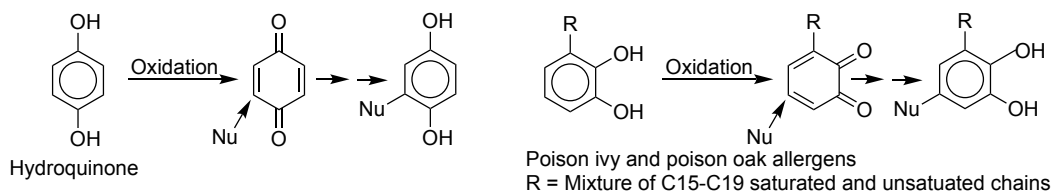


## Michael acceptor electrophiles

### Examples

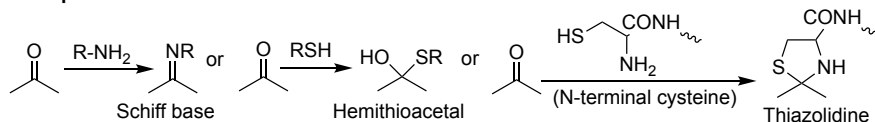


### Pro-Michael acceptors



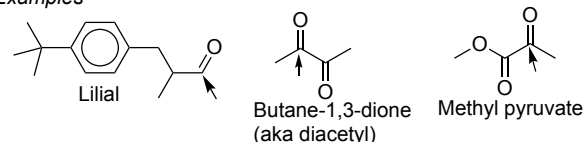
## Schiff base (SB) electrophiles

### General reaction

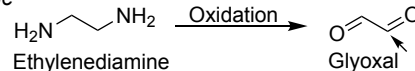


Schiff bases and hemithioacetals are readily hydrolysed in water, regenerating the parent carbonyl compound, but thiazolidines are more stable

### Examples

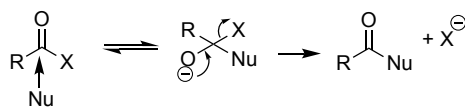


### Pro-SB electrophile

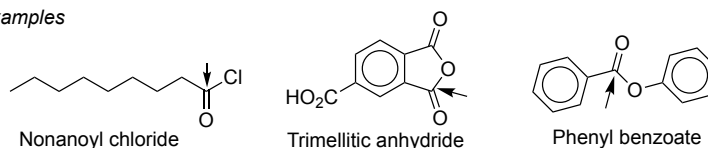


## Acyl transfer electrophiles

### General reaction

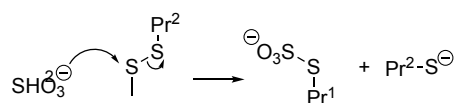


### Examples

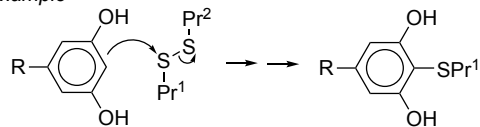


## Nucleophilic sensitizers

General reaction illustrated by reaction of sulphite ion with a cysteine unit of a protein Pr<sup>1</sup>S-SPr<sup>2</sup>



### Example



Cardol, a potent allergen in Cashew nut shell oil. R = mixture of linear C15 saturated, and mono-, di- and tri-unsaturated chains.

Fig. 2 Nucleophile-electrophile mechanisms: reaction mechanistic domains

## Brief overview of reactivity studies

The modification of nucleophilic amino acids by electrophilic skin sensitizers has for many years been investigated in solution. A variety of experimental approaches using amino acids, short peptides containing one or more nucleophilic amino acids and proteins were developed. Examples illustrating each approach can be found in references [33–35]. The principal outcome of the acquired knowledge was the today validated Direct Peptide Reactivity Assay (DPRA), where the sensitizing potency of chemicals is assessed measuring the reactivity with lysine and cysteine containing peptides in solution [36]. For the purpose of predicting sensitization potency of chemicals, simple assays with model nucleophiles seem to be the most useful. Further, to acquire deeper insights into the nature of the modified protein by allergens and involved in the sensitization process, studies involving proteomics and *in cutaneo* haptentation can be extremely helpful.

More recently, studies on reconstructed human epidermis (RHE) and the use of high-resolution magic angle spinning nuclear magnetic resonance (HRMAS-NMR) with  $^{13}\text{C}$ -substituted sensitizers, have provided access to *in situ* reactivity information in a model closer to what may occur *in vivo*. It was shown that amino acids other than the conventional lysine and cysteine can also be reactive, mainly histidine and methionine, and that the reactions are generally faster than in solution [23,37]. However, it is still not known whether the sensitizing potency of a chemical is directly related to a set of nucleophilic sites or to a specific type of reacting amino acid. To get insights into this puzzle, for the first time a quantitative HRMAS-NMR methodology was developed in RHE to establish a chemical reactivity mapping with nucleophilic residues by using methyl methane sulfonate, a sensitizing methylating agent, as proof-of-concept [37]. Interestingly, it was possible to associate a quantitative amount of adducts formed in RHE with a sensitizing dose. This methodology could thus allow further investigation into the links between the number of chemical modifications in the epidermis, skin exposure concentration and sensitization potency. HRMAS-NMR in RHE has also been used to study metabolism processes in the skin, for example in the case of cinnamic alcohol and cinnamaldehyde [23].

However, many sensitizers do not fit the E-Nu model due to their poor or absent electrophilicity and may react with skin proteins by mechanisms involving radical intermediates. Until now, very little is known about the formation of antigenic structures involving radicals in skin sensitization and ACD.

## Skin sensitizers reacting through radical mechanisms

The first essential step in understanding this behaviour is to identify and characterize radical species generated by the targeted compounds that could potentially react with endogenous skin proteins.

## Skin sensitizers and radicals

A non-exhaustive list of skin sensitizers suspected of forming reactive radicals includes hydroperoxides (ROOHs), phenols (PhOH) and benzophenone (BP) derived photoallergens [Table 1].

Compounds with oxidizable allylic positions can autoxidize when exposed to air, by a free radical chain reaction in which hydrogen abstraction followed by oxygen addition forms ROOHs as primary oxidation products. The pioneering work of Karlberg's group in establishing a comprehensive picture of such skin sensitizers, highlighted how ROOHs arising from commonly used fragrance terpenes that autoxidize are frequent causes of ACD [12]. The interest in sensitizing ROOHs increased significantly with the rising prevalence of fragrance allergy due to the worldwide use of fragranced products and odorant terpenes. Sharing the spotlight are those derived from limonene (Lim-OOHs), linalool (Lin-OOHs) and citronellol (Citr-OOHs). Although a 2019 European market surveillance study on fragranced consumer and patient products indicated that concentrations of Lim-OOHs and Lin-OOHs are orders of magnitude below sensitizing doses in animal tests, and no evidence for accumulation in aged products or those used by patients was found [38], there is no doubt ROOHs give frequent positive patch test reactions. The big issue today is therefore to understand how individuals are exposed and sensitized to ROOHs, the sources of which remain elusive.

The reactivity of ROOHs originates from the cleavage of the O-O bond of low dissociation energy (*ca.* 175 kJ.mol<sup>-1</sup>). The cleavage gives rise to unstable oxygen (O), alkoxyl (RO\*) and then peroxy (ROO\*) radicals which undergo radical rearrangements (*i.e.*, intramolecular cyclization, allylic hydrogen abstraction and/or  $\beta$ -scission) due to the presence of double bonds in the terpene chemical structure and form R\* carbon (C) centred radicals. Reactivity studies with amino acids known to participate in radical processes were conducted in solution (aqueous buffer, semiorganic) and followed by nuclear magnetic resonance (NMR) and liquid chromatography coupled to mass spectrometry. It was proved that C-radicals derived from ROOHs react readily with amino acids such as cysteine, histidine and tryptophan when using Fe(II)/Fe(III) redox systems to trigger radical initiation [39]. This was substantial information as it revealed that, depending on the chemical structure, several reactive radical intermediates can arise from these compounds and that different chemical modifications of potentially immunogenic proteins could lead to sensitization.

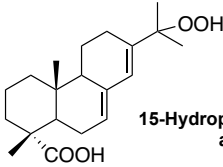
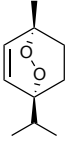
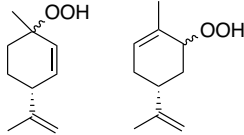
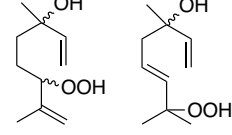
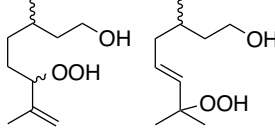
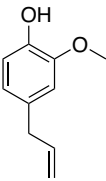
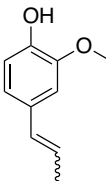
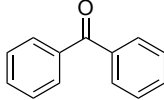
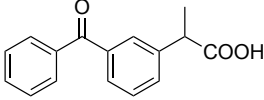
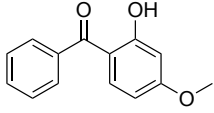
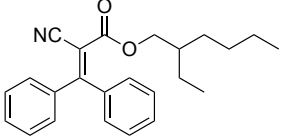
Eugenol and isoeugenol, natural odorant compounds belonging to the group of *ortho*-methoxy phenols having a propenyl group in the *para* position, are known to possess cytoprotective activities as antioxidants but also cytotoxic potential as prooxidants [40,41]. They are indeed important fragrance allergens, compelling the cosmetics and fragrance industries to notify their presence in manufactured products [42]. Both have been the subject of mechanistic studies, where electrophilic

*ortho*-quinone and quinone methide final oxidation products have been implicated as the reactive species towards nucleophilic residues of skin proteins [43]. More recent studies have been interpreted in terms of epoxidation of the double bond as the initial step in the activation of isoeugenol whereas eugenol, in which the double bond is not conjugated with the benzene ring, is activated by demethylation then oxidation to an *ortho*-quinone [44,45]. However, radical mechanisms cannot be ruled out as alternatives to such pathways. The antioxidant activity results from the neutralisation of reactive oxygen species (ROS) arising in the media through the removal of the phenolic hydrogen atom. However, cytotoxicity has been associated to the so-formed phenoxyl radicals (PhO<sup>•</sup>) [46]. This radical is highly delocalized through conjugation with the aromatic ring and not only the oxygen atom but also several ring carbon positions have radical character.

The non-steroidal anti-inflammatory drug ketoprofen and the UV filters oxybenzone and octocrylene are known photoallergens [47]. That is to say compounds that require light (UVA, UVB and/or visible) to become reactive [48]. Both have BP, one of the most photoactive compounds, as a common core [49]. By absorption of a photon, BP is excited from the fundamental singlet state to a triplet state with the biradical form of the molecule (BP<sup>••</sup>) [49,50] that has several potential radical reaction centres as a result of delocalization.

The examples discussed above are shown in Table 1 together with references to the identification of radical intermediates.

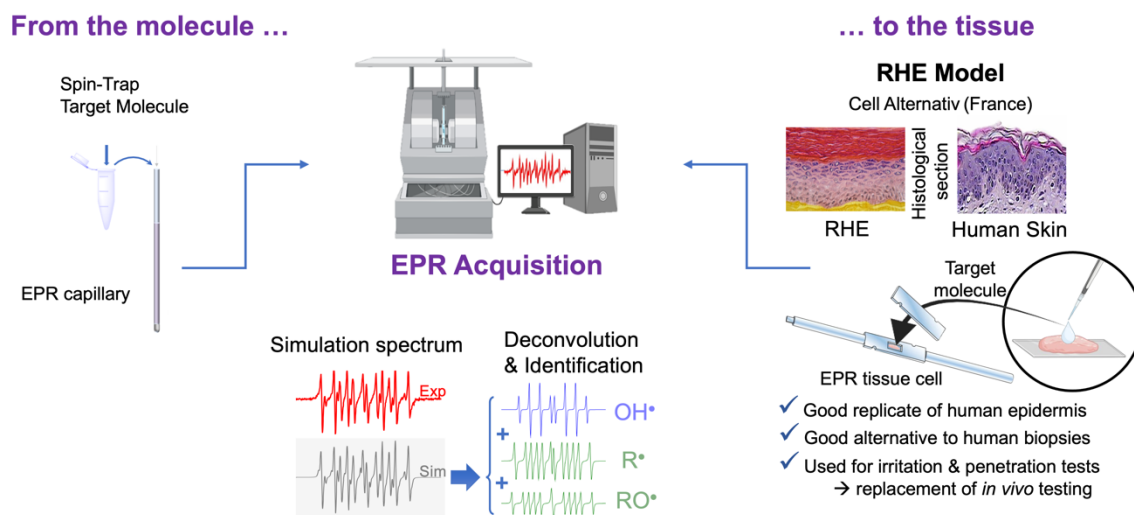
**Table 1.** Skin sensitizers potentially reacting through radical mechanisms and related references on radical's identification

| Compounds                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         | References                                                                                                                                                       |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <p><b>Hydroperoxides/Peroxides</b></p> <div style="display: flex; justify-content: space-around; align-items: center;"> <div style="text-align: center;">  <p>15-Hydroperoxyabiatic acid</p> </div> <div style="text-align: center;">  <p>Ascaridole</p> </div> </div> <div style="display: flex; justify-content: space-around; align-items: center; margin-top: 10px;"> <div style="text-align: center;"> <p>Lim-OOHs</p>  <p>Lim-1-OOH    Lim-2-OOH</p> </div> <div style="text-align: center;"> <p>Lin-OOHs</p>  <p>Lin-6-OOH    Lin-7-OOH</p> </div> <div style="text-align: center;"> <p>Citr-OOHs</p>  <p>Citr-6-OOH    Citr-7-OOH</p> </div> </div> | <p>15-Hydroperoxyabiatic acid: [51–53]<br/> Ascaridole: [54]<br/> Lin-OOHs: [39,55–57]<br/> Lim-OOHs: [39,56,58,59]<br/> Citr-OOHs: [60]<br/> Other: [61–63]</p> |
| <p><b>Phenols</b></p> <div style="display: flex; justify-content: space-around; align-items: center; margin-top: 20px;"> <div style="text-align: center;">  <p>Eugenol</p> </div> <div style="text-align: center;">  <p>Isoeugenol</p> </div> </div>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            | <p>[64]</p>                                                                                                                                                      |
| <p><b>Benzophenone derivatives/Photoallergens</b></p> <div style="display: flex; justify-content: space-around; align-items: center; margin-top: 20px;"> <div style="text-align: center;">  <p>Benzophenone</p> </div> <div style="text-align: center;">  <p>Ketoprofen</p> </div> </div> <div style="display: flex; justify-content: space-around; align-items: center; margin-top: 20px;"> <div style="text-align: center;">  <p>Oxybenzone</p> </div> <div style="text-align: center;">  <p>Octocrylene</p> </div> </div>                                                                                                                                                                                                          | <p>[65]</p>                                                                                                                                                      |

## Electron paramagnetic resonance: main tool to identify radicals from skin sensitizers

A powerful tool for the detection and identification of transient radicals in chemical and biological systems is electron paramagnetic resonance (EPR) (see [66] for EPR fundamentals). While free radicals are often too short-lived or in too low concentrations to be detected directly by EPR in classical experimental conditions (*e.g.* aqueous solution at room temperature), indirect approaches are available: (1) EPR spin trapping (ST) [67] where a spin-trap is used to react with radicals to form more persistent ones (spin-adducts) whose EPR signatures strongly depend on the trapped molecules, and (2) EPR spin-scavenging (SC) [68] where the decay of a paramagnetic probe is monitored while reduced by targeted radicals allowing access to kinetics insights.

Important progress has been accomplished over the last decade in the study of radicals derived from skin sensitizers by EPR-ST particularly. Work in the skin allergy field is carried out “from the molecule”, performing studies in solution, “to the tissue”, by the development of EPR methodologies in combination with RHE 3D models [Fig. 3] [63]. The choice of the spin-trap is of paramount importance, and depends on both the experimental conditions (solvent polarity, temperature, irradiation...) and the nature of the targeted radicals (*e.g.*, C-/O-centred). Today most EPR-ST bio-related investigations use nitron based compounds, such as 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide (DEPMPO), well tolerated *in vivo* at relatively high concentrations [69]. They detect both O- and C-radicals over large ranges of pH, temperature and illumination and are compatible with various organic solvents.



**Fig. 3** EPR-ST methodology developed for the identification of radicals derived from skin sensitizers goes from the test tube to the use of a RHE skin model.

An EPR-ST methodology has been developed to investigate *in situ* the formation of radicals from skin sensitizers in skin keratinocytes, the predominant cell type in the epidermis, using 3D RHE

models from healthy donors. Experiments are based on a topical application procedure to get closer to real-life sensitization [63]. Cumene hydroperoxide was used to establish the proof-of-concept [61]. Subsequently, ascaridole, Lin-OOHs, Lim-OOHs and Citr-OOHs, basic triggers of free radicals, were successfully studied [54,57,59,60]. Complementing these investigations, computational density functional theory (DFT) calculations highlighted potential preferential pathways of radical degradation for ROOHs [59]. Recently, exposure to a solar simulator that mimics natural daylight illumination has been included in the initial assay, providing a starting point for unravelling the chemical mechanisms involved in photoallergic contact dermatitis (PACD), a critical photosensitivity skin disease caused by the combined exposure of photoreactive chemicals in contact with the skin and light (UVA, UVB and/or visible) [48]. Preliminary work focused on BP derivatives being among main indexed PACD culprits [47] and allowed the detection and identification of ROS and C-radicals in photo-exposed RHE for the very first time [65]. The mechanism by which photoallergen-protein binding triggers the adaptive immune response is thought to involve the photo-mediated formation of reactive C-radicals from the photoallergen. However, the link between these radicals formed in the epidermis and their mechanism of action is far from being unravelled. Photoreactivity appeared to be much more intricate than what has been described so far and requires further in-depth investigations. Pilot studies were also carried out on eugenol and isoeugenol, that are belonging to the list of 26 fragrance ingredients that must be labelled in EU cosmetics and household products because of their well-known skin sensitization potential [70]. The experiments brought to light O- and C-radicals arising from both fragrances in photo-exposed RHE [64]. Such finding was unexpected, as neither allergen has ever been reported to be photo-active, nor have radical mechanisms been described. Real-life photo-exposure scenarios appeared to be sufficient to generate radicals in the skin, highlighting the critical need to identify the chemical mechanisms that induce skin sensitization to chromophoric chemicals. Taken together, the above mentioned EPR studies combined with DFT have made it possible to formulate hypotheses about the possible mechanisms of radical degradation for these families of compounds as shown in Table 2.

A future research challenge is to evaluate whether EPR can be used to assess the sensitizing potential of chemicals. Although this has never been studied before, exploring the combined use of EPR-ST and EPR-SC methodologies would be worthwhile. EPR-ST would enable detailed comparisons to be made of the nature of the formed radicals (C- and/or O-centred) in relation to the probed chemical. EPR-SC would enable the molecules under investigation to be ranked by relatively quantifying the production of ROS in the medium and their ability to consume molecular oxygen in solution. These data could then be used to evaluate whether a common trend can be established for each compound category, by comparing them with data from clinical patch tests in humans.

**Table 2.** Examples of radical mechanisms that can be proposed on the basis of EPR studies

|                                                                                                                                                                                                                                                                                       |                                                                                                                                                                |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <h3>Hydroperoxides/Peroxides</h3> <div><p>Lim-1-OOH</p><p>RO•</p><p><math>\cdot\text{CH}_3</math> <math>\beta</math>-scission</p><p>allylic hydrogen abstraction</p><p>intramolecular cyclization</p></div>                                                                           | <h3>Phenols</h3> <div><p>Spin fully delocalized within the aromatic ring</p><p>Isoeugenol</p><p>PhO•</p><p>Delocalization on <i>para</i>-substituent</p></div> |
| <h3>Benzophenone derivatives<br/>Photoallergens</h3> <div><p>Ketoprofen</p><p>Photoionization <math>h\nu</math></p><p>O<sub>2</sub> <math>\rightarrow</math> O<sub>2</sub><sup>-•</sup> <math>\rightarrow</math> HO• + -CO<sub>2</sub></p><p>Benzyl radical</p><p>Biradical</p></div> |                                                                                                                                                                |

## Conclusion

The biological mechanism of skin sensitization is a rather complex multi-step process [Fig. 1], about which much has been learned but much still remains to be learned. However, the skin sensitizing properties (or lack of them) of chemicals depend solely on physico-chemical characteristics that can usually be measured and can in many cases be estimated from the chemical structure. When chemicals can be assigned to their reaction mechanistic domains, estimates of their potency can often be made based on quantitative mechanistic models developed using historical animal test data.

There are still several questions that cannot be answered definitively regarding the chemistry underlying skin sensitization, including: Which protein nucleophiles are involved in sensitization by electrophilic or pro-electrophilic sensitizers? Does the target protein nucleophile vary according to the reaction mechanism? Do the carrier proteins occur in specific locations in the skin specifically for the purpose of acting as carrier proteins, or can sensitization be produced by covalent modification of any skin protein? What are the ranges and limitations of activation mechanisms for chemicals that are not directly reactive, and to what extent are these activation processes metabolic or abiotic?

Although there is still much to learn about the chemical basis of skin sensitization, the present state of understanding is very useful in several ways. The understanding of the relationship between reactivity and sensitization has enabled *in chemico* assays such as the DPRA [71,72] and the kinetic kDPRA [73] to be developed for non-animal prediction of sensitization hazard. These assays enable prediction of potency to a limited extent and there is scope for improving their predictive performance [74]. *In silico* expert systems such as DEREK and the TIMES-SS module in the OECD toolbox are based on structural alerts derived from the reaction mechanistic understanding of sensitization chemistry. These expert systems are regularly updated to reflect new developments in sensitization chemistry. Quantitative understanding of structure-potency trends for the various reaction mechanistic domains, although incomplete, has enabled dermal sensitization threshold (DST) values to be defined and structure-based rules for assigning chemicals to their DSTs to be developed [75–79]. Based on a combination of structural alerts, quantitative mechanistic models and chemistry-based read-across, a chemical can be assigned to one of three categories of DST, the non-reactive, the reactive and the high potency category. If the chemical is to be used at levels below its DST, then no testing is required.

We hope that this brief review has demonstrated the extent to which skin sensitization is a chemically-driven endpoint, and the degree to which current understanding of the underlying chemistry can be applied.

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