

Human Myeloperoxidase-Driven Activation of Skin Sensitizing p-Phenylenediamine-related Aromatic Diamines and Phenylpropanoids – Insights from In Chemico and In Silico Approaches

Hannah Deusinger

Universität Trier

Philipp Peslalz

Wella Company

Karma C. Fussell

Wella Company

Sabine Langhoff

Wella Company

Udo Bock

Universität Trier

Carsten Goebel

Wella Company, BBCG Toxicology & Safety Science Consulting GbR

Brunhilde Blömeke

Bloemeke@uni-trier.de

Universität Trier

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Abstract

Skin sensitization is initiated by the covalent binding of low-molecular-weight chemicals to skin proteins. For prehapten, this requires oxidative activation to generate protein-reactive electrophiles. While abiotic oxidation is established, enzymatic oxidation remains poorly understood. Because neutrophils infiltrate the skin during the induction phase of sensitization and release the heme peroxidase myeloperoxidase (MPO), we investigated whether human MPO contributes to the oxidative activation of selected sensitizers. The study included the hair dye precursor *p*-phenylenediamine (PPD) and its derivatives toluene-2,5-diamine (PTD) and 2-methoxymethyl-PPD (ME-PPD), together with the phenylpropanoid fragrance molecules eugenol and dihydroeugenol, and the aromatic alcohol cinnamyl alcohol. Molecular docking predicted productive binding of all compounds except cinnamyl alcohol, which adopted a non-productive orientation within the MPO active site. Consistent with these predictions, in chemico experiments showed MPO-catalyzed oxidation of the aromatic diamines and phenylpropanoids with comparable catalytic efficiencies ($10\text{--}25 \times 10^3 \text{ mM}^{-1} \times \text{min}^{-1}$), whereas cinnamyl alcohol remained unconverted. High-resolution mass spectrometry revealed distinct metabolite profiles, with the aromatic diamines forming mono-, di-, and trimeric oxidation products, including Bandrowski's Base, whereas phenylpropanoids predominantly yielded monomeric quinone methides and dimers. Protein-binding potential was predicted for several observed metabolites using the OECD QSAR Toolbox. Collectively, these findings demonstrate that MPO-mediated oxidation is governed by substrate chemistry and selectively generates metabolites predicted to be protein-reactive from chemically susceptible sensitizers. Our results support a previously unrecognized role for neutrophil-derived MPO as an oxidative activation system that may influence the local chemical fate of susceptible sensitizers during the induction phase of skin sensitization, extending current concepts of oxidative activation beyond abiotic oxidation and constitutive epidermal metabolism.

Introduction

The molecular initiating event (MIE) of skin sensitization is the formation of stable chemical-protein complexes, most commonly through the covalent binding of electrophilic chemicals to nucleophilic residues in skin proteins. For many low-molecular-weight organic chemicals, electrophilic reactivity first requires oxidative activation to generate protein-reactive intermediates. Depending on whether this activation occurs through abiotic oxidation or enzymatic metabolism, these compounds are classified as prehapten or prohapten, respectively (Karlberg et al. 2013; Lepoittevin 2006). Among hair dye precursors, *p*-phenylenediamine (PPD) represents an extensively studied contact allergen with sensitization prevalences of 0.8 % in the general population (Diepgen et al. 2016) and substantially higher rates among dermatitis patients and occupationally exposed hairdressers (Thyssen and White 2008; Uter et al. 2025). Abiotic oxidation of PPD generates reactive oxidation products, including Bandrowski's Base (BB), which exhibits higher sensitizing potency in the local lymph node assay than PPD itself (White et al. 2006). While the prehapten character of PPD is established (Aeby et al. 2009), it remains unclear whether skin-associated enzymes contribute to the formation of protein-reactive

species. Following topical exposure, non-metabolized PPD was detected within the viable epidermis (Pot et al. 2016), demonstrating that free PPD is available for potential enzymatic reactions. Enzymatic transformation of PPD within the skin has primarily been associated with detoxifying reactions such as N-acetylation by N-acetyltransferase 1 in keratinocytes. In human skin and keratinocyte models, PPD is converted to mono- and diacetylated metabolites (Kawakubo et al. 2000), which exhibit markedly reduced T cell activation potential compared with PPD (Sieben et al. 2001). Both metabolites were classified as non-sensitizing in the local lymph node assay (Aeby et al. 2009). Comparable N-acetylation reactions have also been reported for PPD derivatives. Predominantly mono-acetylated metabolites were identified for toluene-2,5-diamine (PTD) in human skin models (Grohmann et al. 2017), and similar metabolic profiles were reported for 2-methoxymethyl-PPD (ME-PPD) (Goebel et al. 2014) and other PPD analogues (Venkatesan et al. 2022). Whereas these metabolic pathways primarily detoxify aromatic diamines, oxidative activation of exogenous chemicals has mainly been discussed in relation to cytochrome P450-mediated metabolism. Although cytochrome P450 enzymes have traditionally been considered the principal candidates for enzymatic oxidative activation, their activity in human skin is generally low to undetectable (Oesch et al. 2018). Other epidermal oxidoreductases, including alcohol dehydrogenases, may contribute to the activation of selected sensitizers, as exemplified by the oxidation of cinnamyl alcohol to cinnamaldehyde (Cheung et al. 2003). The phenylpropanoids eugenol and dihydroeugenol are also relevant in the context of enzymatic oxidative activation. Both were reflected as prohaptens in the peroxidase peptide reactivity assay, which uses horseradish peroxidase as a model enzyme for oxidative activation (Troutman et al. 2011; Yan et al. 2025). In reconstructed human epidermis models, eugenol-derived protein-reactive intermediates were not detected in untreated skin models but were detected after the addition of horseradish peroxidase (Port-Lougarre et al. 2023). Beyond constitutive metabolism, primarily by keratinocytes, the enzymatic environment of the skin changes during the induction phase of skin sensitization; innate immune cells infiltrate the tissue. Among these, neutrophils are rapidly recruited to the skin (Weber et al. 2015). They release large amounts of the heme peroxidase myeloperoxidase (MPO) into the extracellular environment, where MPO was shown to modulate the induction of contact hypersensitivity (Strzepa et al. 2020). Unlike constitutively expressed epidermal enzymes, MPO therefore becomes available only after immune cell recruitment. In the presence of hydrogen peroxide, MPO catalyzes the one-electron oxidation of a broad range of aromatic diamines and phenolic hydrogen-donor compounds (Davies and Hawkins 2020), raising the intriguing possibility that the developing immune response itself may influence the local chemical fate of susceptible sensitizers. Oxidative activation of eugenol by MPO has been described, leading to a quinone methide-like reactive intermediate and covalent binding to cellular proteins (Thompson et al. 1989). To date, these observations have not been extended to skin sensitizers more broadly.

In the present study, we investigated whether human MPO contributes to the oxidative activation of representative skin sensitizers from two chemically distinct classes, PPD-related aromatic diamines (PPD, PTD, ME-PPD) and phenylpropanoids (eugenol, dihydroeugenol). Using molecular docking, in chemico kinetic analyses, and high-resolution mass spectrometry (HRMS), we characterized substrate

selectivity, catalytic efficiency, and oxidation products, and assessed the protein-binding potential of the resulting metabolites using the OECD QSAR Toolbox. Our results identify MPO as a substrate-selective oxidative activation system capable of generating protein-reactive metabolites from chemically susceptible sensitizers, thereby introducing a previously unrecognized mechanism that may influence the local chemical fate of sensitizers during the induction phase of skin sensitization.

Materials and Methods

Chemicals and Reagents

MPO derived from human leukocytes and catalase from bovine liver were purchased from Sigma Aldrich (Taufkirchen, Germany) with supplier-specified activities. Enzyme concentrations were adjusted based on activity units to ensure comparability between experiments. PPD (purity >99%) was acquired from Lowenstein (New York, USA) and PTD sulfate ($\geq 97\%$) from Dragon Chemicals (Düsseldorf, Germany). ME-PPD (>99%) was synthesized internally by Wella Dye Capability (Hünfeld, Germany). Eugenol (>99%) was obtained from Thermo Fisher (Darmstadt, Germany) and monobasic potassium phosphate, ammonium formate, dihydroeugenol (>99%) and cinnamyl alcohol (>99%) from Sigma Aldrich (Taufkirchen, Germany). Formic acid (LC-MS grade) was purchased from Honeywell (Offenbach, Germany). Potassium hydroxide, methanol and acetonitrile (HPLC- and LC-MS grades) were purchased from Merck (Darmstadt, Germany). Hydrogen peroxide (49.9%, v/v) was manufactured in-house. The product names, catalog numbers, and lot numbers of the used chemicals are provided in Online Resource 1 (Table S1).

Molecular Docking Analysis of Substrate Orientation within the MPO Binding Pocket

Computational docking was performed using Maestro (Schrödinger Release 2024-2, Schrödinger, LLC, New York, USA), a commercially available molecular modeling software. All preparation steps and docking procedures were performed using modules implemented in the software. The crystal structure of MPO (Protein Data Bank ID: 1CXP) was used as the receptor and prepared by removing ligands, ions, stabilizing agents, and crystallographic water molecules. The Protein Preparation Wizard was used to assign bond orders, add hydrogens, create zero-order bonds to metals, form disulfide bonds, and model missing side chains and loops using Prime. Hydrogen-bonding networks were optimized using default settings. All ligands were prepared using the ligand preparation function and relevant protonation states were generated using Epik. Compounds were docked into the heme-centered active site of MPO using Induced Fit Glide Docking with post-docking minimization at 298 K and pH 7.4. Additional docking simulations were performed at pH 5.0, 6.0, and 7.0, adjusting the ligand protonation states and protein preparation accordingly. Binding poses were evaluated based on overall orientation within the binding pocket and docking scores, reflecting the predicted binding affinity of a compound to the enzyme based on energetic and geometric criteria. Molecular visualization was performed separately with UCSF ChimeraX (version 1.10.1; University of California, San Francisco, USA)(Meng et al. 2023).

MPO Activity Determination

Oxidation by MPO was performed in closed vials under defined conditions (0.1M potassium phosphate buffer, pH 7.4 at 38 °C, reaction initiated by addition of 0.1 mM hydrogen peroxide) and applied consistently to all chromatographic analyses. Oxidation of 0.01 to 10 mM PPD or its derivatives by 0.12 U/mL MPO was analyzed after 3 min incubation for PPD and PTD and 5 min for ME-PPD. These reactions were terminated by catalase-mediated peroxide removal (50 U/mL) followed by formic acid adjustment to pH 6 and heating to 90 °C for 2 minutes to ensure enzyme inactivation. Similarly, the oxidation of the phenylpropanoids by 0.24 U/mL MPO was analyzed after 4 min at concentrations up to their solubility limits (1.9 mM for eugenol and 0.75 mM for dihydroeugenol) and stopped by the addition of formic acid (10% final). These conditions were optimized in preliminary experiments to ensure measurable enzymatic conversion, sampling within the initial linear conversion range and enzyme inactivation. All samples were then filtered using 0.45 µm PTFE syringe filters and analyzed immediately. Each experimental condition was performed in triplicate and included enzyme-free controls and test-chemical-free controls.

Substrate depletion was quantified by HPLC-UV using a 1260 Infinity LC system (Agilent Technology, Waldbronn, Germany) managed with OpenLab CDS ChemStation C01.10. Samples were prepared as described above. For separation, a VDSpher PUR 100-C18-M-SE column (150 × 3 mm; 3 µm; VDS Optilab, Berlin, Germany) was used with an injection volume of 10 µL. Quantification of the PPD-related aromatic diamines (PPD, PTD, ME-PPD) was performed using 10 mM ammonium formate, pH 5.9 (eluent A) and acetonitrile (eluent B) with the gradient program 0-4 min: 99-90% A, 4-7 min: 90-80%, 7-7.5 min: 80-20% A, 7.5-11 min: 20% A, 11-11.1 min: 20-99% A, 11.1-15 min: 99% A. The flow rate was set to 0.75 mL/min, the column temperature to 30 °C and detection was performed at 254 nm. The phenylpropanoids (eugenol, dihydroeugenol) were quantified at 280 nm using 0.1% formic acid in water (eluent A) and methanol (eluent B) at 0.55 mL/min and 35 °C with the following gradient program: 0-4 min: 40-10% A, 4-6 min: 10% A, 6-6.1 min: 10-40% A, 6.1-10 min: 40% A. Substrate depletion was determined in triplicate by comparing peak areas of substrates in enzymatic samples to those of the corresponding enzyme-free controls. Depletion data were fitted by non-linear regression using the Michaelis-Menten model (model "kcat") with 1/Y weighting and a constant MPO activity (0.12 U/mL for PPD-related aromatic diamines, 0.24 U/mL for phenylpropanoids). The corresponding molar enzyme concentrations (0.0019 µM and 0.0038 µM) reflect the specific activity of the MPO batch used for these experiments. The maximum reaction velocity $V_{\max}$ and binding affinity K_M were obtained directly from the regression (value ± SEM). The turnover rate k_{cat} was calculated as $V_{\max}$ divided by the molar enzyme concentration and catalytic efficiency as k_{cat} divided by K_M . Data analysis and regression modelling were performed using GraphPad Prism version 10.5.0 (GraphPad Software, Boston, USA).

Metabolite profiling of the five convertible substrates was performed using HPLC-HRMS on an ACQUITY Premier UPLC system (Waters, Milford, USA) coupled to a Xevo G3 quadrupole time-of-flight mass spectrometer (QToF; Waters). Samples were prepared as described above. The chromatographic methods here differ from the HPLC-UV methods; therefore, retention times cannot be directly compared between techniques. A Luna Omega Polar C18 column (150 × 2.1 mm; 1.6 µm; Phenomenex,

Aschaffenburg, Germany) was used for separation with 10 °C sample temperature and an injection volume of 2 µL. Identification of metabolites derived from PPD-related aromatic diamines was performed using 10 mM ammonium formate, pH 5.9 (A) and acetonitrile (B) with a flow rate of 0.3 mL/min at 30 °C and additional UV detection at 254 nm. For PPD, the following gradient program was used: 0-0.5 min: 100% A, 0.5-1.7 min: 100-95% A, 1.7-3.3 min: 95-90% A, 3.3-6.5 min: 90-80% A, 6.5-11 min: 80-20% A, 11-15 min: 20% A, 15-15.1 min: 20-100% A, 15.1-20 min: 100% A. For PTD and ME-PPD, the following gradient was applied: 0-0.5 min: 99% A, 0.5-3.5 min: 99-80% A, 3.5-16.5 min: 80-20% A, 16.5-20 min: 20% A, 20-20.1 min: 20-99% A, 20.1-25 min: 99% A. For the phenylpropanoids, 0.1% formic acid in water (A) and methanol (B) served as mobile phases with a flow rate of 0.2 mL/min at 35 °C and additional UV detection at 280 nm with the gradient program 0-7 min: 60-10% A, 7-12 min: 10% A, 12-12.1 min: 10-60% A, 12.1-20 min: 60% A. In all chromatographic methods, MS detection was performed in positive mode using an electrospray ionization (ESI) source with the capillary voltage set to 3 kV, the source temperature to 120 °C, desolvation temperature to 550 °C, desolvation gas flow to 900 L/h, sample cone voltage to 20 V and cone gas flow to 50 L/h. The collision energy was set to 6 V with a ramp of 10-30 V. Accurate mass screening was performed over a mass range of m/z 50-1200 with leucine enkephalin (m/z 556.2771 for ESI+) as lock mass reference. Instrument control, data acquisition and data processing were performed using Waters Connect 4.0.0 with UNIFY 3.15.1.1238 (Waters, USA). HRMS analyses included enzyme-free and test-chemical-free controls and substrate standards ($n = 3$). Metabolites were postulated based on accurate mass, isotopic distribution, and fragmentation patterns, with structure assignment supported by spectral library comparison and literature data. Only MPO-derived oxidation products that appeared at least twofold above the corresponding control samples and met predefined reliability criteria (mass accuracy, isotopic fit, predicted spectra intensity and fragment matches) were considered for further evaluation.

UV-Vis Spectroscopy for pH-Dependent Screening

UV-Vis spectroscopy was used to assess relative pH-dependent trends in substrate conversion by MPO. For pH-dependent measurements, reactions were adapted to potassium phosphate buffer (0.1M) adjusted to pH 5.0, 6.0, 7.0 and 7.4 at room temperature. MPO was used at a final activity of 0.12 U/mL for all substrates and was initiated by the addition of 0.3 mM hydrogen peroxide. Oxidation product formation was monitored spectrophotometrically at 520 nm for PPD, 515 nm for PTD and ME-PPD and 350 nm for eugenol using an UV-Vis spectrophotometer (Evolution 500 BB, Thermo Fisher, Darmstadt, Germany) managed with Vision Pro (version 4.41). The chosen wavelengths correspond to absorption maxima determined in preliminary spectroscopic scans ($n = 4$) and are, for PPD and eugenol, consistent with reported absorption properties of characteristic oxidation products, including BB and eugenol quinone methide (Thompson et al. 1989; Zhang et al. 2017). The rate of oxidation product formation (AU/min) was converted into molar product formation rates using reported molar extinction coefficients for the PPD-derived BB ($\epsilon = 12880 \text{ M}^{-1}$; (Zhang et al. 2017)). As no extinction coefficient is available for the eugenol-derived quinone methide, a coefficient from a structurally related quinone methide was used (butylated hydroxytoluene, $\epsilon = 28200 \text{ M}^{-1}$ (Willcockson et al. 2013)). Each experiment included enzyme-

free controls and data analysis was performed using GraphPad Prism version 10.5.0 (GraphPad Software).

Prediction of Protein-Binding Potential by QSAR Toolbox

The protein-binding potential of the convertible parent substrates and their MPO-derived metabolites was evaluated using the freely available OECD QSAR Toolbox (version 4.8) in accordance with OECD Guideline No. 497 (OECD 2025). Predictions were generated from SMILES representations of proposed metabolite structures using Defined Approaches for Skin Sensitization – Automated Workflow, integrating experimental data, structural alerts, and read-across from mechanistically relevant analogues, while checking applicability domains across parametric, structural, and mechanistic layers. No metabolic simulations were applied. The 'Protein Binding by OASIS' profiler within the Toolbox was used for evaluation, providing binary alerts (positive/negative) which were interpreted qualitatively as supportive evidence for the presence or absence of structural features associated with protein reactivity.

Results

Cinnamyl Alcohol Displays a Distinct Binding Orientation within the MPO Active Site in Comparison to PPD-related Aromatic Diamines and Phenylpropanoids

Molecular docking was conducted to assess the binding orientation of PPD and its derivatives PTD and ME-PPD as well as the phenylpropanoids eugenol and dihydroeugenol and the aromatic alcohol cinnamyl alcohol, within the binding pocket of MPO. All tested compounds could be docked within the MPO active site with docking scores in a similar range (approximately -4.5 to -5.5). Representative docking poses of PPD, eugenol and cinnamyl alcohol are shown in Fig. 1. For PPD and eugenol, the heteroatom associated with the proposed oxidation site was oriented towards the catalytic heme group. In both cases, π -stacking and hydrogen-bonding interactions with amino acids in the binding pocket were observed, providing potential stabilization of these binding orientations. For PPD but not eugenol, additional interactions involving the second heteroatom and Glu102 were identified. Similar docking poses and docking scores were observed for PTD, ME-PPD and dihydroeugenol across both active sites of MPO (Online Resource 2 Fig. S1). In contrast, cinnamyl alcohol adopted a distinct docking pose within the MPO binding pocket, in which the hydroxyl group is engaged in hydrogen bonding with Glu102, directing the putative oxidation site away from the catalytic heme group and increasing its distance to 7.4 Å compared to PPD and eugenol (4.2 and 5.3 Å, respectively).

MPO Converts PPD-related Aromatic Diamines and Phenylpropanoids with Comparable Catalytic Efficiency

Substrate conversion by MPO was examined experimentally using HPLC-UV to monitor substrate depletion as well as the formation of metabolites simultaneously. MPO dose-dependently converted PPD and its derivatives PTD and ME-PPD and the phenylpropanoids eugenol and dihydroeugenol, whereas no conversion of cinnamyl alcohol was detected under identical conditions (Online Resource 2 Fig. S2-

S4). Substrate depletion data for the five convertible substrates were fitted to Michaelis-Menten kinetics (Fig. 2). The calculated catalytic efficiencies (k_{cat}/K_M) were within a comparable range for all substrates ($10\text{--}25 \times 10^3 \text{ mM}^{-1} \times \text{min}^{-1}$). For PPD, PTD and ME-PPD, the enzymatic turnover rates (k_{cat}) differed between the three compounds, with PTD yielding the highest k_{cat} , followed by ME-PPD and PPD. All three substrates had similar binding affinities for the active site of MPO, ranging from 0.7 to 2.0 mM. For eugenol and dihydroeugenol, Michaelis-Menten analysis yielded distinct k_{cat} and K_M values. Eugenol exhibited a higher k_{cat} and dihydroeugenol showed the lowest K_M among all test substrates. Overall, these differences in k_{cat} and K_M mitigated each other, resulting in comparable catalytic efficiencies across the convertible substrates.

pH Affects Conversion by MPO only for PPD-related Aromatic Diamines Undergoing Changes in Protonation State

To investigate the pH-dependence on substrate conversion by MPO, turnover was compared across the physiologically relevant pH range. PPD and eugenol were selected as representative substrates for both compound classes. UV-Vis spectroscopy was used to enable continuous monitoring of metabolite formation across different pH conditions. Formation of a PPD-derived metabolite monitored at 520 nm and consistent with BB showed a pronounced pH-dependency, with decreased formation under acidic conditions and increasing formation towards neutral pH (Fig. 3a). In contrast, formation of a eugenol-derived metabolite monitored at 350 nm and consistent with eugenol-derived quinone methide was largely unaffected by pH, showing comparable conversion rates across the tested conditions (Fig. 3b). Complementary molecular docking analyses at corresponding pH conditions were also performed for PPD and eugenol. Protonated PPD present at lower pH exhibited a higher docking score (less favorable) relative to its neutral form, whereas eugenol remained unchanged in charge and docking score across pH conditions (Fig. 3c-d). Visualization of the electrostatic surface potential of the MPO binding pocket revealed distinct positively and negatively charged regions surrounding the heme group (Fig. 3e). Additional docking simulations are provided in Online Resource 2 (Fig. S5).

MPO-Derived Metabolites but Not Parent Compounds Possess Structural Protein-Binding Alerts Associated with Skin Sensitization

Characterization of MPO-derived oxidation products was performed with HRMS to profile the reaction mixtures obtained from selected PPD-related aromatic diamines and phenylpropanoids (Online Resource 1 Table S2). Only MPO-derived oxidation products that fulfilled predefined reliability criteria and exceeded the corresponding control samples were included in the subsequent evaluation. Metabolite structures were putatively assigned based on accurate mass, isotopic distribution and fragmentation patterns and remain postulated. MPO-mediated oxidation of the aromatic diamines PPD, PTD and ME-PPD resulted in depletion of the parent compound and formation of monomeric, dimeric and trimeric metabolites (Fig. 4a, Online Resource 1 Table S2). Formation of a metabolite consistent with the stable oxidation product BB (Trimer - 2H) was confirmed for PPD. In contrast to PPD, for which the formation of BB was predominant, PTD and ME-PPD showed a more diverse pattern comprising mono-, di- and

trimeric species. For the phenylpropanoids eugenol and dihydroeugenol, enzymatic oxidation formed monomeric and dimeric products (Fig. 4b, Online Resource 1 Table S2). In both cases, a monomeric product consistent with a quinone methide structure was detected (Monomer - 2H), along with coupling products (Dimer and Dimer - 2H), whereas no higher oligomers were observed. Protein-binding potential associated with skin sensitization was predicted for the postulated MPO-derived metabolites and parent compounds using the OECD QSAR Toolbox. While all parent compounds were predicted to be negative, positive alerts were predicted for several metabolites (Fig. 4, Online Resource 1 Table S3). A consistent structural feature among metabolites associated with positive alerts was the presence of benzoquinone diimine or quinone methide-type motifs, which were flagged by the QSAR toolbox as being susceptible to Michael additions. To account for structural ambiguity, representative metabolites of eugenol and PPD with isomeric structures were evaluated, demonstrating that the nature of the electrophilic linkage affected predicted protein-binding (Online Resource 1 Table S4).

Discussion

The present study examined whether human MPO contributes to the oxidative activation of representative compounds from two chemically distinct classes of skin sensitizers. To address this, molecular docking was used to evaluate substrate positioning within the MPO active site, followed by in chemico oxidation studies. Characterization of the resulting metabolites by HRMS, together with QSAR-based prediction of their protein-binding potential, showed that MPO generates protein-reactive metabolites from chemically susceptible substrates, supporting a previously unrecognized role for MPO in oxidative activation during skin sensitization.

Molecular docking provided a structural rationale for the observed substrate selectivity of MPO, showing that productive binding is determined by the orientation of the reactive functional group relative to the catalytic heme, rather than by the docking score alone. All tested compounds docked within the active site of MPO with comparable docking scores. However, as illustrated by cinnamyl alcohol, conversion depended on the positioning of the reactive functional group toward the catalytic heme (Fig. 1). Positioning of the substrate within the active site has been described as an important determinant of enzymatic catalysis (Griffin et al. 2012) and for heme enzymes, substrate positioning relative to the heme has been linked to active vs. non-active binding states (Seifert et al. 2006). Accordingly, the binding orientation of cinnamyl alcohol away from the heme provides a structural explanation for its lack of enzymatic conversion, consistent with the in chemico findings that cinnamyl alcohol remained unconverted whereas the PPD-related aromatic diamines and phenylpropanoids underwent oxidation. These findings indicate that productive substrate orientation, rather than binding affinity alone, governs MPO-mediated oxidation.

Michaelis-Menten kinetic analysis showed that all five MPO substrates were oxidized with comparable catalytic efficiencies (k_{cat}/K_M : $10\text{--}25 \times 10^3 \text{ mM}^{-1} \times \text{min}^{-1}$), despite differences in their individual kinetic behavior (Fig. 2). Together with the docking results, these findings indicate that MPO substrate selectivity is governed primarily by productive substrate orientation and the ability to undergo oxidation,

whereas catalytic efficiencies are similar once productive catalysis is established. Comparable data for MPO-dependent conversion of the studied substrates are limited. Although peroxidase-catalyzed oxidation has previously been reported for PPD using horseradish peroxidase (Zhang et al. 2017) and for eugenol using avocado peroxidase (Rojas-Reyes et al. 2014), direct comparison with the present study is limited due to differences in enzyme source, analytical methodology, and experimental conditions. Collectively, our findings demonstrate that human MPO selectively catalyzes the oxidation of PPD-related aromatic diamines and phenylpropanoids, providing experimental evidence for MPO-mediated activation of chemically susceptible skin sensitizers.

In vivo, MPO is primarily expressed by neutrophils. In the murine contact hypersensitivity model, exposure to low-molecular-weight organic contact allergens activates innate immune pathways, leading to the rapid recruitment of neutrophils to the exposed skin area within hours. Experimental depletion of neutrophils leads to abrogated contact hypersensitivity, highlighting their critical role (Weber et al. 2015). MPO has been shown to contribute to sensitization, as genetic deficiency or pharmacological inhibition of MPO reduces allergen-specific T cell priming (Strzepa et al. 2020). Consequently, MPO becomes locally available at sites of chemical exposure, where it has the potential to oxidize susceptible substrates. Local enzymatic activation within the skin has been demonstrated for several drugs, including the metabolism of lamotrigine to reactive intermediates in human keratinocytes (Chen et al. 2010; Sharma et al. 2019). In this context, the present findings support the concept that MPO may constitute an additional, substrate-selective oxidative activation pathway capable of generating protein-reactive metabolites from susceptible aromatic diamines and phenylpropanoids during the induction phase of skin sensitization.

Given the strong influence of pH on enzymatic activity, substrate oxidation by MPO was investigated across a physiologically relevant pH range. Oxidation of PPD was strongly pH-dependent, whereas eugenol oxidation remained essentially unchanged. Formation of PPD-derived BB was reduced under acidic conditions and increased toward neutral pH, whereas eugenol conversion rates remained constant (Fig. 3). Molecular docking suggested that these differences arise from changes in substrate protonation rather than alterations in the active MPO site. At lower pH, protonation of PPD (pKa: 6.44 (Corbett 1969)) introduces a positive charge that is predicted to reduce favorable electrostatic interactions within the MPO active site, thereby reducing stabilization of the enzyme-substrate complex and limiting electron transfer. In contrast, eugenol does not change in protonation state across the investigated pH range (pKa: 9.8 (Yuwono et al. 2002)), consistent with its stable conversion rates and docking scores. These findings show that pH-dependent MPO-catalyzed oxidation is governed primarily by substrate protonation rather than by direct effects on the enzyme. Similar protonation-dependent effects on protein-ligand interactions have been described for other small molecules (Kim and McCammon 2016).

In human skin, the epidermal surface pH is mildly acidic (4-5), whereas the viable epidermis and dermis are generally close to physiological pH (7.35-7.45 (Brooks et al. 2025; Hajjar and Zhou 2023)). Under these conditions, the protonation state of substrates may modulate MPO-mediated oxidation. Because repeated washing and detergent exposure increase skin surface pH through disruption of the acid

mantle (Brooks et al. 2025), conditions that favor deprotonation could enhance MPO-mediated oxidation of PPD or its derivatives. These results further demonstrate that MPO-mediated oxidation depends not only on substrate structure but also on physicochemical factors that influence productive substrate-enzyme interactions.

To complement the kinetic analyses, HRMS-based metabolite profiling was performed to characterize MPO-derived metabolites of PPD, PTD and ME-PPD as well as eugenol and dihydroeugenol. Abiotic oxidation of PPD and PTD is known to generate electrophilic quinonediimine intermediates that subsequently form dimers and oligomeric products (Corbett 1969; Dolinsky et al. 1968; Goux et al. 2007; Jahn et al. 2012). Consistent with these reports, MPO-mediated oxidation of the selected aromatic diamines produced a broad spectrum of oxidation products, including mono-, di-, and trimeric species. PPD yields a defined product profile dominated by BB, whereas PTD and ME-PPD generated a more diverse pattern of mono-, di-, and trimeric metabolites (Fig. 4a).

The described MPO-derived metabolites that are identical or structurally closely related to previously reported abiotic oxidation products suggest that MPO catalyzes the formation of oxidation products already known from abiotic oxidation, while markedly accelerating their generation. For PPD, formation of BB and related oligomeric products has been attributed to highly reactive quinonediimine intermediates (Aptula et al. 2009; Corbett 1969), indicating that similar intermediates are generated during MPO-catalyzed oxidation. These findings suggest that MPO accelerates rather than fundamentally alters the oxidative pathway. This interpretation is further supported by the faster (up to eightfold) formation of BB in the presence of MPO than under enzyme-free conditions. BB is known as an oxidation product with greater skin sensitization potency than PPD in the local lymph node assay and oxidized PPD reaction mixtures containing BB induce dendritic cell activation, whereas PPD itself exhibits little to no activity (Aeby et al. 2009). Thus, the identification of BB demonstrates that MPO-mediated oxidation can generate highly potent sensitizing species. In addition, the present study provides the first evidence for MPO-mediated formation of monomeric and trimeric oxidation products of ME-PPD, whereas PTD produced a broader spectrum of structurally related oxidation products.

MPO-mediated oxidation of eugenol and dihydroeugenol generated monomeric quinone methides and dimeric coupling products (Fig. 4b). For eugenol, these findings are consistent with previous reports describing peroxidase-mediated formation of quinone methides and dimeric oxidation products (Bouhlef et al. 2012; Thompson et al. 1989). Quinone methides are highly electrophilic intermediates associated with increased protein reactivity (Thompson et al. 1989). The present study extends these observations to the structurally related dihydroeugenol, which exhibited a comparable product pattern. In contrast to PPD, PTD and ME-PPD, MPO-mediated oxidation of the phenylpropanoids was limited to monomeric and dimeric products, suggesting that their oxidation terminates at an earlier stage of the reaction sequence.

While BB and eugenol quinone methide provide examples of MPO-derived sensitizing species, they represent only a subset of the tentatively assigned metabolites in the present study. Since covalent binding of chemicals to skin proteins represents the MIE of skin sensitization, the protein-binding

potential of the postulated oxidation products was predicted using the OECD QSAR Toolbox. BB and eugenol quinone methide both possess structural alerts associated with protein-binding potential. In addition, several other postulated metabolites exhibited structural alerts for protein-binding, whereas the parent compounds, i.e., PPD, PTD, ME-PPD, eugenol, and dihydroeugenol, showed no structural alerts (Fig. 4). Notably, oxidation products predicted to possess protein-binding potential were consistently associated with quinone methide or quinonediimine functionalities. These electrophilic motifs are absent in the parent substrates and emerge only upon oxidation. Evaluation of isomeric metabolite structures further revealed that the predicted protein-binding potential depends on the nature of the electrophilic linkage (Online Resource 1 Table S4). This data further support that MPO has the potential to oxidize the investigated aromatic diamines and phenylpropanoids, leading to the formation of metabolites with predicted protein-binding potential from otherwise non-reactive parent substrates.

We showed that MPO oxidized PPD, PTD, ME-PPD, eugenol, and dihydroeugenol with comparable catalytic efficiencies. However, the extent of MPO-catalyzed oxidation cannot be directly translated into overall skin sensitization potency. This is illustrated by the no expected sensitization induction level (NESIL), which differs markedly among chemicals despite their similar catalytic efficiencies. For example, ME-PPD (1075 $\mu\text{g}/\text{cm}^2$) has an approximately 26- to 39-fold higher NESIL than PTD (41.3 $\mu\text{g}/\text{cm}^2$) and PPD (27.5 $\mu\text{g}/\text{cm}^2$), respectively (Goebel et al. 2012; Goebel et al. 2014). Likewise, eugenol and dihydroeugenol, although both compounds were converted with comparable catalytic efficiency, eugenol (5900 $\mu\text{g}/\text{cm}^2$) has an approximately 3.5-fold higher NESIL than dihydroeugenol (1700 $\mu\text{g}/\text{cm}^2$) (Api et al. 2016; Api et al. 2021). NESIL values integrate the outcome of multiple biological and toxicological processes occurring between chemical exposure and sensitization induction and therefore, cannot be explained by oxidative activation alone. This is further illustrated by non-animal approaches that assess downstream key events. For example, the cocultured activation test showed that ME-PPD required approximately 27- and 14-fold higher concentrations than PPD and PTD to induce dendritic cell activation (Hennen and Blömeke 2017), despite the comparable MPO-catalyzed oxidation observed here. These findings indicate that MPO-mediated oxidation represents one mechanistic step within the sequence of events leading to skin sensitization rather than a predictor of overall sensitization potency. Instead, the present study provides mechanistic evidence that MPO selectively oxidizes chemically susceptible substrates and generates metabolites with predicted protein-binding potential.

In summary, we show that human MPO selectively oxidizes PPD and its derivatives PTD and ME-PPD, as well as the phenylpropanoids eugenol and dihydroeugenol, whereas cinnamyl alcohol remained unconverted. Substrate orientation within the MPO active site determines productive conversion, as exemplified by the non-productive binding pose of cinnamyl alcohol. Enzymatic conversion of the substrates proceeded with similar catalytic efficiency. MPO-catalyzed oxidation was largely unaffected by physiologically relevant pH changes, except when shifts in substrate protonation altered productive enzyme-substrate interactions. HRMS-based metabolite profiling revealed MPO-derived oxidation products consistent with previously described oxidation products, including BB and the eugenol quinone methide, and revealed several additional metabolites. QSAR-based analysis further indicated that several MPO-derived metabolites possess structural features associated with protein-binding potential relevant

to the MIE of skin sensitization, whereas the corresponding parent compounds lacked such alerts. Collectively, these findings identify MPO as a substrate-selective oxidative activation system capable of generating protein-reactive species from chemically susceptible sensitizers. As MPO is released by infiltrating neutrophils during the induction phase of skin sensitization, MPO-mediated oxidation may influence the local chemical fate of susceptible sensitizers at sites of chemical exposure. Our findings extend current concepts of oxidative activation beyond abiotic oxidation and constitutive epidermal metabolism. Future studies incorporating direct protein adduct analysis and more complex human-relevant skin models will help define the quantitative contribution of MPO-catalyzed oxidation to skin sensitization.

Abbreviations

BB	Bandrowski's Base
ESI	Electrospray Ionization
HPLC	High-Performance Liquid Chromatography
HRMS	High-Resolution Mass Spectrometry
k_{cat}	Turnover Rate
$k_{\text{cat}}/K_{\text{M}}$	Catalytic Efficiency
K_{M}	Michaelis-Menten Constant
ME-PPD	2-Methoxymethyl- <i>p</i> -Phenylenediamine
MIE	Molecular Initiating Event
MPO	Myeloperoxidase
NESIL	No Expected Sensitization Induction Level
OECD	Organization for Economic Co-operation and Development
PPD	<i>p</i> -Phenylenediamine
PTD	Toluene-2,5-diamine
QSAR	Quantitative Structure Activity Relationship
QToF	Quadrupole Time-of-Flight Mass Spectrometer

Declarations

Supplementary Information The online version contains supplementary material.

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Author Contributions B.B., C.G. and U.B. conceptualized the study. H.D., P.P., U.B. and S.L. developed the methodology. P.P. and H.D. performed experimental work and data curation. P.P., H.D., U.B. and K.C.F. conducted formal analysis. H.D. and K.C.F. prepared the figures and visualizations. H.D. and K.C.F. wrote the original draft of the manuscript. All authors contributed to data interpretation, critically reviewed and edited the manuscript, and approved the final version. B.B., C.G. and U.B. acquired funding. B.B. and C.G. performed project administration and supervision.

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Data Availability All data are available upon request.

Conflicts of Interest Philipp Peslalz, Karma C. Fussell and Sabine Langhoff are employees of the Wella Company. Hannah Deusinger and Carsten Goebel were employed by the Wella Company for most of the study period. The Wella Company produces and markets hair coloring products.

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Figures

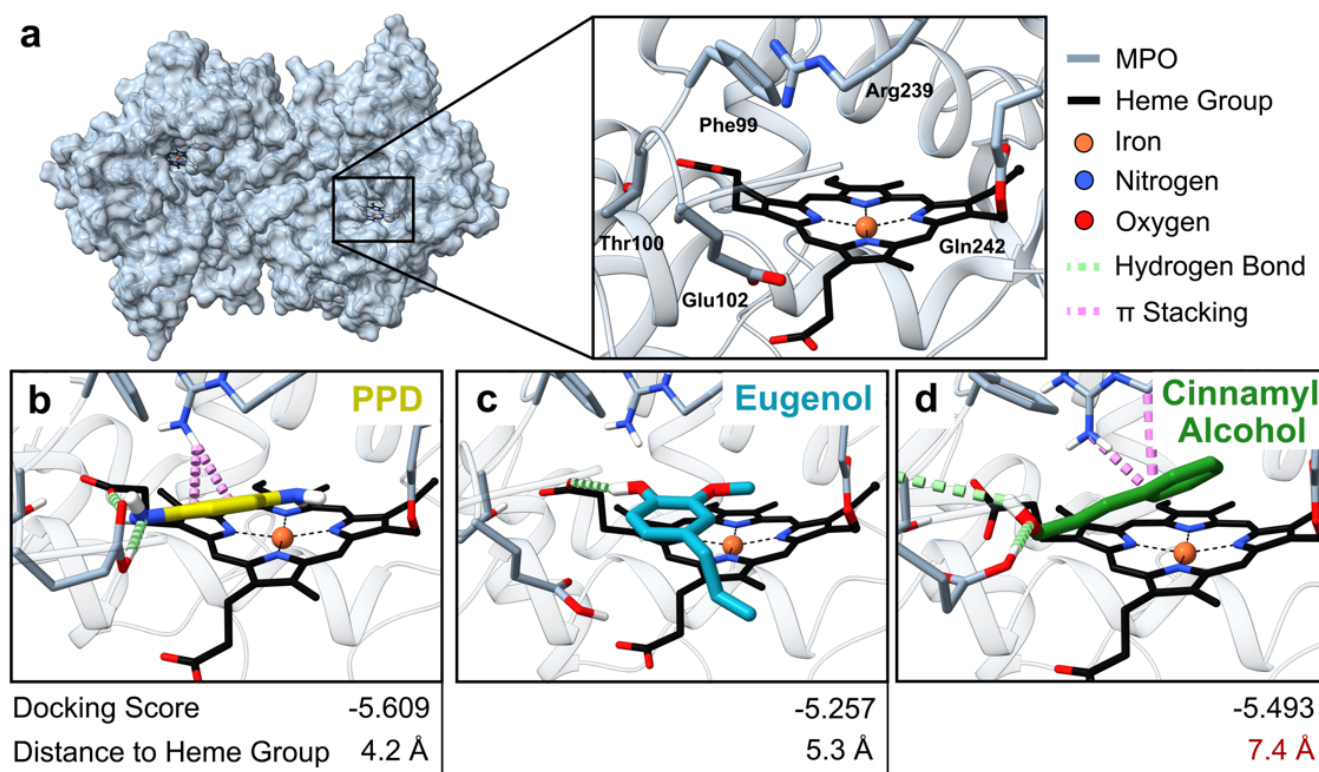


Figure 1

Molecular Docking Reveals Similar Docking Scores but Distinct Binding Orientations of *p*-Phenylenediamine (PPD), Eugenol and Cinnamyl Alcohol within the Binding Pocket of Human Myeloperoxidase (MPO). The 3D structure of MPO (a) reveals two identical heme-containing binding pockets (black) with the amino acids involved in substrate positioning (inset). PPD (b, yellow), eugenol (c, turquoise), and cinnamyl alcohol (d, dark green) were each docked in one binding pocket using induced fit calculations. Interactions between the compound and the enzyme are marked as dotted lines (rose = π stacking, light green = hydrogen-bonding). Docking scores as well as the distance from the putative reactive heteroatom to the catalytic heme group (Å) were determined

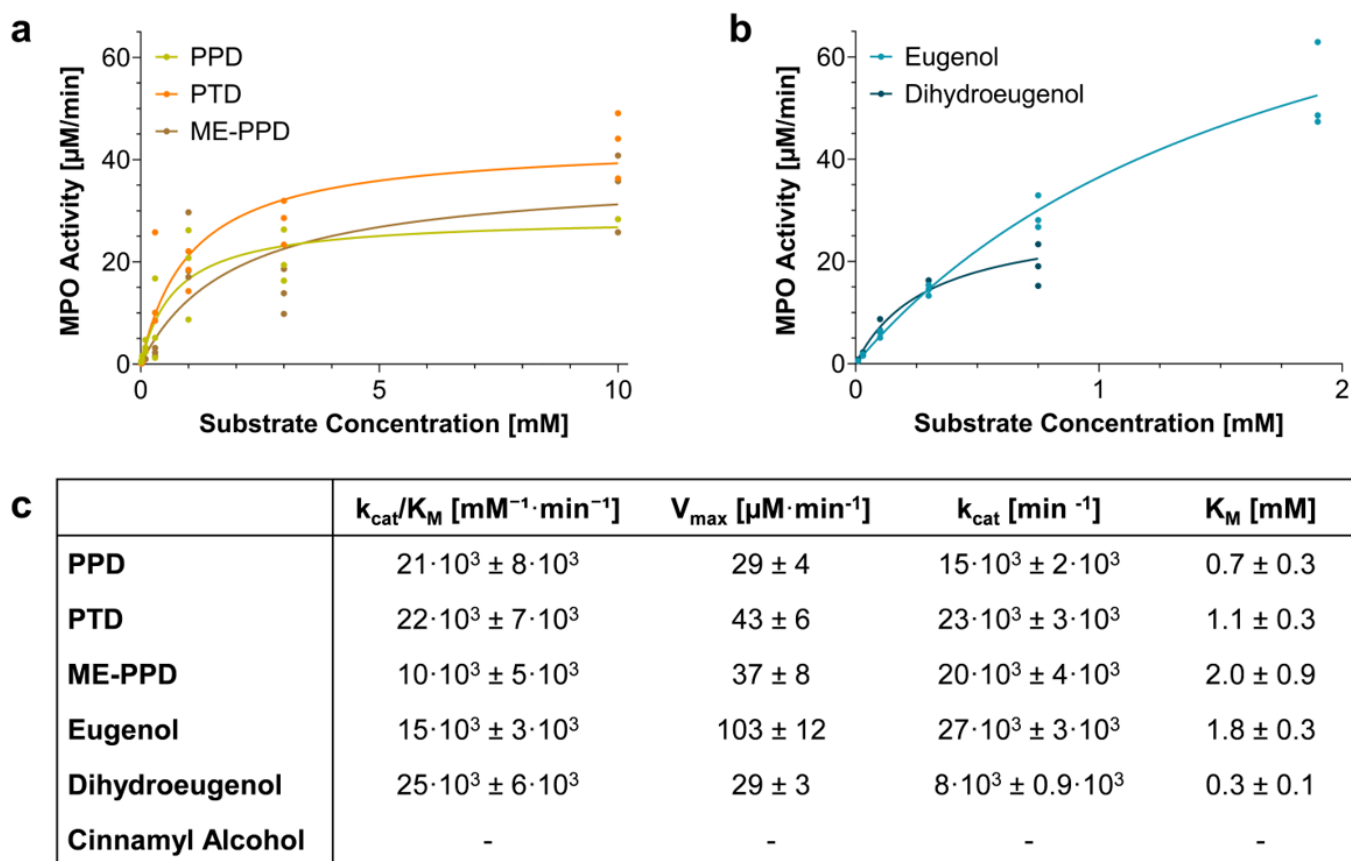


Figure 2

Human Myeloperoxidase (MPO) Converts *p*-Phenylenediamine(PPD)-related Aromatic Diamines and Phenylpropanoids and Reveals Comparable Catalytic Efficiencies Despite Differences in Turnover and Binding Affinity. Substrate depletion was quantified by HPLC-UV with different substrate concentrations ($n = 3$ per 5-7 concentrations due to differences in solubility limits). PPD, toluene-2,5-diamine (PTD), 2-methoxymethyl-PPD (ME-PPD) (a), and eugenol and dihydroeugenol (b) were converted by MPO concentration-dependently, while cinnamyl alcohol showed no detectable conversion and was therefore not included in the kinetic analysis. Depletion curves were fitted to the Michaelis-Menten model to derive maximum reaction velocity V_{max} and binding affinity K_M , from which catalytic efficiency k_{cat}/K_M , and turnover rate k_{cat} were calculated (value $\pm$ SEM, c)

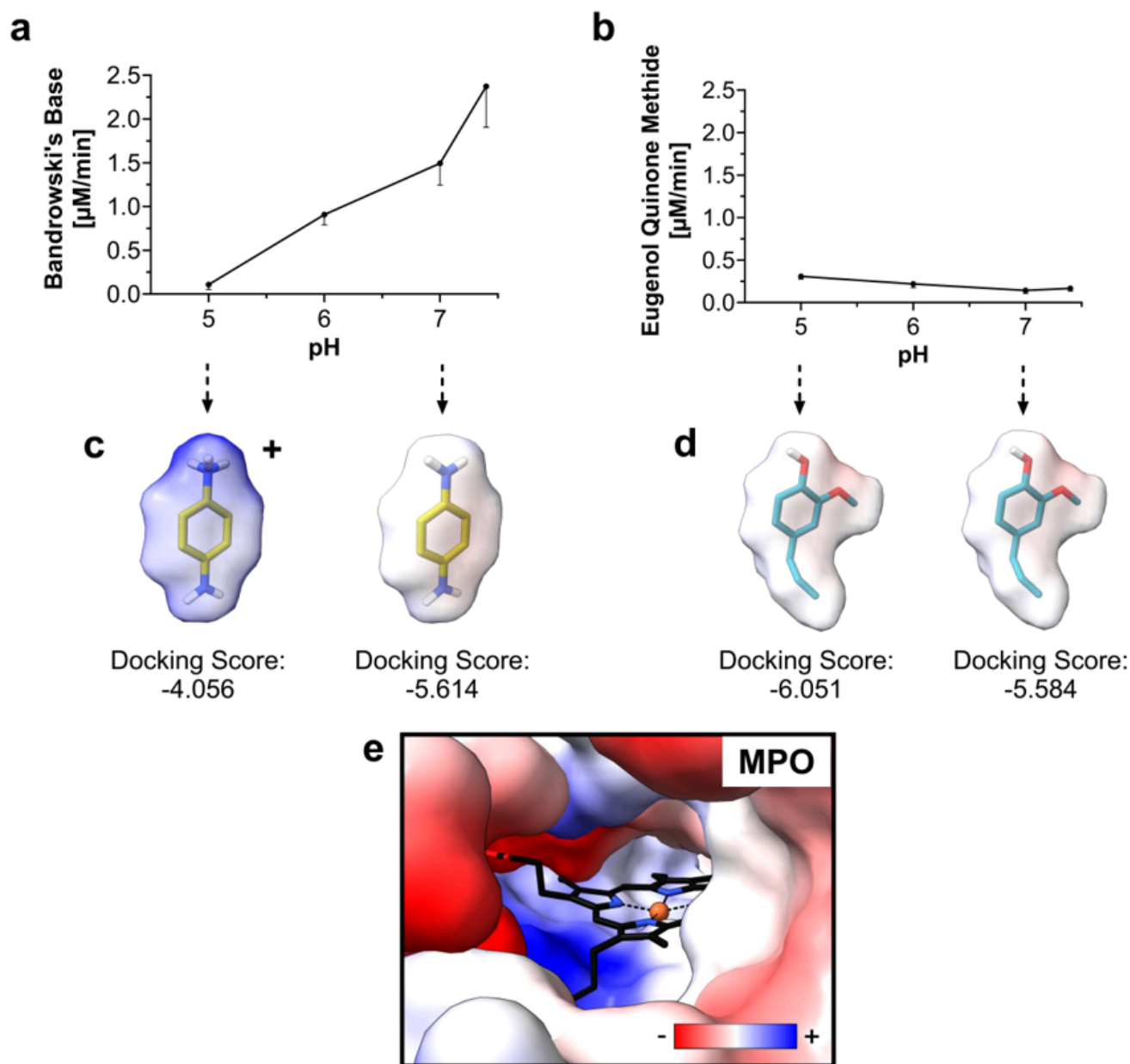


Figure 3

Alterations in pH Influence Conversion of *p*-Phenylenediamine (PPD) by Human Myeloperoxidase (MPO), but not Eugenol. MPO-mediated formation of a PPD-derived metabolite, monitored at 520 nm and consistent with Bandrowski's Base, decreased at acidic pH relative to neutral pH (a), whereas formation of an eugenol-derived metabolite, monitored at 350 nm and consistent with the quinone methide-type species, remained largely unaffected (mean $\pm$ SD, $n = 4$, determined by UV-Vis; b). Molecular docking of PPD and eugenol into the MPO binding pocket showed that docking scores and protonation state vary across pH conditions for PPD but remain comparable for eugenol (c-d). The electrostatic environment of the MPO binding pocket (pH 5.5, (Fiedler et al. 2000)) revealed charged regions surrounding the catalytic heme group (e)

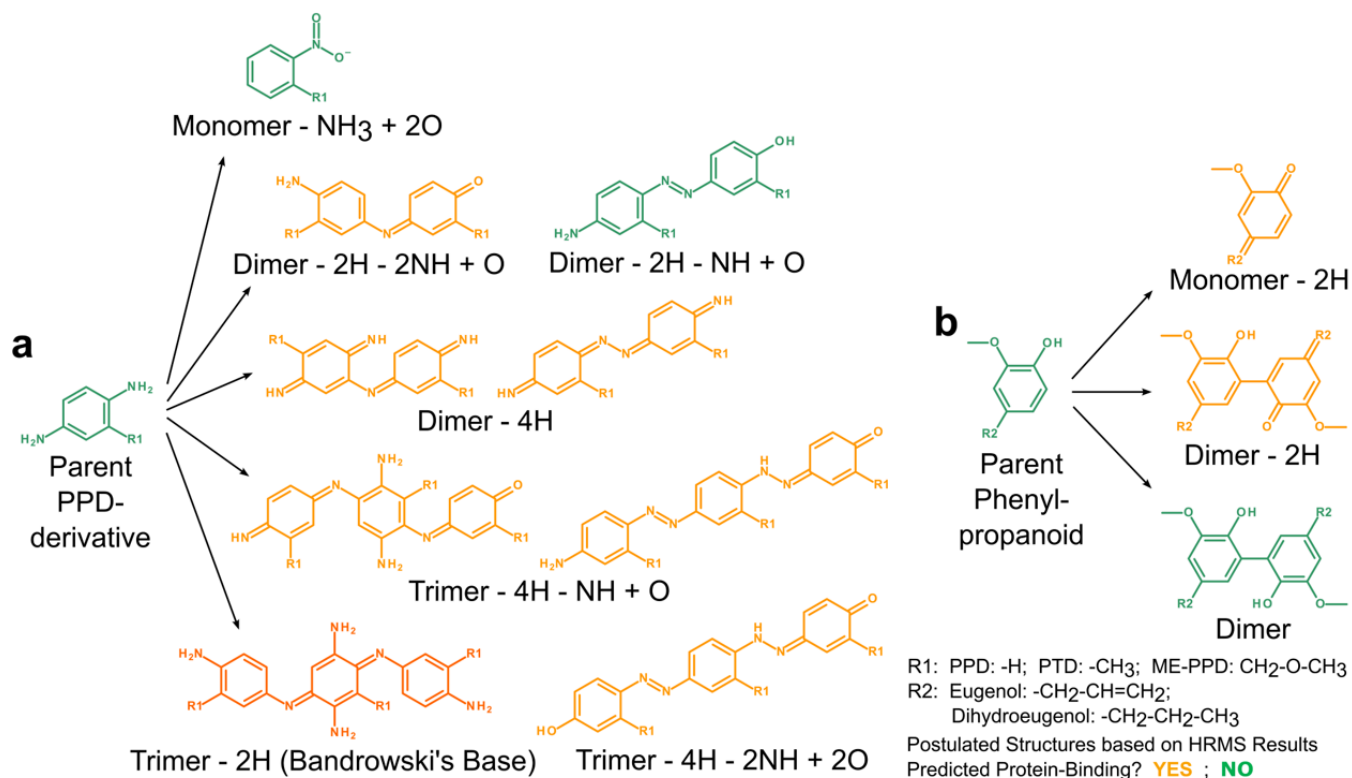


Figure 4

Protein-Binding Potential Associated with Skin Sensitization was Predicted for Human Myeloperoxidase (MPO)-Derived Metabolites of *p*-Phenylenediamine(PPD)-related Aromatic Diamines and Phenylpropanoids, but Not the Parent Compounds. Oxidation products derived from selected aromatic diamines (PPD, toluene-2,5-diamine [PTD], and 2-methoxymethyl-PPD, a) and phenylpropanoids (eugenol and dihydroeugenol, b), postulated from HRMS analysis, are shown. The structures were evaluated based on protein-binding potential using the OECD QSAR Toolbox (Defined Approaches for Skin Sensitization – Automated Workflow, no simulators) and color-coded (orange = protein binding predicted, green = no protein binding). For the aromatic diamines, enzymatic oxidation resulted in a diversity of mono-, di- and trimeric metabolites, including Bandrowski's Base (Trimer - 2H, highlighted with a darker shade). For the phenylpropanoids, oxidation yielded monomeric and dimeric species exhibiting protein-binding alerts. All parent substrates lacked protein reactivity

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