

Reduced Sensitizing Capacity of Epoxy Resin Systems: A Structure–Activity Relationship Study

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Epoxy resins can be prepared from numerous chemical compositions. Until recently, alternatives to epoxy resins based on diglycidyl ethers of bisphenol A (DGEBA) or bisphenol F (DGEBF) monomers have not received commercial interest, but are presently doing so, as epoxy resins with various properties are desired. Epoxy resin systems are known to cause allergic contact dermatitis because of contents of uncured monomers, reactive diluents, and hardeners. Reactive diluents, for example, glycidyl ethers, which also contain epoxide moieties, are added to reduce viscosity and improve polymerization. We have investigated the contact allergenic properties of a series of six analogues to phenyl glycidyl ether (PGE), all with similar basic structures but with varying carbon chain lengths and degrees of saturation. The chemical reactivity of the compounds in the test series toward the hexapeptide H-Pro-His-Cys-Lys-Arg-Met-OH was investigated. All epoxides were shown to bind covalently to both cysteine and proline residues. The percent depletion of nonreacted peptide was also studied resulting in 88% depletion when using PGE and 46% when using butyl glycidyl ether (**5**) at the same time point, thus revealing a large difference between the fastest and the slowest reacting epoxide. The skin sensitization potencies of the epoxides using the murine local lymph node assay (LLNA) were evaluated in relation to the observed physicochemical and reactivity properties. To enable determination of statistical significance between structurally closely related compounds, a nonpooled LLNA was performed. It was found that the compounds investigated ranged from strong to weak sensitizers, congruent with the reactivity data, indicating that even small changes in chemical structure result in significant differences in sensitizing capacity.

Introduction

Allergic contact dermatitis (ACD¹) is a common occupational health issue which causes suffering for the individual as well as a considerable economic loss for the society (*1*). Clinical experience and experimental studies have shown that there is a correlation between the reactivity of a compound and its ability to cause contact allergy. As the compounds (haptens) themselves are too small to induce an adaptive immune response, they must be able to react with macromolecules (proteins) in the skin to form immunogenic complexes (*2*). The details of this process are largely unknown even though extensive research has been carried out. The formation of covalent bonds between electrophilic haptens and nucleophilic sites of skin proteins, forming immunogenic complexes, is a plausible mechanism for the sensitizing effect seen for several clinically relevant chemicals.

Epoxy resins are among the most common causes of occupational contact allergy (*3*). The first cases of epoxy

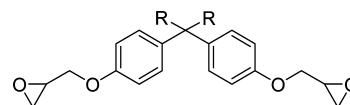


Figure 1. Chemical structures of DGEBA (R = Me) and DGEBF (R = H).

dermatitis were observed in the 1950s; shortly after that, epoxy resins were introduced on the market (*4*). They were then classified as extreme sensitizers in tests on volunteers (*5*), and their usage became strictly regulated to prevent outbreaks of ACD. In spite of this, frequencies of contact allergy to epoxy resins within occupations with increased exposure continue to be high, e.g., 18.2% in tile setters and terrazzo workers, 9.7% in construction and cement workers, 10.2% in workers in the electronics industry, and 8.2% in painters, according to the literature (*3*).

In 2001, about 1,100,000 tons of epoxy resins were sold worldwide (*4*). Of the epoxy resins used, 75–95% are polymerization products of the diglycidyl ether of bisphenol A (DGEBA), while 1% is based on the diglycidyl ether of bisphenol F (DGEBF) (Figure 1). DGEBA and DGEBF cross-react, and the monomers are considered to be the major allergens in epoxy resins (*6*). Epoxy resins are normally used in so-called epoxy resin systems (ERS). These commercial products are combinations of epoxy resins, curing agents, modifiers, and reactive diluents. Cured resins may contain up to 25% of monomers together with reactive diluents (glycidyl ethers),

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¹ Abbreviations: ACD, allergic contact dermatitis; AGE, allyl glycidyl ether; BGE, butyl glycidyl ether; DMSO, dimethyl sulfoxide; DGEBA, diglycidyl ether of bisphenol A; DGEBF, diglycidyl ether of bisphenol F; EC3, estimated concentration required to produce a stimulation index of 3; ERS, epoxy resin systems; GPMT, guinea pig maximization test; LLNA, local lymph node assay; *m*-CPBA, *meta*-chloroperbenzoic acid; PGE, phenyl glycidyl ether; PHCKRM, H-Pro-His-Cys-Lys-Arg-Met-OH; SAR, structure–activity relationship; SI, stimulation index.

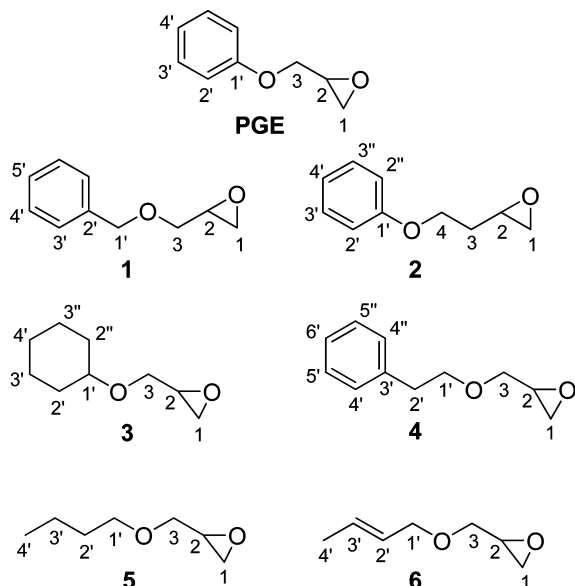


Figure 2. Chemical structures of the compounds studied.

which might also contain epoxy groups. The reactive diluents are added to reduce viscosity and improve polymerization (7). ERS are used in applications where strong, flexible, and lightweight construction materials are required. Also, ERS components other than the epoxy resin monomers have been shown to be potent sensitizers (8). Work with ERS mainly causes hand eczema but also airborne dermatitis due to contents of reactive diluents and hardeners as well as uncured monomers carried by grinding dust. The investigation of a series of epoxy products revealed that approximately 50% contain varying (0.1–20%) amounts of reactive diluents (9), the most common being aromatic glycidyl ethers such as phenyl glycidyl ether (PGE) and aliphatic glycidyl ethers, e.g., butyl glycidyl ether (BGE, **5**) (Figure 2) and allyl glycidyl ether (AGE) (**7**, **10**). PGE was found to be an extreme sensitizer in the guinea pig maximization test (GPMT) since all of the exposed animals in the experiment were sensitized. In addition, cross-reactivity reactions were observed in the animals sensitized to DGEBA or DGEBF, when challenged with PGE (**11**). Furthermore, in a study performed in 2001 (**12**), most patients sensitized to reactive diluents were allergic to PGE.

To prevent ACD, any exposure to the allergenic compounds must be avoided. In order to make proper risk assessment, the availability of reliable methods for the identification of contact allergens and the evaluation of their sensitizing capacity are crucial. Skin sensitization potency related to physicochemical parameters of a hapten can be understood using structure–activity relationship (SAR) data. As the extreme skin sensitizer PGE is a commonly used reactive diluent in ERS, and the structure of DGEBF consists of two identical PGE molecules, we found it adequate to use PGE as a starting point in the present investigation of contact allergy to ERS. In order to analyze how the chemical reactivity and sensitizing capacity depend on the structure of contact allergenic epoxides, a series of structural analogues to PGE (**1–6**) (Figure 2) were synthesized. Their reactivity was evaluated through experiments with a model peptide, and data from both the depletion of nonreacted peptide and the formed reaction products were analyzed. The sensitizing potency of the epoxides within the series was determined using the murine local lymph node assay (LLNA). To investigate if there was a statistically significant difference in sensitizing

potency between the structurally closely related compounds, a modified LLNA with nonpooled lymph nodes was performed (**13**).

To the best of our knowledge, no studies of the allergenic potency of these modified epoxy products have been published to date. We strongly believe that an improved knowledge of their overall properties will provide the basis of future investigations toward the prevention of epoxy resin allergy.

Experimental Procedures

Caution: This study involves skin sensitizing compounds which must be handled with care.

Instrumentation and Mode of Analysis. ^1H and ^{13}C NMR spectroscopy was performed on a Jeol Eclipse 400 spectrometer at 400 and 100 MHz, respectively, using CDCl_3 solutions (residual CHCl_3 δ 7.26 and CDCl_3 δ 77.0 as internal standards). Electron-ionization mass spectral analysis (70 eV) was performed on a Hewlett-Packard 5973 mass spectrometer connected to a gas chromatograph (Hewlett-Packard 6890). The GC was equipped with a cool on-column capillary inlet and an HP-5MSi fused silica capillary column (30 m $\times$ 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 $^\circ\text{C}$ for 1 min, increased by 10 $^\circ\text{C}/\text{min}$, and ended at 250 $^\circ\text{C}$ 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 1500.

LC/MS analyses were performed using electrospray ionization (EIS) on a Hewlett-Packard 1100 HPLC/MS. The system included a vacuum degasser, a binary pump, an autoinjector, a column thermostat, a diode array detector, and a single quadrupole mass spectrometer. The HPLC was equipped with a HyPURITY C18 column (150 $\times$ 3 mm i.d., particle size 3 μm , Thermo Hypersil-Keystone, Thermo Electron Corp., Bellafonte, PA). The mobile phase consisted of 0.005% pentafluoropropanoic acid, 0.1% acetic acid, and 5% acetonitrile in water (solvent A) and 0.005% pentafluoropropanoic acid, 0.1% acetic acid, and 50% water in acetonitrile (solvent B). A linear gradient from 0% to 100% B in 20 min, followed by 10 min of isocratic elution was used. The flow rate was 0.40 mL/min, and the column temperature was set to 40 $^\circ\text{C}$. The electrospray interface was used with the following spray chamber settings: nebulizer pressure, 40 psig; capillary voltage, 3500 V; drying gas temperature, 350 $^\circ\text{C}$; and drying gas flow rate, 10 L/min. Fragmentor voltage was set to 120 V to produce molecular fragments. The mass spectrometer was used in scan mode detecting molecular ions with m/z values ranging from 50 to 1600. Column chromatography was performed on Merck silica gel 60 (230–400 mesh ASTM); in one purification activated neutral aluminum oxide, Brockmann scale 4, (150 mesh) was used. Short path distillation was performed using a Büchi GKR-50 Kugelrohr distiller.

Chemistry. Phenyl glycidyl ether (PGE), benzyl glycidyl ether (**1**), and butyl glycidyl ether (BGE) (**5**) were purchased from Aldrich Chemicals (Stockholm, Sweden). H-Pro-His-Cys-Lys-Arg-Met-OH (98.5%) was purchased from Bachem (Bubendorf, Switzerland). Acetone was purchased from Merck (Darmstadt, Germany) and olive oil from Apoteket AB (Göteborg, Sweden). Unless otherwise indicated, reagents were obtained from commercial suppliers and used without further purification. TLC was performed using silica gel coated aluminum plates developed with 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) followed by heating. The purity of both synthesized and purchased test compounds was >98% (GC/MS) before testing of sensitizing capacity.

2-(2-Phenoxyethyl)oxirane (2). This two-step reaction started with the synthesis of 1-phenoxy-3-butene (**2a**) from phenol and 1-bromo-3-butene as previously described (**14**). 3-Chloroperbenzoic acid (*m*-CPBA) (77% w/w) (1.05 g, 4.27 mmol) was added to a solution of 1-phenoxy-3-butene (0.67 g, 4.53 mmol) in chloroform (25 mL) at 0 $^\circ\text{C}$. The reaction was kept stirring at 0 $^\circ\text{C}$ for 2 h,

then at room temperature for another 12 h. The crude product was taken up in dichloromethane (40 mL) and washed with aqueous sodium hydroxide (2.5 M) (4 × 25 mL). The organic layer was dried (Na₂SO₄), filtered, and the solvent was evaporated. Purification by column chromatography (silica gel, 5% ether/pentane) gave **2** (0.62 g, 90%) as an oil. ¹H NMR δ 1.90–1.98 (1H, m), 2.06–2.14 (1H, m), 2.58 (1H, dd, *J* = 4.9, 2.7 Hz), 2.82 (1H, app t), 3.13–3.17 (1H, m), 4.07–4.16 (2H, m), 6.90–6.97 (3H, m), 7.25–7.30 (2H, m). ¹³C NMR δ 32.5 (C3), 47.1 (C1), 49.7 (C2), 64.5 (C4), 114.5 (C2', C2''), 120.8 (C4'), 129.5 (C3', C3''), 158.7 (C1'). EI-MS (70 eV), *m/z* (%) 164 (62) (M⁺), 146 (12), 133 (100), 105 (61), 91 (10), 77 (39), 51 (14).

Cyclohexyl Glycidyl Ether (3). Compound **3** was prepared from cyclohexanol and epichlorohydrin. A two-necked round-bottom flask equipped with a water condenser, addition funnel, magnetic stirrer, rubber septum, and an argon inlet was charged with cyclohexanol (0.05 mol, 5.08 g) and diluted with ether (13 mL). BF₃OEt₂ (6 mmol, 0.79 mL) was added during stirring, and the reaction mixture was heated to reflux under argon, whereafter epichlorohydrin (0.05 mol, 3.9 mL) was added dropwise (0.22 mL/min). After 6 h of reflux, the solvent was distilled off and the reaction mixture cooled to room temperature. Aqueous sodium hydroxide (50%) (50 mL) was added dropwise via the addition funnel during 30 min. After 3 h, the reaction was terminated by neutralization with aqueous HCl (10%), and the organic phase was washed with brine. The organic phase was separated and dried (Na₂SO₄). Column chromatography (silica gel, 10% ether in pentane) gave **3** in 50% yield. ¹H NMR δ 1.13–1.30 (5H, m), 1.47–1.59 (1H, m), 1.67–1.77 (2H, m), 1.87–1.95 (2H, m), 2.60 (1H, dd, *J* = 5.1, 2.7 Hz), 2.80 (1H, dd, *J* = 5.0, 4.2 Hz), 3.10–3.15 (1H, m), 3.26–3.32 (1H, m), 3.45 (1H, dd, *J* = 11.4, 5.6 Hz), 3.69 (1H, dd, *J* = 11.4, 3.4 Hz). ¹³C NMR δ 24.2 (C3', C3''), 25.8 (C4'), 32.2 (C2', C2''), 44.8 (C1), 51.3 (C2), 68.7 (C3), 78.2 (C1'). EI-MS (70 eV), *m/z* (%) 156 (2) (M⁺), 127 (16), 113 (88), 99 (83), 67 (33), 57 (100).

Phenethyl Glycidyl Ether (4). Compound **4** was prepared from 2-phenylethanol and epichlorohydrin. The synthesis was performed as previously described (15). The crude product was purified by column chromatography (deactivated neutral aluminum oxide and 10% ethyl acetate/hexane), which gave **4** in 51% yield. ¹H NMR δ 2.59 (1H, dd, *J* = 5.0, 2.7 Hz), 2.78 (1H, dd, *J* = 5.0, 4.2 Hz), 2.94 (2H, t, *J* = 7.32 Hz), 3.12–3.15 (1H, m), 3.40 (1H, dd, *J* = 11.6, 5.8 Hz), 3.69–3.79 (3H, m), 7.24–7.32 (5H, m). ¹³C NMR δ 36.4 (C2'), 44.4 (C1), 50.9 (C2), 71.6 (C3), 72.5 (C1'), 126.3 (C6'), 128.5 (C4', C4''), 129.0 (C5', C5''), 138.8 (C3'). EI-MS (70 eV), *m/z* (%) 178 (0.1) (M⁺), 117 (5), 105 (66), 104 (100), 91 (62), 77 (18), 65 (15), 57 (17).

2-Butenyl Glycidyl Ether (6). Compound **6** was synthesized from crotyl alcohol and epichlorohydrin as previously described (16) producing **6** in 42% yield. ¹H NMR δ 1.69–1.72 (3H, m), 2.60 (1H, dd, *J* = 5.0, 2.7 Hz), 2.79 (1H, dd, *J* = 4.9, 4.4 Hz), 3.12–3.16 (1H, m), 3.37 (1H, dd, *J* = 11.4, 5.8 Hz), 3.67 (1H, dd, *J* = 11.4, 3.2 Hz), 3.92–4.01 (2H, m), 5.54–5.62 (1H, m), 5.69–5.77 (1H, m). ¹³C NMR δ 17.7 (C4'), 44.6 (C1), 51.1 (C2), 70.6 (C1'), 71.9 (C3), 127.3 (C3'), 130.2 (C2'). EI-MS (70 eV), *m/z* (%) 128 (6) (M⁺), 113 (6), 99 (21), 83 (41), 69 (72), 55 (100).

Reactions of PGE and Epoxides 1–6 with the Model Peptide H-Pro-His-Cys-Lys-Arg-Met-OH (PHCKRM). All solvents were degassed with argon prior to use. Solutions of PGE in dimethyl sulfoxide (DMSO) (40 mM, 100 μL) together with potassium phosphate buffer (100 mM, pH 7.4) (200 μL) were added to a vial purged with argon containing PHCKRM in DMSO (40 mM, 100 μL). Accordingly, final concentrations of PGE and the model peptide in the reaction mixture were 10 mM and 1 mM, respectively. The reaction was kept under argon at room temperature and was monitored with HPLC-ESI-MS for 24 h. Samples were collected and analyzed every 40 min. Epoxides **1–6** were treated identically, and their reactions were investigated using the method described above.

Experimental Animals. Female CBA/Ca mice, 8 or 9 weeks of age, were purchased from B&K Sollentuna, Sweden. The mice were

housed in hepa-filtered airflow cages and kept on standard laboratory diet and water ad libitum. The local ethics committee in Gothenburg approved the study.

Sensitizing Capacity of PGE and 1–6 in Mice. The local lymph node assay (LLNA) (17) was used to assess the sensitizing capacity. Mice in groups of three were treated by topical application on the dorsum of both ears with the test compound (25 μL) dissolved in acetone/olive oil (AOO) (4:1 v/v) or with a vehicle control. All solutions were freshly prepared for every application. Each compound was tested in five different concentrations. The test concentrations used were as follows: PGE and **1**, 0.01, 0.1, 1, 10, and 20% (w/v); corresponding molar concentrations for PGE, 0.67 mM, 6.7 mM, 67 mM, 0.67 M, and 1.3 M; and for **1**, 0.61 mM, 6.1 mM, 61 mM, 0.61 M, and 1.2 M. Epoxide **2** was tested in the following concentrations: 0.1, 1, 10, 20, and 30% (w/v); corresponding molar concentrations, 0.61 mM, 6.1 mM, 61 mM, 0.31 M, and 1.8 M. Epoxides **3–6** were tested in the following concentrations: 0.01, 0.1, 1, 10, and 30% (w/v); corresponding molar concentrations for **3**, 0.64 mM, 6.4 mM, 64 mM, 0.64 M, and 1.9 M; for **4**, 0.56 mM, 5.6 mM, 56 mM, 0.56 M, and 1.7 M; for **5**, 7.6 mM, 76 mM, 0.76 M, 1.5 M, and 2.3 M; and for **6**, 0.78 mM, 7.8 mM, 78 mM, 0.78 M, and 2.3 M. Treatments were performed daily for three consecutive days (day 0, 1, and 2). Sham treated control animals received the vehicle alone. On day 5, all mice were injected intravenously via the tail vein with [methyl-³H]thymidine (2.0 Ci/mmol, Amersham Biosciences, UK) (20 μCi) in phosphate-buffered saline (PBS, containing 137 mM NaCl, 2.7 mM KCl, and 10 mM phosphate buffer, pH 7.4) (250 μL). After 5 h, the mice were sacrificed, the draining lymph nodes were excised and pooled for each group, and single cell suspensions of lymph-node cells in PBS were prepared using cell strainers (Falcon, BD labware, 70 μm pore size). Cell suspensions were washed twice with PBS, precipitated with TCA (5%), and left in the refrigerator overnight. The samples were then centrifuged, resuspended in TCA (5%) (1 mL), and transferred to scintillation cocktail (10 mL) (EcoLume, INC Radiochemicals, USA). The [methyl-³H]thymidine incorporation into DNA was measured by β-scintillation counting on Beckman LS 6000TA Instruments. Results are expressed as mean dpm/lymph node for each experimental group and as stimulation index (SI), i.e., 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. The sensitizing capacity was classified to the following: <0.1%, extreme; ≥0.1 to <1%, strong; ≥1 to <10%, moderate; and ≥10%, weak (18).

LLNA with Nonpooled Lymph Nodes. Single-cell suspensions of the lymph nodes from individual mice were prepared, and the [methyl-³H]thymidine incorporation was measured to investigate if a statistically significant difference in the sensitizing capacity at equal molar concentrations of PGE, **3**, and **6** could be demonstrated. The concentration was selected on the basis of results from the LLNA experiments with pooled lymph nodes on these epoxides. The test compounds dissolved in acetone/olive oil (AOO) (4:1 v/v), were topically applied (25 μL) on the dorsum of both ears to 3 groups of mice (*n* = 9 per group). A sham treated control group of 9 mice received the vehicle alone. All solutions were freshly prepared before application. Treatment and measurements were performed according to the ordinary LLNA method, but the single-cell suspensions from the lymph nodes of each mouse were treated separately. Results are expressed as stimulation index (SI), i.e., the ratio of individual test animal/mean of the control group. An identical experiment was performed with epoxides **1**, **2**, and **4**. The sensitizing capacity of PGE and **4** was also compared using this nonpooled LLNA method, but with two treatment groups and a control group (*n* = 12).

Statistical Analysis. The nonparametric Mann–Whitney *U* test was used as a statistical method. To obtain global significance at a level of *P* < 0.05, the significance levels were corrected for multiple comparisons using the Bonferroni correction. For each comparison, a value of *P* < 0.017 was considered statistically significant.

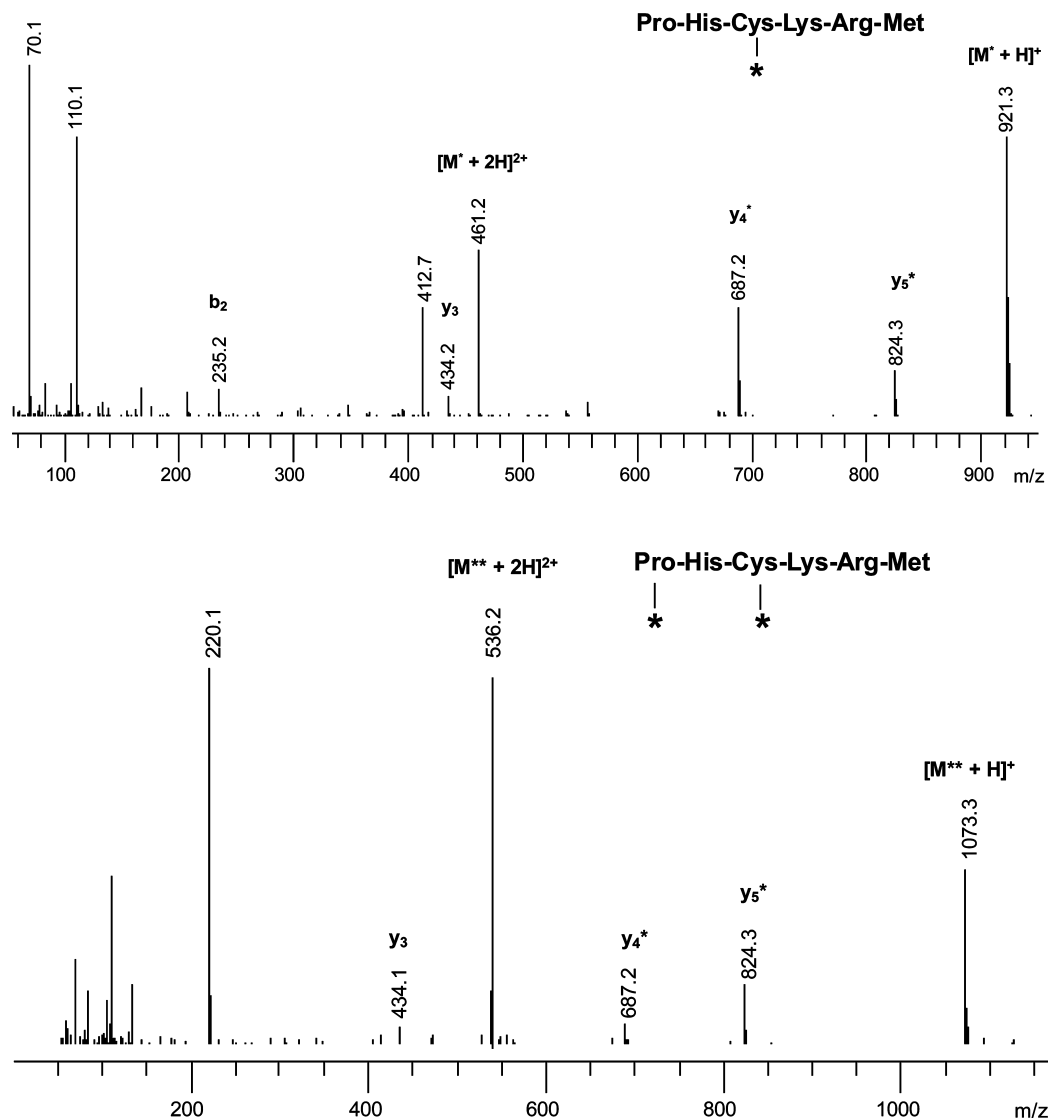


Figure 3. HPLC-MS spectra (positive ESI mode) of peptide-PGE adducts. The haptened fragment ions are denoted with an asterisk (*). The mass spectrum on top corresponds to the adduct formed between the epoxide PGE and the peptide, where PGE is attached to the cysteine residue (t_R = 12.7 min). The mass spectrum at the bottom corresponds to a diadduct formed when PGE is attached to both the cysteine and proline residues (t_R = 14.6 min).

Results and Discussion

In the present study, the chemical reactivity of a series of PGE analogues has been correlated to their sensitizing capacity in the LLNA. The series consists of six structurally modified PGE analogues, **1–6**, of which five are glycidyl ethers (Figure 2). Similarity in chemical structure and hydrophobicity within the series was considered important to allow an accurate comparison as possible of the allergenic activity in relation to structure and to avoid errors originating from possible variations in skin penetration capacity. As compared to PGE, epoxides **1**, **2**, and **4** (Figure 2) contain an identical core structure but have varying lengths of the carbon chains between the ether oxygen and the aromatic ring system (**1** and **4**) or a prolonged carbon chain between the ether oxygen and the epoxide moiety (**2**). Epoxides **3**, **5**, and **6** lack an aromatic system, but **3** differs from **5** and **6** by still being cyclic, and **6** differs from **5** by containing an allylic double bond.

Synthesis. All compounds except **2** were prepared according to literature procedures using the appropriate alcohol and epichlorohydrin as starting materials, resulting in the desired glycidyl ether via a base promoted S_N2 reaction. Compound **2** was synthesized from 4-phenoxybut-1-ene (**2a**) (**14**), which was

epoxidized with *m*-CPBA to produce **2** (schemes of the synthetic pathways are depicted in Supporting Information).

Reactivity of PGE and Epoxides 1–6 toward the Model Peptide H-Pro-His-Cys-Lys-Arg-Met-OH (PHCKRM). To investigate the reactivity of PGE and epoxides **1–6**, their reactions with the nucleophilic hexapeptide PHCKRM were analyzed. This peptide has previously been used to assess the reactivity of contact allergens (**19–22**). A representative HPLC-MS spectrum (positive ESI mode) of adducts formed when incubating PGE with the peptide is illustrated in Figure 3 (haptened fragments are denoted *).

A cysteine adduct was rapidly formed in all of the reactivity experiments of the epoxides and the peptide PHCKRM as indicated by the appearance of $[M^* + H]^+$ and $[M^* + 2H]^{2+}$ (* denotes one haptination) ions in the mass spectra (Table 1). In addition, signals corresponding to the y_5^* , y_4^* , y_3 , y_2 , and b_2 fragments were observed, showing that the cysteine residue of the peptide had been modified. The results were in good agreement with the well-known high reactivity of cysteine (**23**). Gradually, a diadduct was formed indicated by the appearance of $[M^{**} + H]^+$ and $[M^{**} + 2H]^{2+}$ (** denotes two haptentions). On the basis of the y_5^* , y_4^* , y_3 , and b_2 fragments,

Table 1. Monoadducts Formed in the Reactivity Experiments with the Epoxides and PHCKRM^a

epoxide	[M* + H] ⁺ m/z	[M* + 2H] ²⁺ m/z	y ₅ [*] m/z	y ₄ [*] m/z	y ₃ m/z	y ₂ m/z	b ₂ m/z
PGE	921.3	461.2	824.2	687.2	434.1	nd ^b	235.1
1	935.4	468.2	845.4	701.3	434.1	nd	235.1
2	935.4	468.2	nd	701.3	434.1	nd	235.1
3	928.4	462.2	830.4	693.3	434.1	306.1	235.1
4	949.4	475.3	852.3	715.3	434.1	nd	235.1
5	901.5	451.2	804.3	667.4	434.1	306.0	235.1
6	899.4	450.2	802.3	665.4	434.1	nd	235.1

^a The reactivity experiments of PGE and epoxides 1–6 toward the model peptide PHCKRM were performed as described in Experimental Procedures. The reactions were monitored with HPLC-ESI-MS. Haptenated fragments are denoted (*). ^b nd: not detected.

Table 2. Diadducts Formed in the Reactivity Experiments with the Epoxides and PHCKRM^a

epoxide	[M** + H] ⁺ m/z	[M** + 2H] ²⁺ m/z	y ₅ [*] m/z	y ₄ [*] m/z	y ₃ m/z	b ₂ m/z
PGE	1071.4	536.2	824.2	687.2	434.1	nd ^b
1	1099.4	550.4	nd	nd	nd	nd
2	1099.4	550.4	nd	701.3	434.1	235.1
3	1083.5	542.3	830.4	693.3	434.1	nd
4	1127.2	564.3	852.3	715.3	434.1	nd
5	1032.4	516.2	804.3	667.4	nd	nd
6	1027.4	514.3	802.3	665.4	nd	nd

^a The reactivity experiments of PGE and epoxides 1–6 toward the model peptide PHCKRM were performed as described in Experimental Procedures. The reactions were monitored with HPLC-ESI-MS. (**) Denotes two haptenations. ^b nd: not detected.

the new products were assigned to be cysteine and proline diadducts (Table 2) (structures of the hapten–peptide complexes are shown in the Supporting Information). A high correlation between reactivity toward a model peptide and sensitizing potency should not automatically be expected since factors such as skin penetration and metabolism of the hapten are not taken into account, but previous studies have shown that peptide reactivity is correlated with sensitization potency (24). Table 3 summarizes the reactivity data for PHCKRM with the epoxides, showing that PGE formed the highest amount of cysteine adducts with PHCKRM already after 40 min, while 5 was the least reactive, forming the lowest amount at this time point. However, after 1200 min all epoxides had reacted to almost an equal level, only PGE formed a significantly higher amount of the cysteine/proline diadduct. At the end of all experiments, i.e., after 24 h, all peptide had reacted (data not shown).

The various reactivity experiments were also used to determine the depletion of unreacted peptide, a method that has previously been used for reactivity assessments (25). Figure 4 summarizes the depletion data for PHCKRM, using the 40 min time point for analysis. As expected, PGE was the most reactive epoxide causing 88% depletion. For the epoxides 1–4, the extent of depletion varied within a range of 61 to 69%, whereas epoxides 5 and 6 reached 46 and 54% depletion, respectively. Hence, a large difference in the rate of depletion was observed between the fastest, PGE, and the slowest, 5, reacting epoxide (Figure 4).

Sensitizing Capacity of the Epoxides. The murine LLNA (17) was used to evaluate the sensitizing capacity of PGE and epoxides 1–6 (Table 4) (for the complete set of data, see Supporting Information). All tested epoxides fulfilled the criteria for a sensitizer according to the LLNA. According to the classifications suggested by Kimber et al. (18), only PGE was a sensitizer of strong potency with an EC₃ value of 0.031 M (0.46% w/v), whereas epoxides 1–4 were sensitizers of moderate potency with EC₃ values of 0.15 M (2.5% w/v), 0.13 M

Table 3. Reactivity of PGE and Epoxides 1–6 toward the Model Peptide PHCKRM^a

test epoxide	reaction time (min)	Cys-adduct ^b (%)	Pro-Cys diadduct ^b (%)
PGE	40	86	0
	80	95	4
	1200	57	43
1	40	69	0
	80	77	2
	1200	73	19
2	40	62	0
	80	85	0
	1200	77	23
3	40	62	0
	80	74	3
	1200	85	15
4	40	62	0
	80	78	2
	1200	86	14
5	40	44	0
	80	62	0
	1200	80	15
6	40	53	0
	80	72	0
	1200	76	17

^a The reactivity experiments were all performed in DMSO/potassium phosphate buffer at pH 7.4 (1:1), with a 10-fold excess of hapten. The reactions were kept at room temperature and monitored with HPLC-ESI-MS, and aliquots were withdrawn and analyzed every 40 min for 24 h. ^b The sum of nonreacted peptide, mono-, and diadducts was set to 100%.

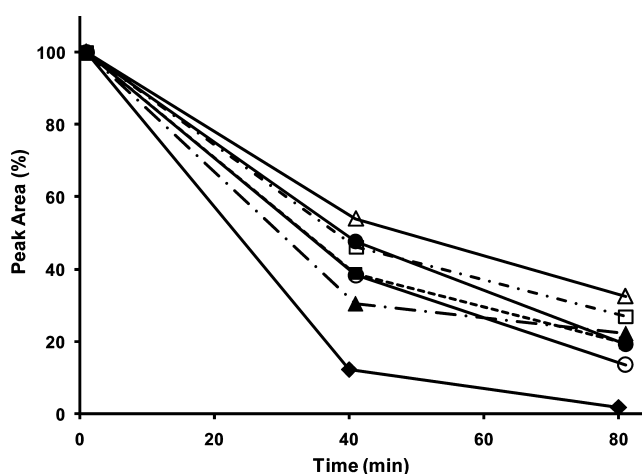
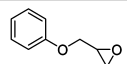
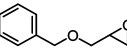
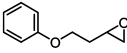
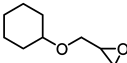
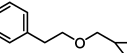
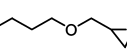
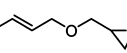


Figure 4. Depletion (consumption rate) of the model peptide H-Pro-His-Cys-Lys-Arg-Met-OH (PHCKRM) obtained with PGE (◆), 1 (▲), 2 (○), 3 (●), 4 (■), 5 (△), and 6 (□), normalized to 100%. All epoxides reacted rapidly, and at the end of all of the experiments, i.e., after 24 h, all unreacted peptide was consumed.

(2.2% w/v), 0.33 M (5.2% w/v), and 0.083 M (1.5% w/v), respectively. Epoxides 5 and 6 were classified as sensitizers of weak potency with EC₃ values of 2.2 M (28% w/v) and 1.1 M (14% w/v), respectively (Figure 5). These results indicate that the sensitizing capacity decreased when the chain length between the epoxide moiety and the ether oxygen increased, as well as when the distance between the ether oxygen and the aromatic system increased. The fact that epoxides 5 and 6 were both classified as sensitizers of weak potency (EC₃ ≥ 10%) revealed that the presence of an aromatic moiety increased the sensitizing ability of the compound. Epoxide 3, which is a saturated analogue of PGE, gave a higher EC₃ value than the epoxides containing an aromatic moiety but showed a lower EC₃ value than the acyclic epoxides 5 and 6.

LLNA with Nonpooled Lymph Nodes. To establish whether there was a statistically significant difference between the

Table 4. Results from the LLNA of PGE and Epoxides 1–6^a

Test compounds	Chemical structure	EC3 value ^b (% w/v)	EC3 value ^b (M)
PGE		0.46	0.031
1		2.5	0.15
2		2.2	0.13
3		5.2	0.33
4		1.5	0.083
5		28	2.2
6		14	1.1

^a Groups of mice were treated with the test compound in five different concentrations, on the dorsum of both ears for three consecutive days. Sham treated control animals received the vehicle alone. On day five, all mice were injected intravenously with [methyl-³H]thymidine, and its respective incorporation into the lymph nodes was measured. The increase in thymidine incorporation relative to vehicle-treated controls was derived for each experimental group and recorded as stimulation indexes (SIs). The EC3 values (the estimated concentration required to induce an SI of 3) were calculated using linear interpolation. ^b Complete data are given in Supporting Information.

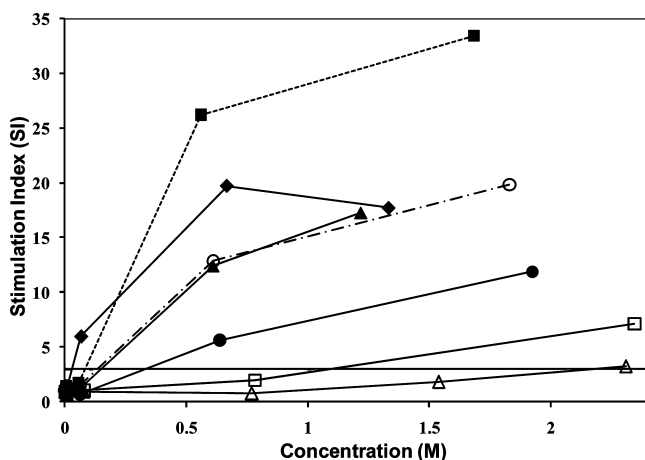


Figure 5. Dose–response curves for PGE (◆), 1 (▲), 2 (○), 3 (●), 4 (■), 5 (△), and 6 (□), tested in the murine LLNA. The concentrations are given in molar. The horizontal line marks an SI of 3, the cutoff limit for a compound to be a sensitizer.

sensitizing potencies of closely related epoxides, an extension of the LLNA was applied. This modified method (17) has previously been used within the group (13). Results from the three modified LLNA experiments are given in Figures 6A–C (for complete data, see Supporting Information). The first experiment was performed using 0.72 M of PGE, 3, and 6. Statistical analysis revealed that PGE was significantly more potent in inducing lymph node cell proliferation than the nonaromatic 3 and the acyclic 6 (Figure 6A). In addition, epoxide 3 was found to be significantly more potent in inducing

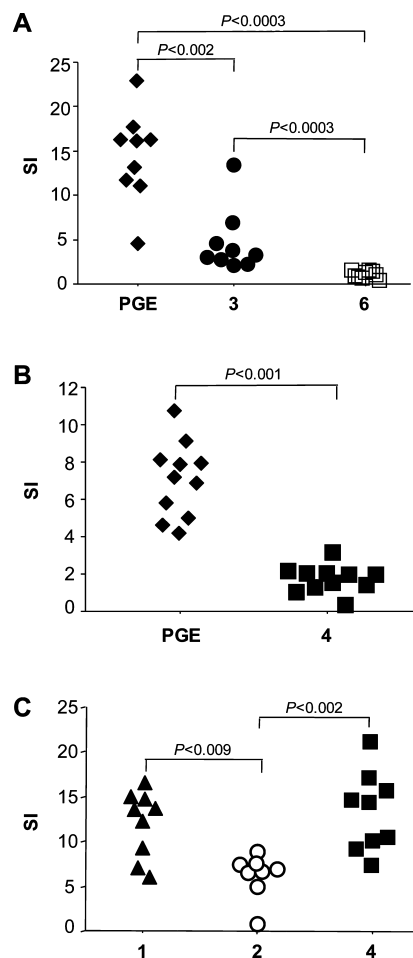


Figure 6. Results from the modified LLNA experiments using single-cell suspensions of the local lymph nodes from individual mice. (A) Statistical analysis showed that when using 0.72 M of either epoxide, PGE (◆) was more potent in inducing lymph node cell proliferation than both 3 (●) and 6 (□), giving a significant difference. Furthermore, epoxide 3 (●) was significantly more potent in inducing cell proliferation than 6 (□). (B) Statistical analysis showed that when using 0.065 M of either epoxide, PGE (◆) was significantly more potent in inducing lymph node cell proliferation as compared to 4 (■). (C) Statistical analysis showed that when using 0.60 M of either epoxide, both 1 (▲) and 4 (■) were significantly more potent in inducing lymph node cell proliferation than 2 (○). No statistically significant difference was seen between the results obtained for 1 (▲) and 4 (■).

cell proliferation than 6. The present results agree with our previously reported observation that conformational restriction seems to affect the sensitizing activity of haptens (26). It is also known that permeability through the lipid membrane is dependent on the number of rotating bonds and thereby molecular flexibility (27).

When using 0.065 M of PGE and epoxide 4, statistical analysis indicated that PGE was significantly more potent in inducing cell proliferation as compared to 4 (Figure 6B). The results show that when prolonging the distance between the aromatic system and the ether oxygen, the sensitizing potency is decreased.

The statistical analyses of a third investigation using 0.60 M of epoxides 1, 2, and 4 revealed that both 1 and 4 were significantly more potent in inducing lymph node cell proliferation as compared to 2 (Figure 6C). This observation indicates that a prolonged distance between the ether oxygen and the epoxide moiety affects the sensitizing potency more than a similar distance alteration between the ether oxygen and the aromatic system. Furthermore, no statistically significant dif-

Table 5. Reactivity and Depletion Studies of PHCKRM, PGE, and Epoxides 1–6, Listed According to Sensitizing Potency Category^a

LLNA category	epoxide	react. ^b (%)	depl. ^b (%)
strong	phenyl glycidyl ether (PGE)	86	88
moderate	benzyl glycidyl ether (1)	69	69
	2-(2-phenoxyethyl)oxirane (2)	62	62
	cyclohexyl glycidyl ether (3)	62	67
	phenethyl glycidyl ether (4)	62	61
weak	butyl glycidyl ether (5)	44	46
	butenyl glycidyl ether (6)	53	54

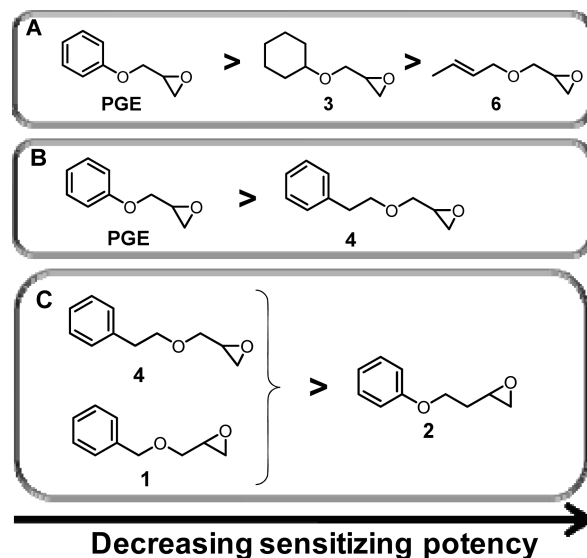
^a The reactivity experiments were all performed in DMSO/potassium phosphate buffer at pH 7.4 (1:1), with a 10-fold excess of hapten. The reactions were kept at room temperature and monitored with HPLC-ESI-MS. ^b The 40 min time point was used both for analysis of the depletion of PHCKRM and the formation of cysteine adducts.

ference was observed between the results obtained for **1** and **4**. Thus, the structural modification of **1** and **4**, i.e., prolongation with one or two carbons, respectively, between the ether oxygen and the aromatic system, results in comparable stimulation indexes at the chosen concentration (Figure 6C).

The results from the reactivity experiments and the depletion study of PHCKRM are compared with the sensitizing capacities obtained in the LLNA (Table 5). Our results from the reactivity experiments were congruent with the classification based on the LLNA experiments. PGE was the strongest contact allergen and reacted fastest with PHCKRM, while epoxide **5** (BGE) was the slowest to react with PHCKRM and showed the highest EC3 value (i.e., was the weakest allergen) in the test series. Thus, the reactivity experiments, when the formation of monoand diadducts was analyzed, differed between the strong, moderate, and weak allergens and showed congruence with the results obtained in the LLNA (Table 3). These results demonstrate that studies on the reactivity of a model peptide are applicable for correlation with the sensitizing capacity of the type of compounds investigated. Furthermore, the depletion of nonreacted peptide was in good agreement with the results from both the reactivity and the LLNA experiments for the compounds investigated. However, solely used, the results from this approach should be interpreted with caution since it is assumed that the loss of unreacted peptide is due to the reaction with the test chemical, even though peptide dimerization or other oxidative processes also might be involved (28).

A compilation of our results from the three nonpooled LLNA experiments is summarized in Figure 7. The obtained results disclose the following order of allergenicity, starting with the most potent sensitizer: PGE > **3** > **6**. This illustrates the importance of an aromatic ring system for the sensitizing capacity of epoxy resin analogues, as the weaker sensitizers **3** and **6** both lack this structural element. PGE was shown to be more sensitizing than **4**, whereas no significant difference was obtained for the sensitizing capacities of **1** and **4**. Thus, the following order can be disclosed, starting with the most potent sensitizer: PGE > (**1** and **4**) > **2** (Figure 7). This observation indicates that a prolonged distance between the ether oxygen and the epoxide moiety affects the sensitizing potency more than a similar distance alteration between the ether oxygen and the aromatic system.

In summary, ERS can be prepared from numerous chemical compositions. To prevent outbreaks of ACD, not only the polymerizing capacity is of interest but also the sensitizing capacity of epoxy compounds. In the present study, we have synthesized a series of PGE analogues (**1**–**6**) to investigate their reactivity and sensitizing capacity. The compounds range from strong to weak sensitizers, indicating that even small

**Figure 7.** An illustrative compilation of the results from the nonpooled LLNA experiments.

changes in chemical structure result in significant differences in sensitizing capacity. In the present study, the PGE analogues without an aromatic moiety showed the lowest ability to sensitize. Along with results from previous studies (16) of the polymerizing properties of **1**, **2**, and **6**, our observations suggest that epoxy resins with decreased ability to cause ACD can be developed. The gained knowledge may support the prevention of ACD from epoxy resins; however, further mechanistic investigations are necessary for the prevention of occupational dermatitis.

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Supporting Information Available: Complete LLNA tables from experiments using both pooled and nonpooled lymph nodes, mechanism of the hapten–peptide complex formation, and schemes of the synthesis of epoxides **2**–**4**, and **6**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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