Oximes: Metabolic Activation and Structure−Allergenic Activity Relationships

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Metabolic activation of chemicals (prohaptens) in the skin can cause allergic contact dermatitis. We have explored structure−allergenic activity relationships for seven potential oxime prohaptens using the local lymph node assay and a GSH trapping screen with liver microsomes. The general structure−allergenic activity relationships found were that an α,β-unsaturation is necessary for an oxime to be a prohapten and that increased steric hindrance around this double bond leads to reduction in sensitizing capacity. We also found that sensitizing oximes can be distinguished in vitro from nonsensitizers by monitoring of monooxidized (+16 Da) GSH conjugates in the GSH trapping screen. However, care should be taken when interpreting data from GSH trapping screens, as nonsensitizers may also form GSH conjugates via alternative mechanisms. This investigation emphasizes the importance of considering cutaneous bioactivation in toxicity assessment of chemicals used in contact with the skin.

Introduction

Biotransformation of xenobiotic chemicals, such as drugs, food additives, and cosmetics, can give rise to reactive and toxic intermediates.[1] This phenomenon is known as bioactivation or metabolic activation, and the processes involved are often oxidative, catalyzed by members of the cytochrome P450 (P450[a]) enzyme superfamily.[2] The reactive metabolites formed can interact with cells in numerous ways, including covalent modification of cellular components (e.g., proteins and DNA), which in turn may lead to tissue necrosis, carcinogenicity, and immunotoxicity.[1] Bioactivation in the skin can lead to development of allergic contact dermatitis (ACD), the clinical symptom of contact allergy.[3,4] ACD is the most frequent manifestation of immunotoxicity in humans,[5] and it is estimated that 10–15% of the adult European population is sensitized to one or more chemicals.[6,7] ACD is classified as a T-lymphocyte-mediated delayed-type or type-IV hypersensitivity reaction and is caused by the binding of reactive chemicals (haptens) to carrier macromolecules, such as proteins in the skin.[4] To prevent ACD in a sensitized individual, a life-long avoidance of the allergenic compound is necessary. Thus, it is important to identify contact allergens and to evaluate their sensitizing potency to make proper risk assessments.

The development of assays for prediction of skin sensitizing potential is a task of great importance. Currently, the only reliable methods to determine allergenic activity are based on in vivo tests, one of which is the local lymph node assay (LLNA).[8] The LLNA has been evaluated and accepted by the U.S. Food and Drug Administration (FDA) and is recommended by the Organisation for Economic Co-operation and Development (OECD) as a stand-alone test method for determination of sensitizing capacity. However, there is an urgent need for alternative nonanimal-based methods due to European Union legislation such as REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) and the seventh Amendment to the Cosmetics Directive.[9,10] One of the challenges in the development of alternative assays is the identification and potency assessment of prohaptens, which become transformed to skin sensitizers via bioactivation in the skin.[11–14] By definition, prohaptens may also be activated before skin contact (e.g., by air oxidation[15–17]), though, in the following sections, the term prohapten will refer only to allergens that are activated via skin metabolism. Alternative methods for the prediction of contact allergy that do not include metabolic activation will not identify prohaptens as sensitizers. For the development and evaluation of robust alternative methods, it is important that the mechanisms involved in prohapten activation are investigated and that structure–activity relationships (SARs) for prohaptens are explored. The information obtained from SAR studies can also be used in in silico predictive approaches and in the design of non- or only weakly-sensitizing compounds with minimal loss in desired activity (e.g., biological, technical, or cosmetic).

Currently, the glutathione (GSH) trapping screen with hepatic subcellular fractions is one of the most commonly used in vitro techniques for investigation of potential bioactivation and subsequent formation of reactive metabolites/intermediates. It is used widely in drug discovery[18,19] and has also been used in studies of prohaptens.[13,14] This assay is based on the ability of the GSH thiol group to efficiently react with and trap electrophilic compounds formed primarily by phase I metabolic conversions. The conjugates formed can be identified using LC-MS techniques, where detection of the *m/z* 272 fragment (corresponding to deprotonated γ-glutamyl-dehydroalanyl-glycine) in the negative ionization mode has been shown to be a highly efficient tool to detect GSH conjugates of different structural classes.[20] Additional information regarding the nature and chemical structure of the reactive metabolites trapped can then be gained from subsequent tandem MS analysis. Incubations of contact allergens with GSH alone has also been utilized in assays for the correlation of the electrophilic character with sensitizing capacity.[21–23]

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[a] Abbreviations: ACD, allergic contact dermatitis; P450, cytochrome P450; ESI, electrospray ionization; FDA, Food and Drug Administration; FCAT, Freund's complete adjuvant test; GSH, glutathione; HLM, human liver microsome; LLNA, local lymph node assay; MLM, mouse liver microsome; MW, microwave irradiation; OECD, organisation for economic co-operation and development; SAR, structure−activity relationship; SIM, selected ion monitoring.

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Scheme 1. Proposed Mechanism of Cutaneous Metabolic Activation of *R*-Carvoxime

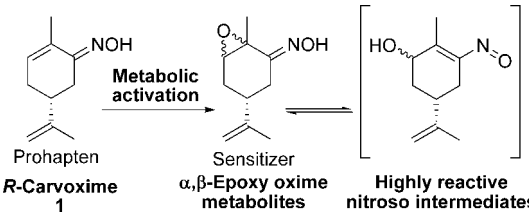
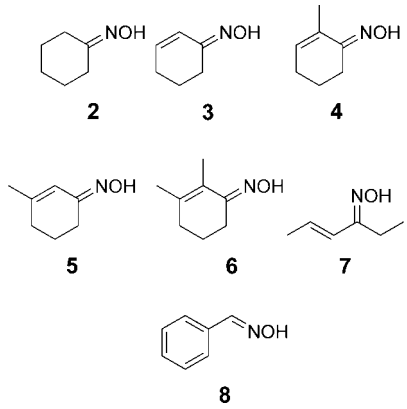


Chart 1. Structures of Oximes Studied for Structure−Allergenic Activity Relationships

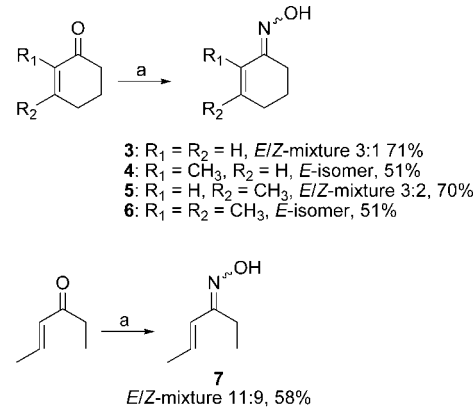


The present study concerns oximes as prohaptens. Oximes have numerous industrial applications, for example, as anti-skinning agents in paint, blocking agents in the polymer industry, and chelators in the metals industry. Certain oximes have also been shown to have anti-inflammatory,[24] antiallergic,[25] antidotal,[26] and antiepileptic[27] properties. In addition, oximes are common metabolic intermediates formed from benzyl- and alkylamines via, for example, P450-mediated oxidation.[28] However, despite the wide use of oximes, their contact allergenic activity has been poorly studied. We have previously shown that the α,β-unsaturated *R*-carvoxime (**1**, Scheme 1), is a prohapten of strong sensitizing potency.[29] Studies performed in guinea pigs revealed that a β-methyl substituted analogue of **1** had retained allergenic activity, whereas a saturated analogue of **1** was inactive.[29] In subsequent mechanistic studies, it was found that **1** is metabolically activated by epoxidation of the α,β-unsaturation (Scheme 1) and that the α,β-epoxy oxime metabolites formed were sensitizers of extreme potency and highly chemically reactive toward a nucleophilic model peptide.[14] The reason for the high reactivity and sensitizing capacity of the α,β-epoxy oxime metabolites was proposed to be due to the formation of allylic nitroso intermediates by tautomerisation (Scheme 1).[14] In the present study, we have explored SARs for oximes and investigated the possibility of correlating in vitro studies of metabolic activation of oximes with their sensitizing potency. For this reason, a series of seven oximes (**2**–**8**, Chart 1) were screened for sensitizing capacity using the LLNA and reactive metabolite-trapping studies were carried out using the GSH trapping screen together with human and mouse liver microsomes (HLMs or MLMs).

Results and Discussion

Design of Oximes Used in SAR Investigation. To investigate structure−allergenic activity relationships for oximes, a series of seven compounds (**2**–**8**) was designed. Similarity within the series in chemical structure, molecular weight, and hydrophobicity was considered important to, as accurately as possible, allow comparison of allergenic activity in relation to structure and to avoid variations in skin penetration capacities. Oximes **2**–**6** contain the same cyclic structure but have a varying substitution pattern at the α- and β-positions, and **2** is a saturated analogue of **3**. To study the influence of conformational flexibility on the sensitizing capacity, compound **7**, an acyclic analogue of **3**, was included in the series. To investigate if an oxime conjugated with an aromatic ring could induce skin sensitization, benzaldoxime (**8**) was also included. α,β-Unsaturated oximes have previously been shown to be poor electrophiles.[29] Therefore, any sensitizing effect observed is expected to be caused by conversion of **2**–**8** into reactive intermediates in the skin.

Scheme 2. Synthesis[a] of oximes **3**–**6** and **7**



[a] Reagents and conditions: $NH_2OH \cdot HCl$, NaOAc, MeOH/water 9:1, MW, 150 °C, 5 min.

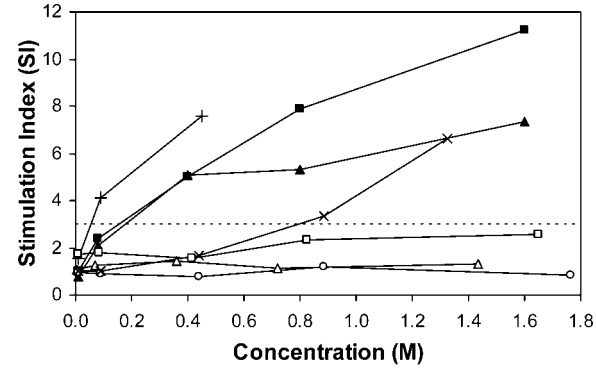


Figure 1. Dose−response curves for oximes **2** (○), **3** (+), **4** (■), **5** (▲), **6** (Δ), **7** (×), and **8** (□) tested in the local lymph node assay (LLNA). The horizontal, dotted line marks a stimulation index (SI) of **3**, the cutoff limit for a compound to be considered a sensitizer.

Synthesis. Oximes **3**–**7** were synthesized using microwave-assisted heating in moderate to good yields (51–71%) from their corresponding ketones and hydroxylamine hydrochloride (Scheme 2). The *E*-isomer was preferentially formed in all cases and exclusively for **4** and **6**, as this minimizes steric interactions between the hydroxyl group of the oxime and the proximal hydrogen or methyl substituent in the α-position. The microwave-assisted heating allowed the preparation of **3**–**7** with reduced reaction times compared to previously reported procedures.[30–33]

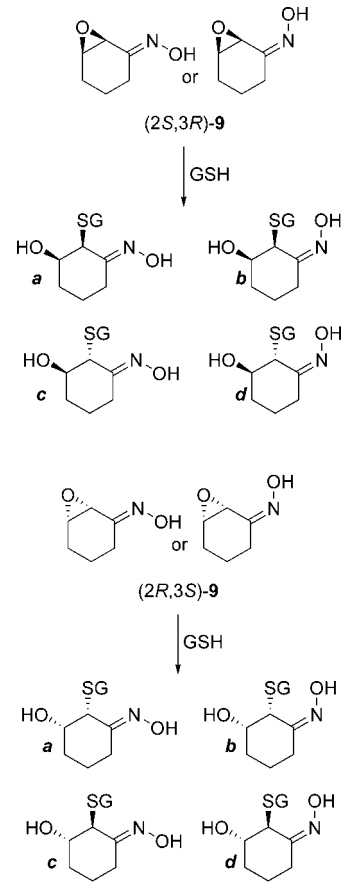
Skin Sensitizing Potency. Currently, the murine LLNA[34] is the method of choice for identification and potency assessment of contact allergens and was used to determine the sensitizing potency of oximes **2**–**8** (Figure 1, Table S1, Supporting Information). Oxime **2** was inactive at the concentrations tested

(0.10–20% w/v) and was classified as a weak sensitizer/nonsensitizing compound. This result is in agreement with a previous study of a saturated analogue of **1**, which was shown to be a nonsensitizer in guinea pigs.[29] The nonsubstituted oxime **3** was found to be a strong sensitizer (EC3: 0.055 M, 0.61% w/v), thus verifying that an α,β-unsaturation is necessary for an oxime to be a sensitizer. The α- and β-methyl substituted oximes **4** and **5**, respectively, were weaker sensitizers compared to **3** (**4**: 0.16 M, 2.0% w/v; **5**: 0.18 M, 2.2% w/v) and were classified as moderate sensitizers. In comparison, the α-methyl substituted oxime **1** has previously been tested in the LLNA and was found to be a strong sensitizer (36 mM, 0.60% w/v).[29]The difference observed in sensitizing capacity between **1** and **4** might be due to a difference in skin penetration, as the only structural disparity between these compounds is the pendant isopropenyl group in the 5-position of **1**. The α,β-dimethyl substituted oxime **6** was found to be a weak sensitizer/nonsensitizing compound as it was inactive at the concentrations tested (0.10–20% w/v). Previous sensitization experiments in guinea pigs showed that a similar α,β-dimethyl substituted α,β-unsaturated oxime, the β-methyl analogue of **1** is a sensitizer, although the number of responding animals were lower compared to a corresponding experiment with **1**.[29] The differences between the LLNA and the FCAT test procedures could account for this discrepancy. In the FCAT,[35] the test compound is administered intradermally together with an immunostimulatory adjuvant (which increases the sensitivity at the point of sensitization), whereas, in the LLNA,[8] the test compound is applied topically. The LLNA results for **3**–**6** show that increased alkyl substitution around the double bond of α,β-unsaturated oximes leads to a reduction in their sensitizing capacity. This phenomenon is probably due to the methyl groups inducing steric hindrance at the structural moiety that is important for the metabolic activation, thus partly or completely preventing the metabolic activation reaction.

The conformationally more flexible and nonsubstituted α,β-unsaturated oxime **7** was notably less sensitizing (EC3: 0.79 M, 9.0% w/v; classification: moderate sensitizer) compared to its cyclic analogue **3**. The importance of considering conformational flexibility together with metabolic activation has been acknowledged for mutagenic chemicals,[36,37] however, it has not yet been suggested for contact allergenic prohaptens. However, a similar reactivity pattern has previously been observed in our laboratory when investigating SARs for potentially prohaptenic alkenes.[13] We found that the monoterpene β-phellandrene is a moderate sensitizer in the LLNA (EC3: 0.41 M), whereas its acyclic 3,4-seco analogue was found to be inactive (EC3 > 1.8 M).[13] The chemical structures of the compounds tested were, in both this and the present study, carefully chosen to avoid variations in skin penetration capacities. Hence, the differences observed in sensitizing capacity for cyclic versus acyclic prohaptens could be due to an inherent dissimilarity in the metabolic activation processes of such compounds. However, the hypothesis that noncyclic prohaptens are less sensitizing compared to their cyclic analogues needs further verification before general SARs can be formulated.

Benzaldoxime (**8**) failed to induce an EC3 value at the concentrations tested (0.10–20% w/v), thus showing that oximes conjugated with an unsubstituted phenyl ring are weak or nonsensitizing compounds. It was not considered necessary to retest **2**, **6**, and **8** in higher concentrations, as the chosen concentration interval allows a distinction between strong, moderate, and weak contact allergens. In addition, false positive results can be obtained for compounds (e.g., irritants) that typically when tested in high concentrations are able to induce a cell proliferation not involving the formation of memory T-cells.[38] This can result in difficulties in distinguishing between weak sensitizers and nonsensitizing compounds.

Scheme 3. Formation of Glutathione (GSH) Conjugates from the Isomers of the α,β-Epoxy Oxime **9**[a]



[a] Enantiomeric pairs of GSH conjugates are indicated as *a*, *b*, *c*, and *d*.

Preparation of GSH Conjugates of α,β-Epoxy Oxime 9. Compound **9** is the proposed α,β-epoxy oxime metabolite of **3** (Scheme 3). GSH conjugates of **9** were prepared to aid the development of an LC-MS method for the analysis of incubation mixtures for GSH conjugates formed from putative α,β-epoxy oxime metabolites of **2**–**7**. Using LC-MS, four major products were found in the reaction between **9** and GSH (Figure 2A). According to mass accuracy analysis and tandem MS, each of these products were found to be GSH conjugates of **9**, as they exhibited a molecular ion at *m*/*z* 435.1550 (±10 ppm) and a fragmentation pattern that support a conjugate formed by addition of GSH to **9** (Figure 3, Scheme 4). A full interpretation of the MS fragmentation pattern is provided in the Supporting Information (Scheme S1). The presence of ions at *m*/*z* 306 and 360 corresponding to loss of glycine and pyroglutamate from the GSH moiety, respectively (Scheme 4), showed that **9** had been trapped by the thiol function of GSH. We have previously shown that the conjugation reaction between GSH and the putative α,β-epoxy oxime metabolites formed from **1** proceeds exclusively via addition of the GSH thiol function to the α-position of the α,β-epoxy oxime.[14] This addition can take place at both faces of the proposed nitroso intermediate formed from the α,β-epoxy oxime (Scheme 1) and may also lead to inversion of the oxime stereochemistry.[14] When these reactivity patterns are applied to the reaction between GSH and **9** (which is a mixture of four diastereomers), a total of eight distinct conjugates may be formed (Scheme 3). As these eight conjugates

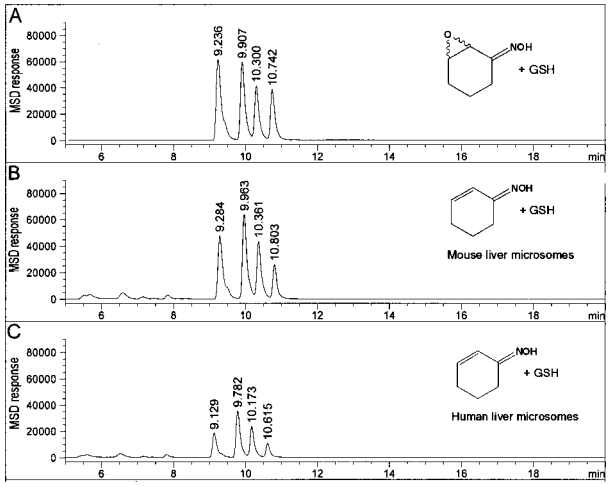


Figure 2. Chromatograms of glutathione (GSH) conjugates formed in reaction mixtures of α,β-epoxy oxime **9** and GSH (A) and in mouse (B) and human (C) liver microsomal incubations of oxime **3**. Analysis was performed in the negative ionization mode and the *m/z* 433 ion was monitored.

consist of four enantiomeric pairs, only four peaks will appear using standard nonchiral chromatographic techniques (Figure 2A). Therefore, GSH conjugates formed from **9** most likely correspond to the GSH conjugates shown in Scheme 3. The involvement of a nitroso intermediate in the reaction between **9** and GSH was indicated by the change in *E*/*Z* ratio of the oxime moiety, from 4:1 for **9** (as determined by ^{1}H NMR, Figure S1, Supporting Information) to approximately 1:1 (Figure 2A, LC-MS data).

GSH Trapping Experiments. Oximes **2**–**8** were incubated with MLMs or HLMs in the presence of GSH to identify reactive metabolites and to investigate the possibility of predicting skin sensitization capacity of oximes using the GSH trapping screen. Even though the metabolic activity of the skin differs from that of the liver, the same overall metabolic processes are considered to occur.[39]The molecular weights of the GSH conjugates formed in the MLM and HLM incubations of oximes **2**–**8** are shown in Table 1. Chromatograms of the conjugates formed in MLM incubations are shown in Figure 4. The results from the MLM and HLM incubations are qualitatively similar; however, greater amounts of metabolites were overall formed from the MLMs as compared to the HLMs (data not shown).

1. Detection of GSH Conjugates Formed from α,β-Epoxy Oxime Metabolites. Analogously to the reaction between GSH and **9**, it was assumed that conjugates formed by trapping of α,β-epoxy oxime metabolites with GSH would have a molecular weight of +16 Da (one oxygen atom) in addition to the combined masses of the parent oxime and GSH (Scheme 5). P450-mediated hydroxylation, which also results in monooxidized metabolites was not considered to be an important bioactivation pathway for the compounds studied. As aliphatic alcohols are nonelectrophilic, the *C*-hydroxylated metabolites are likely equally poorly reactive toward GSH as the parent oxime itself. In addition, aliphatic alcohols are nonallergenic.[40] Four peaks containing ions matching this criterion were identified in the MLM and HLM incubations of **3** (Figure 2B,C). These peaks were all absent in the NADPH-free control. The retention times of the compounds formed matched the retention times of the four GSH conjugates formed in the reaction between **9** and GSH. To examine the presence of GSH-trapped α,β-epoxy oxime **9** in the microsomal incubations, the incubation mixtures were spiked with synthetically prepared conjugates, and the mass spectra of the metabolically formed GSH conjugates were compared with those of the synthetically prepared. Both the spiking and the mass spectra could confirm that **3** is metabolically converted to **9** by both HLMs and MLMs (data not shown). The mass spectra of all four compounds formed in the incubation mixtures contained the same fragments (e.g., *m/z* 417, 288, and 255; Scheme 4) as were found in the mass spectra of the synthetically formed GSH conjugates. As **3** was shown to be a strong sensitizer in the LLNA and α,β-epoxy oximes have previously been shown to be sensitizers of extreme potency,[14] these results indicate that the allergenic activity observed for **3** is a result of its metabolic conversion to **9** in the skin.

Substantial amounts of ions matching the +16 Da criterion were also found in the MLM and HLM incubations containing the α,β-unsaturated oximes **4**, **5**, and **7** (Table 1, Figure 4C,D,F). The MLM incubation mixtures of **4**, **5**, and **7** were therefore reanalyzed using tandem MS for fragmentation pattern comparison with the +16 Da conjugates formed from **3**. All +16 Da conjugates exclusively formed in the NADPH-containing incubations of **4**, **5** and **7** were found to exhibit fragments of the same origin as found for oxime **3**, thus confirming that the compounds detected were GSH-trapped monooxidized metabolites, most likely α,β-epoxy oximes. In addition, compounds **4**, **5**, and **7** were all moderate sensitizers in the LLNA. As oximes **4**, **5**, and **7** are structurally related to oximes **1** and **3**, it is likely that these oximes exert their allergenic activity via metabolic activation to α,β-epoxy oximes. Oxime **6** was inactive in the LLNA, which was proposed to be due to a partial or complete prevention of metabolic epoxidation of the α,β-unsaturated double bond. Hence no, or only small amounts of, +16 Da GSH conjugates were expected to be formed in the liver microsomal incubations of **6**. Indeed, only small amounts of +16 Da conjugates could be detected in the incubations with **6** (Figure 4E), thus confirming this hypothesis. Compounds **2** and **8** were also inactive in the LLNA. As **2** is a saturated oxime, no +16 Da GSH conjugates were expected to be identified in the incubations, unless **2** was activated via an alternative pathway. It is possible that oxime **8** could form a monooxidized reactive metabolite via epoxidation of the aromatic ring, however this was not expected to occur as **8** was inactive in the LLNA. No +16 Da GSH conjugates were identified in the incubations with **2** and **8** (Figure 4A and G), thus confirming that these compounds cannot be bioactivated to form reactive +16 metabolites.

2. Detection of Additional GSH Conjugates Formed in Liver Microsomal Incubations. Despite the fact that a good correlation between sensitizing oximes (**3**, **4**, **5**, and **7**) and the presence of +16 Da conjugates in the GSH trapping screen had been observed, we found it of interest to study the formation of additional conjugates. Monitoring of the *m/z* 272 ion in the negative ion mode has previously been demonstrated as a useful tool for detection of unknown GSH conjugates of different structural classes.[20] Even though the +16 Da conjugates were the predominant GSH conjugates found in the MLM and HLM incubations of oximes **2**–**8**, various additional GSH conjugates could be identified when using SIM detection of the *m/z* 272 ion (Figure 4H−N). The molecular weights of the GSH conjugates identified were obtained from the total ion chromatogram run in scan mode and are indicated in Table 1 and Figure 4H−N. Conjugates corresponding to an addition of GSH to the corresponding ketone (−15 Da) of the oximes were identified in the MLM incubations of **3**–**7** and HLM incubations

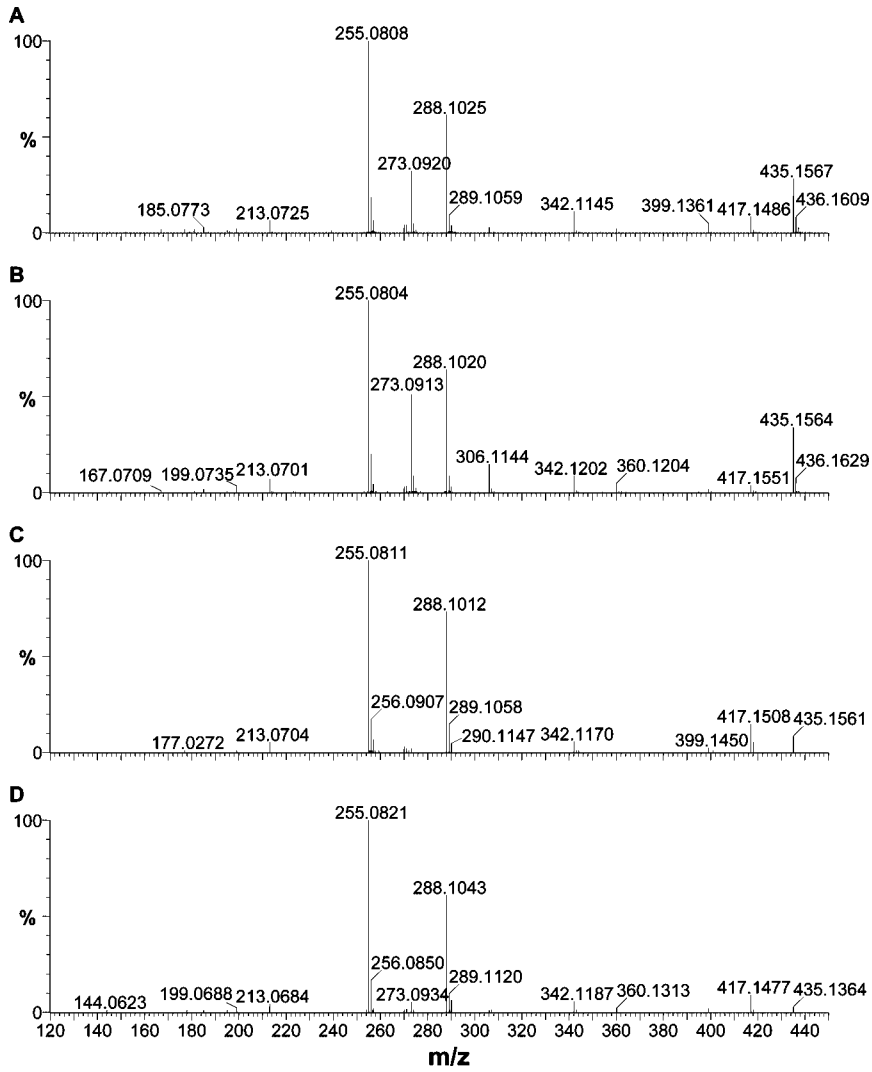


Figure 3. Tandem MS spectra of glutathione conjugates formed from α,β-epoxy oxime **9**: A, retention time 9.2 min; B, 9.9 min; C, 10.3 min; and D, 10.7 min.

of **3**–**5** and **7**. These conjugates were also observed in the NADPH-free controls. Oxime hydrolysis to the corresponding ketone analogues may occur both enzymatically and nonenzymatically and has previously been observed for **2**.[41] α,β-Unsaturated ketones, which can be formed from **3**–**7**, are soft electrophiles and react rapidly and efficiently with soft nucleophiles, such as GSH. Hence, the absence of –15 Da conjugates in the incubations with **2** and **8** may be due to the corresponding ketones of these oximes not being sufficiently reactive toward GSH. α,β-Unsaturated ketones, but not nonconjugated ketones, are also potential skin sensitizers.[29,42] Carvone, the corresponding ketone of **1**, is a considerably weaker sensitizer (EC3: 0.86 M) than both **1** (EC3: 0.036 M) and the α,β-epoxy oxime metabolites formed from **1** (EC3: 0.00088–0.0011 M).[14,29] Thus, it is likely that the main contribution to the activation of α,β-unsaturated oximes occurs via the epoxide rather than the ketone pathway. The impact of the ketone metabolites on the sensitizing potency of oximes is most likely minimal as ketones are usually not sufficiently potent. However, previous studies in guinea pigs sensitized to **1** showed that about 40% (6/15 animals) responded when tested with carvone, indicating that the alternative route exists.[29]

Conjugates corresponding to a loss of 2 Da in molecular weight relative to the weight of the parent oximes and GSH were identified in all incubations. Such conjugates may be formed after dehydration (loss of water, 18 Da) of a +16 Da conjugate. This would explain the appearance of −2 Da conjugates in incubations with oximes **3**–**7**, but not the observation of −2 Da conjugates in incubations with oximes **2** and **8** as these oximes cannot be metabolically converted to α,β-epoxy oximes. However, the formation of −2 Da conjugates from oximes does not seem important for the development of contact allergy to oximes, since **2**, **6**, and **8** were inactive in the LLNA.

3. Inherent Reactivity of Oximes 2–8 Toward GSH. Conjugates corresponding to a direct addition of GSH to the oxime (±0 Da) were identified in the incubations of oximes **3**, **5**, and **7**. Similarly to the −15 Da and some of the +16 Da GSH conjugates, these conjugates were also present in the NADPH-free controls (marked with an asterisk in Figure 4). To investigate their origin, reactions of oximes **2**–**8** and GSH in buffer without liver microsomes were carried out. In general, only minor amounts of GSH conjugates were identified. Oximes are poor electrophiles, but do react with good nucleophiles, such as the thiol function of GSH.[29] Conjugates resulting from a direct reaction between the oxime and GSH were identified in incubations with **3**, **4**, **5**, and **7**. GSH conjugates formed from the corresponding ketone of the oximes were found in incubations with **3** and **7**. Trace amounts of conjugates with a mass corresponding to a mass gain of one oxygen atom (+16 Da) in

Scheme 4. Proposed Structures for Major Tandem MS Fragments of Glutathione Conjugates of α,β-Epoxyoxime **9**

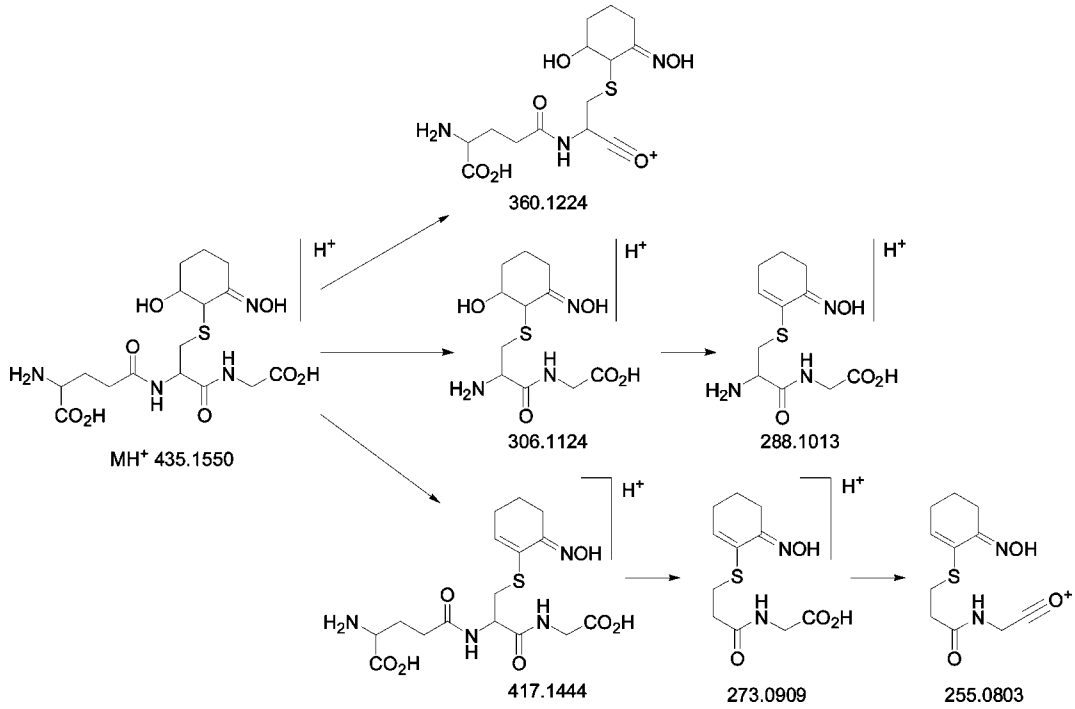


Table 1. LC-MS Analyses of the Liver Microsomal Incubations of Oximes **2**–**8** with Glutathione (GSH); Molecular Weights (Da) of Identified GSH Conjugates

experiments[a]			experiments[a]		
substrate	MLM	HLM	substrate	MLM	HLM
2	434 (+14)	434 (+14)	**6**	462 (+16)	462 (+16)
	418 (−2)	418 (−2)		444 (−2)	444 (−2)
				432 (−15)	
3	434 (+16)	434 (+16)			
	418 (±0)	418 (±0)	**7**	436 (+16)	436 (+16)
	416 (−2)	416 (−2)		420 (±0)	420 (±0)
	403 (−15)	403 (−15)		418 (−2)	418 (−2)
				405 (−15)	405 (−15)
4	448 (+16)	448 (+16)			
	430 (−2)	430 (−2)	**8**	442 (+14)	426 (−2)
	417 (−15)	417 (−15)		426 (−2)	
5	448 (+16)	448 (+16)			
	432 (±0)	432 (±0)			
	430 (−2)	430 (−2)			
	417 (−15)	417 (−15)			

[a] The numbers given within parentheses denote the change in molecular weight relative to the weight of the parent oximes and GSH. Conjugates formed in the reaction between an α,β-epoxy oxime metabolite and GSH are assumed to have gained 16 Da. Conjugates formed from a ketone metabolite (formed via hydrolysis of the corresponding oxime) are assumed to have lost 15 Da. MLM = mouse liver microsomes, HLM = human liver microsomes.

addition to the combined masses of the oxime and GSH were detected in incubations with **4** and **7**. These conjugates probably result from autoxidation of the sulfide moiety to form the corresponding sulfoxide of the adducts formed in a direct reaction between the oxime and GSH (±0 Da), as previously has been shown for **1**.[29] Taken together, these experiments show that α,β-unsaturated oximes have an inherent capacity to form conjugates with GSH without metabolic activation. However, the importance of the inherent electrophilicity of α,β-unsaturated oximes for their allergenic activity is likely to be minimal as the chemical reactivity of these oximes toward biological nucleophiles is very low in comparison with that of contact allergens of equal or lower sensitizing capacity. As mentioned above, in comparison to **1**, the α,β-unsaturated ketone carvone is considerably more reactive toward sulfur nucleophiles, but is a >20-fold weaker sensitizer in the LLNA compared to **1**.[29,42] Furthermore, the formation of +16 Da conjugates via *S*-oxidation of ±0 Da conjugates does not appear to be important in the assessment of skin sensitizing oximes using the GSH trapping screen, as all the sensitizing oximes studied (**3**, **4**, **5**, and **7**) afforded significant amounts of unique +16 Da conjugates in the NADPH-containing incubations only.

In summary, we have explored structure−allergenic activity relationships for oximes and investigated the possibility of correlating in vitro studies of metabolic activation of oximes with their sensitizing potency. The general structure-allergenic activity relationships were that an α,β-unsaturation is necessary for an oxime to be a prohapten and that increased steric hindrance around this double bond leads to reduction in sensitizing capacity. In addition, we have shown that an aromatic aldoxime was a weak/nonsensitizing compound and that an acyclic α,β-unsaturated oxime was significantly less sensitizing compared to its cyclic analogue. We recommend that these SARs are applied in the design of novel commercially used chemicals and that they should be incorporated in in silico predictive databases. We also found that sensitizing oximes can be distinguished in vitro from nonsensitizers by monitoring of the formation of monooxidized (+16 Da) GSH conjugates in the GSH trapping screen. However, care should be taken when interpreting data from the GSH trapping screen as nonsensitizers may also form significant amounts of GSH conjugates via alternative mechanisms. Hence, this investigation highlights the significance of a solid mechanistic understanding of prohapten activation in the development of novel nonanimal-based assays for prediction of contact allergenic activity.

Experimental Section

Caution: *Skin contact with* **3**–**5**, **7**, *and* **9** *must be avoided. These compounds are skin-sensitizers (***3**–**5** *and* **7***) or probable skin-sensitizers (***9***) and must therefore be handled with care.*

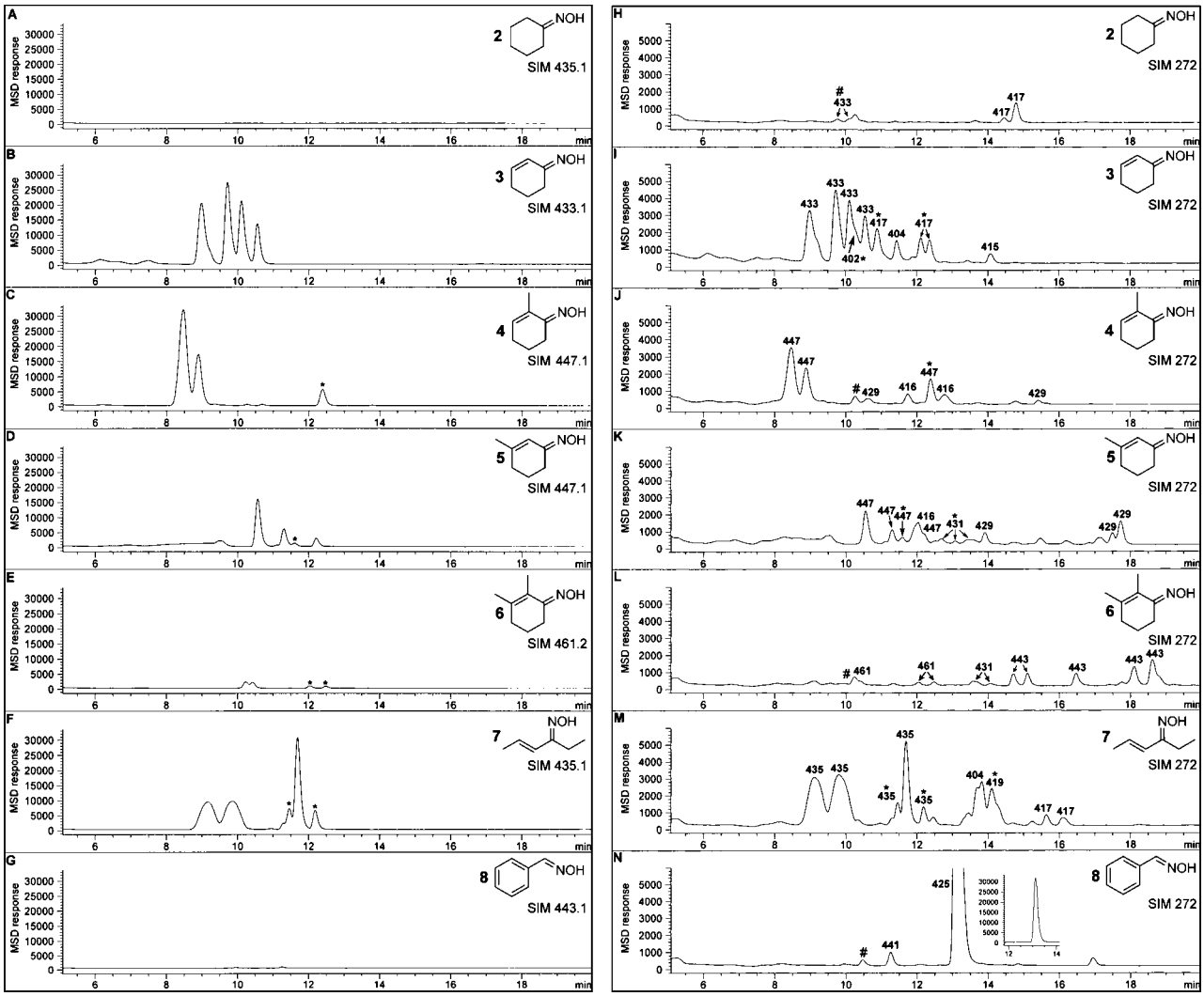
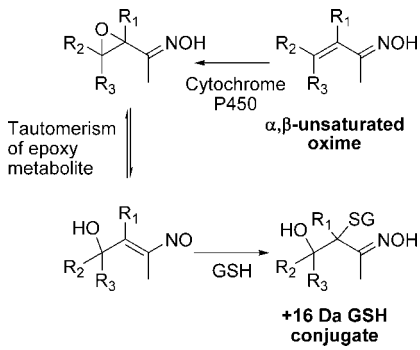


Figure 4. Chromatograms of glutathione (GSH) conjugates formed in mouse liver microsomal incubations of oximes **2** (A and H), **3** (B and I), **4** (C and J), **5** (D and K), **6** (E and L), **7** (F and M), and **8** (G and N). Analysis was performed in the negative ionization mode, and the following ions were monitored: *m*/*z* 435.1 (A and F), 433.1 (B), 447.1 (C and D), 461.2 (E), 443.1 (G), and 272 (H−N). In chromatograms H−N, the mass of identified molecular ions corresponding to the GSH conjugates detected are indicated. Peaks marked with an asterisk (*) were detected also in the NADPH-free controls. Peaks marked with a hash (#) correspond to an impurity in the GSH buffer.

Scheme 5. Formation of +16 Da Glutathione (GSH) Conjugates via Metabolic Conversion of α,β-Unsaturated Oximes[a]



[a] R_1, R_2, and R_3 = alkyl or H.

Chemicals and Biochemicals. Unless otherwise indicated, reagents were obtained from commercial suppliers and used without further purification. Cyclohexanone oxime (**2**) and *syn*-benzaldoxime (**8**) were purchased from Fluka. 2-Methylcyclohex-2-enone, 2,3-dimethylcyclohex-2-enone and 2,3-epoxycyclohexanone oxime (**9**) were synthesized as previously described.[43,44] The reactions were monitored using thin layer chromatography on silica-plated aluminum sheets (Silica gel 60 F254) developed using anisaldehyde dip (2.1 mL of acetic acid, 5.1 mL of anisaldehyde, and 7 mL of H_2SO_4 in 186 mL of ethanol) or permanganate dip (1.0 g $KMnO_4$, 6.7 g K_2CO_3, and 1.7 mL of 5% aqueous NaOH in 100 mL of water) followed by heating. Column chromatography was performed on wet packed silica (Silica gel 60, 230–400 mesh). The purity of synthesized and purchased test compounds was determined to be ≥98% (GC/MS) before testing. Pooled HLMs from male and female donors, female CD-1 MLMs, and an NADPH-regenerating system were purchased from BD Biosciences (Woburn, MA).

Instrumentation. The microwave-assisted reactions were carried out using a Biotage Initiator instrument. Purity analyses were performed on a Hewlett-Packard gas chromatograph (model 6890) connected to a mass spectrometer (Hewlett-Packard model 5973). The GC was equipped with a HP-1MS fused silica capillary column (30 m × 0.25 mm, 0.25 μm, Agilent Technologies, Palo Alto, CA). Helium was used as carrier gas and the flow rate was 1.2 mL/min. The temperature program started at 35 °C for 1 min, increased by 10 °C/min, and ended at 250 °C for 5 min. For mass spectral analysis, the mass spectrometer was used in the scan mode detecting ions with *m*/*z* values ranging from 50 to 500.

LC-MS analyses were performed using atmospheric pressure electrospray ionization (ESI) on a Hewlett-Packard 1100 HPLC-MS. The system included a vacuum degasser, a gradient pump, an

autoinjector, a column thermostat, a diode array detector, and a single quadropole mass spectrometer. The electrospray interface was used with the following spray chamber settings: nebulizer pressure, 40 psig; capillary voltage, 3.5 kV; drying gas temperature, 350 °C; and drying gas flow rate, 10 L/min.

Determination of accurate mass was performed using ESI on a Micromass Q-Tof 2 instrument (a hybrid quadropole-time-of-flight mass spectrometer). The mass spectrometer was connected to a Hewlett-Packard 1100 HPLC system, which included a vacuum degasser, a gradient pump, a column thermostat, and a HTS PAL autoinjector. The following mass spectrometric source conditions were used: capillary voltage, 3.1 kV; cone voltage, 35 V; MCP, 2350 V; TOF, 9.10 kV; source temperature, 120 °C; and desolvation temperature, 320 °C. Leucine enkephalin at *m/z* 556.2771 was used as lock mass and the instrument was calibrated with sodium formate. A measured mass with a ≤10 ppm deviation from the theoretical mass was considered accurate.

LC-MS Methods for Analysis of GSH Conjugates. The microsomal incubations, the reactivity experiments, and the mixture of **9** and GSH were analyzed for GSH conjugates in the negative ionization mode. For each sample, 20 μL (reactivity experiments), 10 μL (liver microsomal incubations), or 0.5 μL (GSH conjugates of **9**) were injected onto a HyPURITY 3 μm C18 150 mm × 3.0 mm column (Thermo Electron Corp., Bellafonte, PA), and the analytes were eluted with a gradient flow at 0.4 mL/min. The flow was diverted to waste for the first 5 min of each run. Mobile phase A consisted of 0.005% pentafluoropropanoic acid, 0.1% formic acid, and 3% acetonitrile in water and mobile phase B of 0.005% pentafluoropropanoic acid, 0.1% formic acid, and 50% acetonitrile in water. A linear gradient of 0–50% B in A in 20 min was followed by a second gradient of 50–100% B in 1 min and ended with 4 min of isocratic elution at 100% B. The column was equilibrated with 100% A for 10 min between each run. The detector was used in the scan mode (% cycle time 40, fragmentor voltage 70 eV), detecting ions at *m/z* 100–500, and in the SIM mode, detecting the deprotonated γ-glutamyl-dehydroalanyl-glycine ion at *m/z* 272 (% cycle time, 30; fragmentor voltage, 120 eV) and the deprotonated molecular ion of a GSH-trapped α,β-epoxy oxime metabolite (% cycle time 30, fragmentor voltage 70 eV, *m/z* of $[M_{\text{GSH conjugate of epoxy oxime}} - H]^-$ of oximes **2–8**: 435.1 (**2** and **7**), 433.1 (**3**), 447.1 (**4** and **5**), 461.2 (**6**), and 443.1 (**8**)).

Accurate mass analysis in the positive ionization mode was performed on the mixture of **9** and GSH (ca. 5 μM) and selected samples from the liver microsomal incubations (diluted 1:100). For each sample, 20 μL were injected onto an ACE 3 μm C18 100 mm × 2.1 mm column (Advanced Chromatograpy Technologies, Aberdeen, U.K.). Mobile phase A consisted of 0.1% formic acid in water and mobile phase B of 100% acetonitrile. Each run was 25 min using a gradient flow of 0.3 mL/min. The analytes were initially eluted isocratically with 5% B in A for 5 min, followed by a gradient of 5–30% B in 15 min and a second gradient of 30–95% B in 1 min. After this, the flow was held at 95% B in A for 2 min, whereafter it returned to 5% B in 0.1 min, where it stayed for 1.9 min. The flow was split post-column to lead 0.12 mL/min into the mass detector. MS spectra were acquired in the positive ionization mode using a scan range between *m/z* 100–700 Da or using the following tandem MS settings: set mass, *m/z* 435; scan range, *m/z* 100–600; collision energy, 15 eV.

General Procedure for Preparation of Oximes Using Microwave-Assisted Heating. To a solution of ketone (ca. 1 M) in methanol/water 9:1 in a 10–20 mL microwave reaction vial were added hydroxylamine hydrochloride (1.1 equiv) and sodium acetate (1.2 equiv). The vial was sealed and the mixture was heated in a microwave cavity at 150 °C for 5 min and was then allowed to cool to room temperature. The mixture was filtered, diluted with water (80 mL), and extracted with chloroform (3 × 50 mL). The organic phases were washed with saturated aqueous sodium bicarbonate (2 × 50 mL), water (50 mL), and brine (50 mL), dried over magnesium sulfate, and concentrated under reduced pressure.

Cyclohex-2-enone Oxime (3). Cyclohex-2-enone (1.54 g, 16 mmol) was reacted as described in the general procedure. The crude product was purified by column chromatography on silica gel (25% ethyl acetate in hexanes) affording a 3:1 *E*/*Z* mixture of **3** as a colorless solid (1.27 g, 71%). NMR spectral data were consistent with literature data for **3**.[30]

(*E*)-2-Methylcyclohex-2-enone Oxime (4). 2-Methylcyclohex-2-enone (1.76 g, 16 mmol) was reacted as described in the general procedure. The crude product was purified by recrystallization from hexanes affording **4** as colorless needles (1.01 g, 51%). NMR spectral data were consistent with literature data for **4**.[31]

3-Methylcyclohex-2-enone Oxime (5). 3-Methylcyclohex-2-enone (1.76 g, 16 mmol) was reacted as described in the general procedure. The crude product was purified by column chromatography on silica gel (40% ethyl acetate in hexanes) affording a 6:4 *E*/*Z* mixture of **5** as a colorless solid (1.75 g, 70%). NMR spectral data were consistent with literature data for **5**.[32]

(*E*)-2,3-Dimethylcyclohex-2-enone Oxime (6). 2,3-Dimethylcyclohex-2-enone (1.50 g, 12 mmol) was reacted as described in the general procedure. The crude product was purified by recrystallization from hexanes affording **6** as a pale yellow solid (0.86 g, 51%). NMR spectral data were consistent with literature data for **6**.[31]

Hex-4-en-3-one Oxime (7). Hex-4-en-3-one (1.50 g, 15.3 mmol) was reacted as described in the general procedure. The crude product was purified by column chromatography on silica gel (15% ethyl acetate in hexanes), affording a 11:9 *E*/*Z* mixture of **7** as a colorless oil (1.01 g, 58%). NMR spectral data were consistent with literature data for **7**.[33]

Incubation of Oximes 2–8 with GSH and Liver Microsomes. Each microsomal incubation contained HLMs or MLMs (1 mg/mL of protein), substrate (200 μM), GSH (5.0 mM), 100 mM potassium phosphate buffer (pH 7.4), and an NADPH-regenerating system (1.3 mM $NADP^+$, 3.3 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase, and 3.3 mM magnesium chloride) in a total volume of 250 μL. The incubations were performed in duplicate and initialized by addition of the NADPH-regenerating system after 3 min of preincubation at 37 °C. Control experiments were run in the absence of the NADPH-regenerating system. After 2 h, the incubation mixtures were terminated by addition of acetonitrile (250 μL) and centrifuged at 3000 rpm and 4 °C for 10 min. The supernatants (450 μL) were concentrated to dryness with a stream of nitrogen. The samples were reconstituted with 1% acetonitrile in water (100 μL), mixed for 10 min at 300 rpm and analyzed using the LC-MS analysis method described above. Incubations run with MLMs, which contained ions with a mass corresponding to one or more +16 Da GSH conjugates, were reanalyzed for accurate mass.

Reaction of α,β-Epoxy Oxime 9 with GSH. Compound **9** (2.5 mg, 0.02 mmol) and GSH (6 mg, 0.02 mM) were added to potassium phosphate buffer (1 mL, 100 mM, pH 7.4). The mixture was shaken for 5 min and was subsequently diluted with buffer (3 equiv) and analyzed using LC-MS as described above. A chromatogram of the conjugates formed is shown in Figure 2A. The exact masses of the conjugates formed (singly protonated molecular ions, MH^+) as determined by Q-Tof analysis were 435.1568 (ret. time 9.2 min), 435.1565 (9.9 min), 435.1561 (10.3 min), and 435.1574 (10.7 min), which corresponds to mass deviations of <10 ppm from the calculated mass (435.1550 Da). To confirm the presence of these conjugates in liver microsomal incubations of **3**, the major GSH conjugates formed from **9** were separated and fractions containing the individual conjugates were collected. The same LC method as described above was used, but with the following modification: a linear gradient of 0–25% eluent B in A in 20 min was followed by a second gradient of 25–100% of B in A for 1 min and ended with 4 min of isocratic elution at 100% B. For each run, 10 μL of a 20 mM solution of GSH conjugates of **9** was injected. The combined fractions were lyophilized to dryness.

Reaction of Oximes 2–8 with GSH. A mixture of oxime (200 μM) and GSH (5 mM) in potassium phosphate buffer (100 mM, pH 7.4) was gently shaken in a water bath at 37 °C for 2 h. These mixtures were analyzed using the LC-MS analysis method described above.

Sensitization Experiments using the Local Lymph Node Assay (LLNA).[34] All animal procedures were approved by the local ethics committee. The procedures were performed according to the OECD guidelines (Guideline No. 429; Skin Sensitization: Local Lymph Node Assay). Female CBA/Ca mice, 8 weeks of age, were purchased from Harlan (Horst, Netherlands) or Scanbur (Sollentuna, Sweden). The assay was performed using the test concentrations shown in Table 1. Briefly, groups of female CBA/Ca mice ($n = 3$ or 4) received 25 μL of the test compound dissolved in vehicle (acetone/olive oil 4:1) on the dorsum of the ears daily for three consecutive days. Control animals were treated in the same way with vehicle alone. All mice were injected intravenously five days after the first treatment, with 250 μL of phosphate-buffered saline containing 20 μCi of [^{3}H]thymidine. Five hours later, draining auricular lymph nodes were excised and pooled for each group, and a single cell suspension of lymph node cells was prepared. The thymidine incorporation was measured by β-scintillation counting. Results are expressed as mean dpm/lymph node for each experimental group and as stimulation index (SI), that is, test group/control group ratio. Test materials that at one or more concentrations caused an SI greater than 3 were considered to be positive in the LLNA. EC3 values (the estimated concentration required to induce an SI of 3) were calculated by linear interpolation.[45] The sensitizing potency of the test compounds was classified according to the following: <0.1, extreme; ≥0.1–<1, strong; ≥1–<10, moderate; ≥10–<100, weak.[46,47]

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Supporting Information Available: Full data of the LLNA responses of compounds **2**–**8**, a ^{1}H NMR spectrum of oxime **9**, and a full interpretation of the MS fragmentation pattern of GSH conjugates of **9**. This material is available free of charge via the Internet at http://pubs.acs.org.

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