Chemical Research in Toxicology

Article

pubs.acs.org/crt

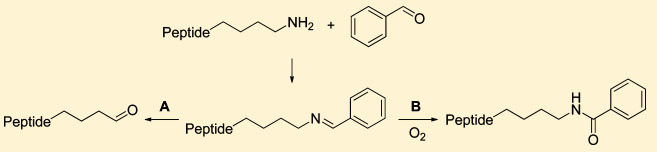
Chemical Reactivity and Skin Sensitization Potential for Benzaldehydes: Can Schiff Base Formation Explain Everything?

Andreas Natsch,* Hans Gfeller, Tina Haupt, and Gerhard Brunner

Givaudan Schweiz AG, Ueberlandstrasse 138, CH-8600 Duebendorf, Switzerland

Supporting Information

ABSTRACT: Skin sensitizers chemically modify skin proteins rendering them immunogenic. Sensitizing chemicals have been divided into applicability domains according to their suspected reaction mechanism. The widely accepted Schiff base applicability domain covers aldehydes and ketones, and detailed structure−activity-modeling for this chemical group was presented. While Schiff base formation is the obvious reaction pathway for these chemicals, the *in silico* work was followed up by limited experimental work. It remains unclear whether hydrolytically labile Schiff bases can form sufficiently stable epitopes to trigger an immune response in the living organism with an excess of water being present. Here, we performed experimental studies on benzaldehydes of highly differing skin sensitization potential. Schiff base formation toward butylamine was evaluated in acetonitrile, and a detailed SAR study is presented. *o*-Hydroxybenzaldehydes such as salicylaldehyde and the oakmoss allergens atranol and chloratranol have a high propensity to form Schiff bases. The reactivity is highly reduced in *p*-hydroxy benzaldehydes such as the nonsensitizing vanillin with an intermediate reactivity for *p*-alkyl and *p*-methoxy-benzaldehydes. The work was followed up under more physiological conditions in the peptide reactivity assay with a lysine-containing heptapeptide. Under these conditions, Schiff base formation was only observable for the strong sensitizers atranol and chloratranol and for salicylaldehyde. Trapping experiments with $NaBH_3CN$ showed that Schiff base formation occurred under these conditions also for some less sensitizing aldehydes, but the reaction is not favored in the absence of *in situ* reduction. Surprisingly, the Schiff bases of some weaker sensitizers apparently may react further to form stable peptide adducts. These were identified as the amides between the lysine residues and the corresponding acids. Adduct formation was paralleled by oxidative deamination of the parent peptide at the lysine residue to form the peptide aldehyde. Our results explain the high sensitization potential of the oakmoss allergens by stable Schiff base formation and at the same time indicate a novel pathway for stable peptide-adduct formation and peptide modifications by aldehydes. The results thus may lead to a better understanding of the Schiff base applicability domain.



INTRODUCTION

Skin sensitizers are reactive exogenous molecules with the ability to chemically modify skin proteins.[1] The modified proteins represent novel nonself epitopes to the immune system and thus trigger a specific T-cell mediated immune response. On the basis of this mechanistic understanding, the relationship between reactivity of molecules toward proteins and their sensitization potential has attracted increased interest and was used to develop experimental assays to predict the skin sensitization potential of chemicals to replace the current animal tests.[2] Gerberick et al.[3] developed a peptide depletion assay using different heptapeptides. This assay, later coined the DPRA (direct peptide reactivity assay), has gone through thorough prevalidation at ECVAM and it gives a rapid overall estimate of reactivity of test chemicals toward peptides. Chipinda et al.[4] used nitrobenzenthiol as a simple surrogate for a reactive cysteine in a peptide and developed a kinetic profiling approach to compare sensitizers. A large database on glutathione depletion has also been used to establish a correlation between reactivity and skin sensitization.[5] The link between sensitization and reactivity has also led to the development of a number of *in silico* approaches to predict which chemicals are reactive and thus sensitizers and to quantify their sensitization potency based on *in silico* SAR-modeling.[6] In one such paradigm, chemicals are first classified into so-called applicability domains according to their suspected reaction mechanism, which is inferred from functional groups present in the molecules.[7] Experimental or *in silico* reactivity indices and physicochemical parameters for chemicals within an applicability domain are then used to model their *in vivo* sensitization potential.[8]

One widely accepted applicability domain contains all chemicals with the theoretical capability to form Schiff bases.[7,9,10] The sensitization potential of these molecules has been modeled based on the Taft σ^* and $\Sigma\sigma^*$ constants used as reactivity indices and cLogP as physicochemical parameter, and a QSAR was developed which predicts the sensitization potential of the chemicals in this applicability domain nicely.[9] However, this was not followed up by experimental work to prove Schiff base formation of these chemicals toward peptides or proteins. However, using the peptide reactivity approach, Gerberick et al.[11] had screened a significant number of aldehydes and ketones for their reactivity toward a test peptide

Received: June 18, 2012

   dx.doi.org/10.1021/tx300278t | *Chem. Res. Toxicol.* XXXX, XXX, XXX−XXX

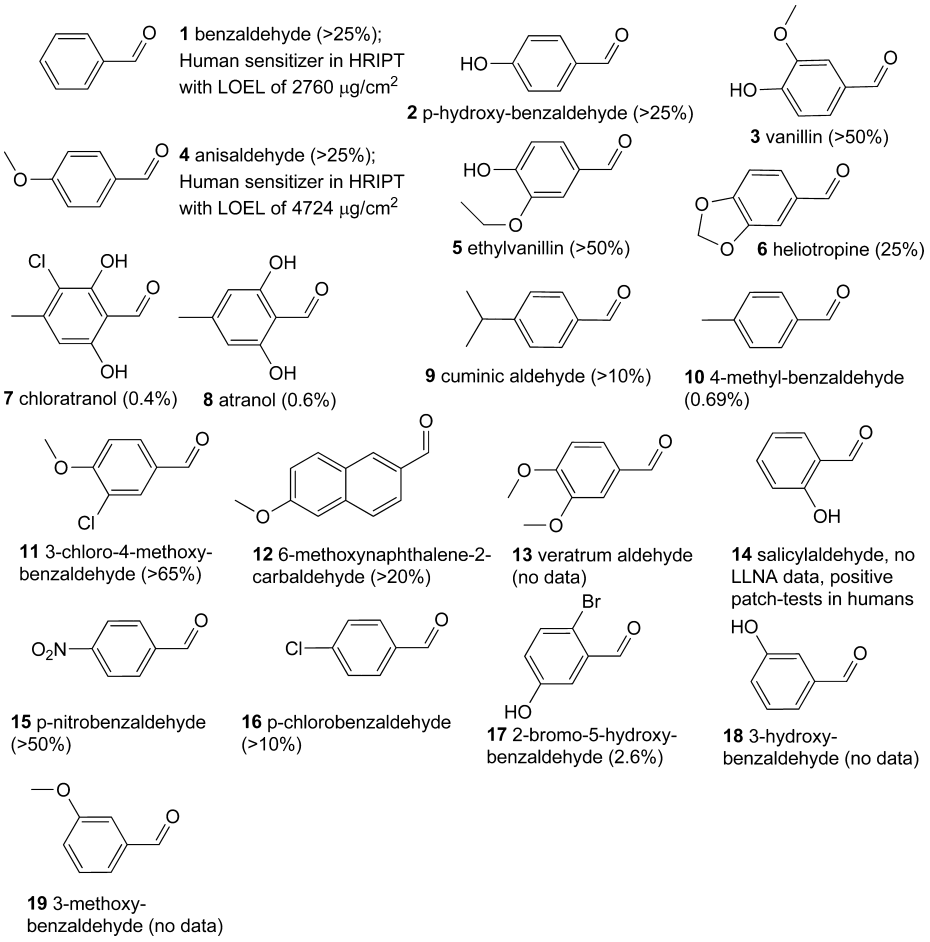


Figure 1. Structure of the tested benzaldehydes. In parentheses is shown the sensitization potential as an EC3 value in the LLNA or, if available, other information regarding the skin sensitization potential. For **13**, **18**, and **19**, no data regarding the skin sensitization potential are available, but these chemicals were included to complete the SAR study for reactivity.

with a lysine residue. The sensitizing aldehydes α-hexyl cinnamic aldehyde, hydroxycitronellal, lilial, cyclamen aldehyde, benzaldehyde, and farnesal did not lead to significant peptide depletion,[11] although the reactions were run at pH 10.2 to keep the lysine residue in its unprotonated form and in the presence of a large excess of test chemical (50-fold over peptide). Similarly, for the same aldehydes we could not observe adduct formation in a peptide reactivity assay with a heptapeptide containing both Cys and Lys residues.[12] These chemicals only promoted the formation of disulfide bridges at the Cys residue. However, under much more concentrated reaction conditions (80 mM peptide and 200 mM test chemical), Reichhardt et al.[13] reported Schiff base formation with the ϵ-NH_2-group of Lys-Tyr for the two aldehydes formaldehyde and benzaldehyde. Furthermore, supplementary information from Aleksic et al.[14] reports Schiff base formation in a peptide reactivity assay for cyclamen aldehyde, hydroxycitronellal, benzaldehyde, and α-hexyl cinnamic aldehyde. The test conditions in this assay were selected to favor Schiff base formation by applying a 100-fold excess of test chemical and pH 10. Still, in these cases peptide depletion was only marginal, and the reaction was monitored by the more sensitive approach of following adduct formation.

Although Schiff base formation is an obvious reaction mechanism for small aldehydes to modify skin proteins, there is an open question which to our surprise has attracted little attention: In water, Schiff base formation is a reversible reaction reaching an equilibrium, and the Schiff base would be expected to persist only as long as the free aldehyde is present in significant quantities. Since modified peptides need to be transported by migrating dendritic cells to local lymph nodes to trigger the immune response, and since the locally and topically applied aldehyde will not be present all along the migration route and in the lymph node, it is not inherently clear how Schiff bases could form sufficiently stable epitopes which are not hydrolyzed prior to T-cell presentation.

In summary, there is a certain discrepancy between the theoretical and *in silico* work postulating Schiff base formation as a key mechanism for skin sensitization by aldehydes and ketones and the experimental data on stable adduct formation by the Schiff base mechanism in the now widely used peptide reactivity assays such as the DPRA.

Benzaldehydes (BAs) represent an interesting class of chemicals to study with respect to their skin sensitization potential. This class comprises chloratranol (**7**) and atranol (**8**), the allergens in oakmoss, for which very low thresholds for elicitating immune responses in sensitized patients have been reported and which are the true culprit of relatively frequent positive patch test reactions of dermatitis patients to oakmoss absolute.[15,16] At the other extreme are vanillin (**3**) and ethylvanillin (**5**), which are widely used in flavor and fragrance compositions and which are generally considered nonsensitizers in animal tests and in humans. BA (**1**) was for a long time

dx.doi.org/10.1021/tx300278t | *Chem. Res. Toxicol.* XXXX, XXX, XXX−XXX

reported as a classical nonsensitizer based on animal tests but was recently discovered to be a sensitizer in a human repeat insult patch test (HRIPT; RIFM, personal communication). This result contrasts with a published theoretical consideration arguing for slow Schiff base formation for BA due to a resonance loss effect.[17] The broad span in *in vivo* sensitizing potency in humans and the conflicting evidence from some modeling studies made the BAs an interesting case to experimentally study the Schiff base applicability domain in more detail. Here, we thus report a SAR study for reactivity of differently substituted BAs with butylamine followed by detailed analysis of reactivity in a slightly modified DPRA protocol, and we show that in water, Schiff base formation leads to stable adducts only for *o*-hydroxy BAs. At the same time, Schiff base formation appears to be the first reaction step in a sequence involving oxidative events leading to stable peptide adducts and peptide modifications by other benzaldehyde derivatives and other sensitizing aldehydes.

MATERIALS AND METHODS

Caution: *Some of the test chemicals are mild to strong skin sensitizers in the LLNA, and any skin contact should be avoided.*

Chemicals. All fragrance chemicals were obtained from Givaudan Schweiz AG. Other BA derivatives were obtained from Sigma−Aldrich (Buchs, Switzerland). Atranol and chloratranol were synthesized according to the following procedure: O-Alkylation (MeI, K_2CO_3, acetone)[18] of 5-methyl-1,3-benzenediol (orcinol) led to orcinol dimethyl ether (47%). Subsequent *t*-BuLi deprotonation followed by quenching with DMF cleanly afforded atranol dimethyl ether (67%) that was monochlorinated in good yield (85%) into chloroatranol dimethyl ether using SO_2Cl_2 in CH_2Cl_2 (0 to 20 °C). De-O-methylation of the two formyl diethers using BBr_3 in CH_2Cl_2 (−70 to 20 °C) provided atranol and chloroatranol (13% and 80% yield, respectively). The structures of all tested BAs are shown in Figure 1; this Figure also gives the sensitization potential from the LLNA and if available human evidence for sensitization risks. The LLNA data came from different sources as indicated in Table S1 in Supporting Information. The majority of LLNA and HRIPT data were kindly provided by RIFM (Research Institute for Fragrance Materials).

Reactions with Butylamine Monitored by GC-FID and GC/MS Analysis. The test chemicals (100 μL) dissolved at 2 mM in acetonitrile were mixed with an equal volume of butylamine dissolved at 20 mM in acetonitrile. After 6 h of incubation at 22 °C, the reactions were extracted with 200 μL of sodium phosphate buffer (pH 7.5, 20 mM), 50 mg of NaCl, and 200 μL of MTBE. The organic phase was analyzed with GC/MS. A combination of a Hewlett-Packard 6890 gas chromatograph and an Agilent 5973 MSD mass spectrometer (70 eV EI mode, ion source temperature 230 °C, Agilent Technologies, Santa Clara, CA, USA) was applied. A capillary column of 12 m length × 0.22 mm i.d. coated with 0.25 μm of BPX5 (5% phenyl polysilylphenylene-siloxane, SGE Analytical Science Pty Ltd., Ringwood, Australia) was used. Sample volumes of 1.0 μL were injected in the splitless mode at an injector temperature of 230 °C. Temperature of the column oven was initially set to 35 °C for 2 min and subsequently increased by 20 °C/min to 240 °C, then by 35 °C/min to 270 °C, and held for 3 min. For quantitative analysis, samples were injected on an Agilent 6890N equipped with a flame-ionization detector and a BGB-5 capillary column (BGB Analytik, Böckten, Switzerland) of 30 m length × 0.32 mm i.d. and 0.25 μm coating with 5% diphenyl−95% dimethylpolysiloxane. The column was held at 40 °C for 1 min and subsequently increased by 8 °C/min to 280 °C and held at this temperature for 3 min.

Reactions with Butylamine in a Dose−Response Analysis. The test chemicals were dissolved at 10 mM in acetonitrile, and serial dilutions down to 0.625 mM were prepared in microtiter plates. To 100 μL of these dilutions, an equal volume of butylamine dissolved at 0.5 mM in acetonitrile was added. Samples were incubated for 3 or 6 h at 22 °C, and the reaction was stopped by adding 50 μL of a 4 mM solution of fluram (Fluka, Buchs, Switzerland). The fluorescence due to the reaction of the free amine groups with fluram was measured (bottom-read fluorescence; excitation at 381 nm, emission at 470 nm; Flexstation, Molecular Devices).

Peptide Reactivity Experiments. The Lys-peptide (Ac-RFAA-KAA, MW 775.4) was obtained from Genscript Inc. (Piscataway, NJ, USA). It has a purity of 98.1%. It was used at a final concentration of 0.5 mM in all experiments. All peptide reactivity tests at pH 7.5 were run at 36 °C in 75% phosphate buffer (20 mM) and 25% acetonitrile with shaking at 150 rpm. Different concentrations of test chemicals dissolved in acetonitrile were used as specified in the Results section. In order to perform *in situ* reduction of formed Schiff bases, the reactions were performed in the presence of 50 mM sodium cyanoborohydride ($NaBH_3CN$).

To determine chemical modifications of peptides, reactions were carried out in a final volume of 1 mL in HPLC vials. LC/MS analysis was performed as described before on a Finnigan LCQ classic mass spectrometer (Thermo Finnigan, San Jose, CA, U.S.A.) operated in the ESI(+) mode.[12] The ion source was operated at 5 kV, the sheath gas flow rate was set to 60 arb and the aux gas flow rate to 20 arb. The temperature of the capillary was kept at 220 °C. Mass spectra were recorded from 400 to 2000 amu. A ZORBAX Eclipse XDB-C18 column, 2.1 mm ID, 150 mm, 5-Micron (Agilent Technologies) was used. The mobile phase consisted of H_2O and methanol, each containing 0.1% formic acid (v/v). The solvent flow was 250 μL/min.

HPLC Separation of Modified Peptides. The peptide adducts from the reaction of BA with the Lys-peptide were isolated by reversed-phase HPLC on a YMC Pack ODS-AQ column (250 mm length, 3.0 mm diameter, 3.0 μm particles) with a gradient from with 10%−100% methanol in 0.1% formic acid in water.

NMR Analysis of Modified Peptides. All NMR experiments were performed on a Bruker Avance III 600 MHz FT-NMR spectrometer equipped with a MicroCryoprobe 1.7 mm TCI (Triple Inverse Cryoprobe) with a Z-Gradient coil. The sample (45 μg) was dissolved in 35 μL of D_2O 99.96% D (euriso-top, D215B). The residual H_2O signal was used as the chemical shift reference at 4.79 ppm. ^{1}H NMR with presaturation: spectral width = 20.5 ppm, acquisition time = 2.7 s, recycle time = 1.0 s, time domain = 64 K points, size of real spectrum = 128 K points, window function = Gaussian multiplication, line broadening −0.2, Gaussian factor 0.2, number of scans = 16, attenuation for presaturation = 60.7 dB, and temperature = 308 K. Gradient selected HSQC, spectral width = 10.8 ppm (^{1}H) × 200.0 ppm (^{13}C); acquisition time, 158 ms; and recycle time, 2.0 s; time domain = 2 K (^{1}H) × 512 (^{13}C) points, size of real spectrum = 1024 × 1024 points, window function = squared sine-bell (phase shift = 2) in both dimensions, number of scans = 32, and temperature = 308 K. Gradient selected HMBC: spectral width = 9.4 ppm (^{1}H) × 200.0 ppm (^{13}C), acquisition time = 181 ms, recycle time = 2.0 s, time domain = 2 K (^{1}H) × 512 (^{13}C) points, size of real spectrum = 2048 (^{1}H) × 1024 (^{13}C) points, window function = sine-bell (phase shift = 4, ^{1}H), squared sine-bell (phase shift = 2, ^{13}C), number of scans = 128, and temperature = 308 K. 1D-selective TOCSY: spectral width = 9.4 ppm (^{1}H) × 200.0 ppm (^{13}C), acquisition time = 2.7 s, recycle time = 2.0 s, time domain = 32 K points, size of real spectrum = 32 K points, window function = exponential multiplication, line broadening of 0.3, number of scans = 128, mixing time = 20−120 ms, and temperature = 298 K.

RESULTS

Schiff Base Formation with Butylamine in Acetonitrile and Analysis by GC and GC-FID. First, the reactivity of the test chemicals dissolved at 1 mM in the presence of a 10-fold excess of butylamine in ACN was studied. For **1**, **2**, **3**, **5**, **7**, **8**, **11**, **12**, **13**, and **14**, the reaction products were analyzed by GC-MS. For all tested chemicals with the exception of **7** and **8**, one new chromatographic signal besides the parent BA signals was observed. The molecular ions and the fragmentation patterns in the associated mass spectra could be interpreted or directly

dx.doi.org/10.1021/tx300278t | *Chem. Res. Toxicol.* XXXX, XXX, XXX−XXX

identified by reference mass spectra comparison (available for parent BA **1**, **3**, and **5**) as the respective Schiff base products. For **7** and **8**, only the Schiff base and no parent aldehyde were detected, and as a byproduct, low levels of *N*-butylidene-1-butanamine were observed.

Conversion rates after 6 h were determined by GC/FID for the differentially substituted BAs in Figure 1 (Table 1). Schiff base formation was almost quantitative for the *o*-hydroxy BAs (i.e., the oakmoss allergens **7** and **8** as well as for **14**) Comparatively low levels of Schiff base formation were observed for *p*-hydroxy BAs as compared to *o*-hydroxy BAs. Low levels of Schiff bases were found for *p*-methoxy BAs. Conversion rates for *p*-NO_2-, *p*-alkyl- BAs, and BA were inbetween.

Table 1. Binding of Benzaldehydes by an Excess of Butylamine

no.	name	% free aldehyde[a]	% bound aldehyde
8	atranol	0	100[b]
7	chloratranol	0	100[b]
14	salicylaldehyde	3.9 ± 1.9	96.1 ± 1.9[b]
1	benzaldehyde	35.9 ± 11.9	64.1 ± 11.9[b]
17	2-bromo-5-hydroxy-benzaldehyde	47.6 ± 1.6	52.4 ± 1.6
15	4-nitrobenzaldehyde	54.4 ± 3.4	45.6 ± 3.4
10	4-methylbenzaldehyde	59.7 ± 6.9	40.3 ± 6.9
18	3-hydroxybenzaldehyde	61.5 ± 2.1	38.5 ± 2.1
16	*p*-chlorobenzaldehyde	81.6 ± 2.9	18.4 ± 2.9
19	3-methoxybenzaldehyde	82.2 ± 2.1	17.8 ± 2.1
2	4-hydroxybenzaldehyde	84.7 ± 2	15.3 ± 2[b]
9	cuminic aldehyde	86.5 ± 4.4	13.5 ± 4.4
3	vanillin	88.1 ± 2	11.9 ± 2[b]
5	ethylvanillin	92.8 ± 3.7	7.2 ± 3.7[b]
11	3-chloro-4-methoxybenzaldehyde	94.2 ± 1.2	5.8 ± 1.2[b]
4	4-methoxybenzaldehyde	95.1 ± 0.1	4.9 ± 0.1
6	heliotropine	96.4 ± 0.1	3.6 ± 0.1
12	6-methoxynaphtalene-2-carbaldehyde	96.8 ± 1.7	3.2 ± 1.7[b]
13	veratrumaldehyde	97.9 ± 1.9	2.1 ± 1.9[b]

[a]1 mM of test chemical was reacted with 10 mM butylamine. The peak areas from GC-FID analysis of the parent aldehyde and the reaction product were integrated, and the sum was set to 100%. [b]In these cases, GC-MS analysis was used to verify that the structure of the bound fraction indeed is the Schiff base.

Dose–Response Analysis to Quantify Reactivity with Butylamine. The experimental procedure chosen above is well suited to monitor formation of new products, but it is not ideal for a quantitative structure–activity study of the reaction rate. Since the reaction was stopped by organic extraction to separate the free butylamine and since some of the test chemicals have relatively high water solubility, the extraction step is not quantitative. Furthermore, a large excess of butylamine was used, which may, in general, facilitate the reaction. We thus performed a dose–response analysis without an extraction step and with determination of the free amine at the end of a fixed incubation time with a fluorescent probe. Dilutions of the test chemicals (0.312 mM to 5 mM) were reacted with 0.25 mM butylamine, and the unbound butylamine was quantified after 3 h and 6 h. Figure 2 presents the logarithmic dose–response curves for compounds **1**, **3**, **4**, **7**, **10**, and **14**. Table 2 presents all the data. For each chemical, linear regression was performed from data plotted as shown in Figure 2. The slope, which is proportional to the rate constant,[19] is given in Table 2 for reactions stopped after 3 and 6 h. In addition, the % depletion of 0.25 mM butylamine by 5 mM of the test chemical is also listed, as these data may be easier to read.

In this study, the *o*-hydroxy BAs **14** (salicylaldehyde), **7** (atranol), and **8** (chloratranol) as well as **1** (benzaldehyde) had the highest reactivity. Residual reactivity is observed for *p*-methoxy BAs and no significant reactivity for *p*-hydroxy BAs such as vanillin. The dramatic difference in reactivity between these latter chemicals and chemicals such as atranol and chloratranol supports the hypothesis that the differential ability to bind amine nucleophiles may explain their highly differing sensitization potential.

Peptide Reactivity Assays: Quantifying Schiff Base Formation with Trapping by $NaBH_3CN$. Next, we performed a modified version of the DPRA assay to observe Schiff base formation in a mainly aqueous milieu (75% buffer and 25% ACN). We used a pH of 7.5 and a low amount of test chemicals (1 mM) and trapped the formed Schiff bases by *in situ* reduction with $NaBH_3CN$. This low amount of test peptide was selected since $NaBH_3CN$ trapping does facilitate the reaction, and with higher amounts of test chemical, double

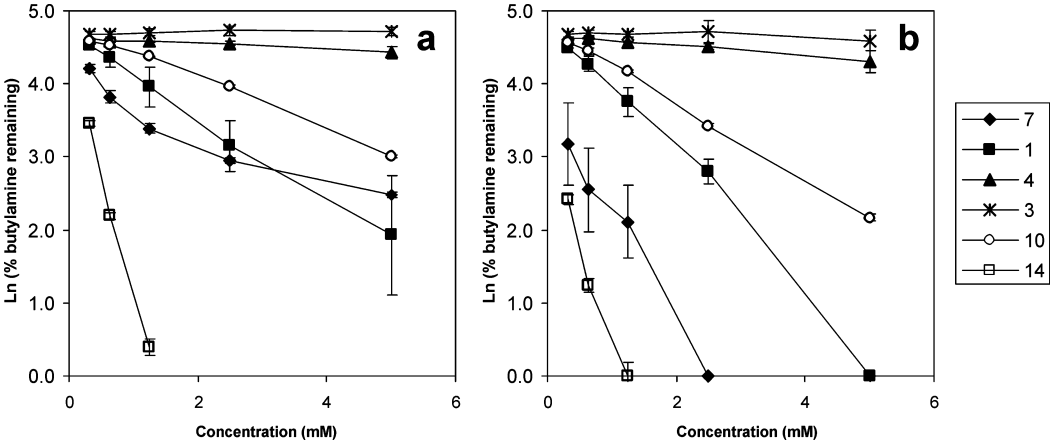


Figure 2. Dose–response analysis of butylamine binding by benzaldehyde derivatives. Test chemical (0.3125–5 mM) was incubated with 0.25 mM butylamine for (a) 3 h and (b) 6 h, and the unbound butylamine was determined with the amine-specific probe fluorescamine.

dx.doi.org/10.1021/tx300278t | *Chem. Res. Toxicol.* XXXX, XXX, XXX–XXX

Table 2. Dose−Response Analysis of Butylamine-Binding by Benzaldehyde Derivatives

		3 h incubation		6 h incubation	
		% depletion at 5 mM	slope	depletion at 5 mM	slope
14	salicylaldehyde	99.8 ± 0.6	−3.20	97.2 ± 3.6	steep
1	benzaldehyde	93.3 ± 3.2	−0.56	95.4 ± 2.6	−0.77
16	4-chlorobenzaldehyde	89.5 ± 2.6	−0.49	91.3 ±	−0.52
7	chloratranol	88 ± 0.4	−0.34	95.3 ± 1.2	−1.08
10	4-methylbenzaldehyde	76.2 ± 7.2	−0.31	90.9 ± 3.6	−0.52
8	atranol	74.1 ± 1.5	−0.29	94.3 ± 0.8	−0.59
19	3-methoxybenzaldehyde	68.2 ± 1.2	−0.26	90.4 ± 0.2	−0.50
15	4-nitrobenzaldehyde	61.3 ± 3.8	−0.20	54.5 ± 15	−0.17
17	2-bromo-5-hydroxy-benzaldehyde	59.9 ± 3.1	−0.21	90.8 ± 0	−0.51
9	cuminic aldehyde	55.6 ± 4.8	−0.18	81 ± 5.9	−0.36
4	4-methoxybenzaldehyde	16.3 ± 7.8	−0.04	25.9 ± 11.1	−0.07
12	6-methoxynaphtalene-2-carbaldehyde	9.4 ± 1.4	−0.03	15 ± 1.3	−0.05
11	3-chloro-4-methoxybenzaldehyde	8.3 ± 1.4	−0.03	14.9 ± 3.4	−0.05
6	heliotropine	6.2 ± 0.4	−0.02	8.8 ± 1.6	−0.02
18	3-hydroxybenzaldehyde	5.4 ± 11.6	−0.02	26.4 ± 18.6	−0.07
13	veratrumaldehyde	<2	no reaction	9.4 ± 4.4	−0.03
5	ethylvanillin	<2	no reaction	<2	no reaction
2	4-hydroxybenzaldehyde	<2	no reaction	<2	no reaction
3	vanillin	<2	no reaction	<2	no reaction

adducts at the Lys and Arg residues in the Lys-peptide are favored. Here, we were first interested in the SAR for modifications at the Lys residue. Table 3 lists the results both expressed as depletion of the Lys-peptide and relative formation

Table 3. Formation of Reduced Schiff Bases with the Lys-Peptide with $NaBH_3CN$-Trapping

		% depletion[a]	% adduct-16[b] [rel. %]
17	2-bromo-5-hydroxybenzaldehyde	34.2 ± 1.3	47.3 ± 8.7
18	3-hydroxybenzaldehyde	13.6 ± 2.2	23.7 ± 3.3
16	4-chlorobenzaldehyde	13.5 ± 1.4	21.8 ± 2.3
19	3-methoxybenzaldehyde	13.4 ± 2.3	30.5 ± 3
8	atranol	10.8 ± 1.5	complex adduct
1	benzaldehyde	10.5 ± 1.2	17.6 ± 3.2
7	chloratranol	10 ± 5.6	complex adduct
9	cuminic aldehyde	9 ± 5.3	23 ± 3
10	4-methylbenzaldehyde	8.8 ± 1.9	12.4 ± 1.4
15	4-nitrobenzaldehyde	8.5 ± 2.5	14.4 ± 1.3
12	6-methoxynaphtalene-2-carbaldehyde	7.2 ± 2.3	9.4 ± 2.9
14	salicylaldehyde	6.4 ± 1.1	8.5 ± 1.3
11	3-chloro-4-methoxybenzaldehyde	5.3 ± 0.9	6.9 ± 0.7
6	heliotropine	3.2 ± 2.5	1.8 ± 0.3
5	ethylvanillin	2.3 ± 3.5	0.8 ± 0.1
3	vanillin	2.2 ± 4.9	0.7 ± 0.1
13	veratrumaldehyde	0.9 ± 4.2	1.7 ± 0.3
4	4-methoxybenzaldehyde	−0.2 ± 6.4	2.1 ± 0.3
2	4-hydroxybenzaldehyde	−0.6 ± 2.4	0.8 ± 0.3

[a]0.5 mM peptide was reacted with 1 mM test chemical at pH of 7.5 in the presence of 50 mM of $NaBH_3CN$, and the % depletion of the parent peptide was calculated [b]The peak of the reaction product with the theoretical mass of the reduced Schiff base ($M_{peptide} + M_{test\_chemical} - 18 + 2$) was integrated and quantified relative to the control peak of the Lys-residue; since the response factor for the different adducts is not known, this is an approximation. In most cases, the response factor appears higher for the adducts.

of a new peak with the theoretical mass of the reduced SB (referred to as adduct-16). For all chemicals with the exception of atranol and chloratranol, the reduced SB appears as the main reaction product. Relatively high amounts were detected for **17**. Somewhat lower reactivity was apparent for benzaldehyde and the *m*-substituted **21** and **19** and for the *p*-substituted compounds **16**, **9**, **10**, and **15**. Similar to the results from the butylamine experiments, the lowest reactivity under these conditions was observed for the *p*-hydroxy and *p*-methoxy derivatives such as vanillin. However, these quantitative results must be treated with some caution: $NaBH_3CN$ is also able to reduce the test chemical, and thus, we have two competing reactions, and not a constant reservoir of test chemical is present. These results indicate that SB formation can occur with the Lys-peptide in the DPRA if the SBs are trapped *in situ*. For atranol and chloratranol, a more complex picture of adduct formation was observed in the presence of $NaBH_3CN$. For chloratranol, the main peak has the mass consistent with a direct Schiff base (not reduced), while for atranol, several peaks which could not be interpreted were observed.

Peptide Reactivity Assay: Peptide Modifications Observed in the Absence of $NaBH_3CN$. The most relevant question of course is how BAs react in the presence of water and in the absence of *in situ* reduction, as this would be closest to the conditions we can expect in the living organism. We thus performed peptide reactivity assays under modified DPRA conditions (50 mM test chemical, 0.5 mM peptide, no *in situ* reduction, and pH of 7.5 instead of 10.5 as in the original DPRA). Interestingly, significant formation of an adduct with a base ion of the theoretical m/z of a direct SB (labeled as adduct-18) could only be detected for the *o*-hydroxy-aldehydes **7**, **8**, and **14** (Figure 3 shows the LC-MS analysis for atranol **7** as an example, and Table 4 lists the quantitative results). No significant peptide depletion and no, or only traces, of reaction products were observed for most of the *m*- and *p*-substituted hydroxy- and methoxy BAs. These results would indicate that Schiff base formation is not favored (i.e., the equilibrium is far on the left side) in the aqueous DPRA conditions in the

dx.doi.org/10.1021/tx300278t | Chem. Res. Toxicol. XXXX, XXX, XXX−XXX

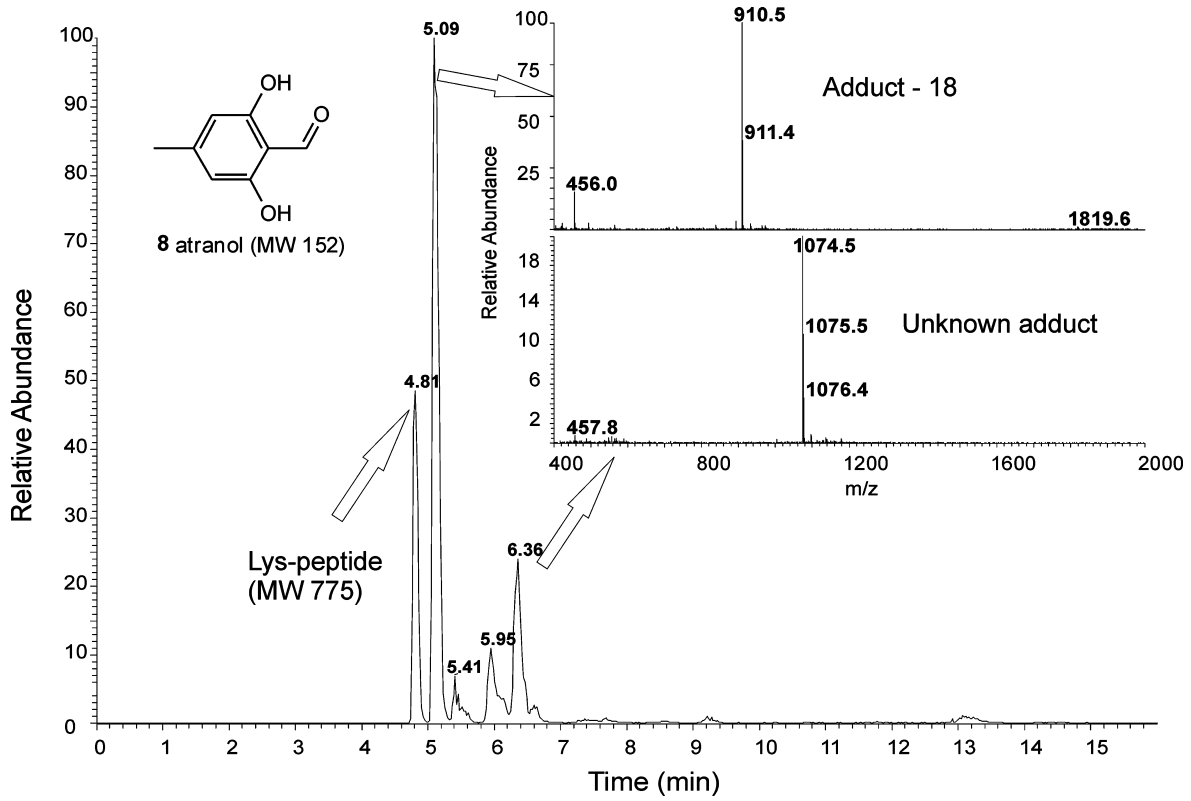


Figure 3. LC-MS analysis of 50 mM atranol **8** incubated with 0.5 mM of the Lys-peptide at pH 7.5 for 24 h.

Table 4. Formation of Adducts and Peptide Modifications with the Lys-Peptide at pH 7.5 without $NaBH_3CN$-Trapping

		% peptide depletion[a]	adduct −18 [rel. %][b]	Lys-aldehyde [rel. %]	adduct −2 [rel. %]	other adducts
7	chloratranol	60.5 ± 0.3	91.7 ± 12.8	n.d.	n.d.	several double adducts, mass 1080–1264
8	atranol	60.1 ± 0.2	95.4 ± 3.9	n.d.	n.d.	several double adducts, mass 1060–1074
16	4-chlorobenzaldehyde	35.8 ± 6.8	n.d.	81 ± 8.1	199.4 ± 7.5	
14	salicylaldehyde	27.3 ± 2.6	73.2 ± 4.1	n.d.	n.d.	
9	cuminic aldehyde	12.5 ± 2.1	n.d.	19.7 ± 3.1	39.6 ± 11.6	
1	benzaldehyde	8.8 ± 2.3	n.d.	36.4 ± 5	19 ± 2.4	
10	4-methylbenzaldehyde	5.1 ± 3.5	n.d.	20.3 ± 11.4	12.2 ± 4	
11	3-chloro-4-methoxybenzaldehyde	4.4 ± 4.2	n.d.	n.d.	12 ± 5	traces of peptide +28
5	ethylvanillin	3.1 ± 2.3	0.7 ± 0	n.d.	n.d.	traces of adduct-16
3	vanillin	2.2 ± 1.9	n.d.	n.d.	n.d.	traces of peptide +28 and adduct-16
13	veratrumaldehyde	1.9 ± 4.4	n.d.	n.d.	n.d.	traces of adduct-38
18	3-hydroxybenzaldehyde	0.9 ± 0.5	n.d.	n.d.	n.d.	
6	heliotropine	0.9 ± 0.7	n.d.	n.d.	n.d.	
15	4-nitrobenzaldehyde	0.5 ± 3.6	n.d.	n.d.	n.d.	
4	4-methoxybenzaldehyde	−0.2 ± 4.6	n.d.	n.d.	n.d.	low amount peptide +28
2	4-hydroxybenzaldehyde	−0.5 ± 3.4	n.d.	n.d.	n.d.	
12	6-methoxynaphtalene-2-carbaldehyde	−0.5 ± 3.3	n.d.	n.d.	n.d.	
19	3-methoxybenzaldehyde	−0.9 ± 3.1	n.d.	n.d.	n.d.	
17	2-bromo-5-hydroxybenzaldehyde	−3.8 ± 3.1	n.d.	0.3 ± 0	n.d.	

[a]0.5 mM of test peptide was incubated with 50 mM of test chemical for 24 h at pH 7.5. [b]The peaks of the reaction products were quantified relative to the control peak of the Lys-residue; since the response factor in the MS analysis for the different adducts is not known, this is a relative value.

absence of the trapping reagent with the exception of the potent sensitizers with *o*-hydroxy groups.

However, we observed two unexpected chromatographic peaks in the reactions with a number of different test chemicals, in particular **1**, **16**, and the *p*-alkyl BAs **9** and **10**: One identical peak (further referred to as peptide-1) was present in incubations with all these test chemicals. It contained two base ions at m/z 775.4 and m/z 807.4 in the ESI^+-spectrum (see Figure 4). Because of the mass difference of 32 between these two ions, we suspected that the ion at 807.4 could be due to the addition of methanol from the eluent used in the chromatographic separation, and LC-MS analysis was repeated

dx.doi.org/10.1021/tx300278t | *Chem. Res. Toxicol.* XXXX, XXX, XXX–XXX

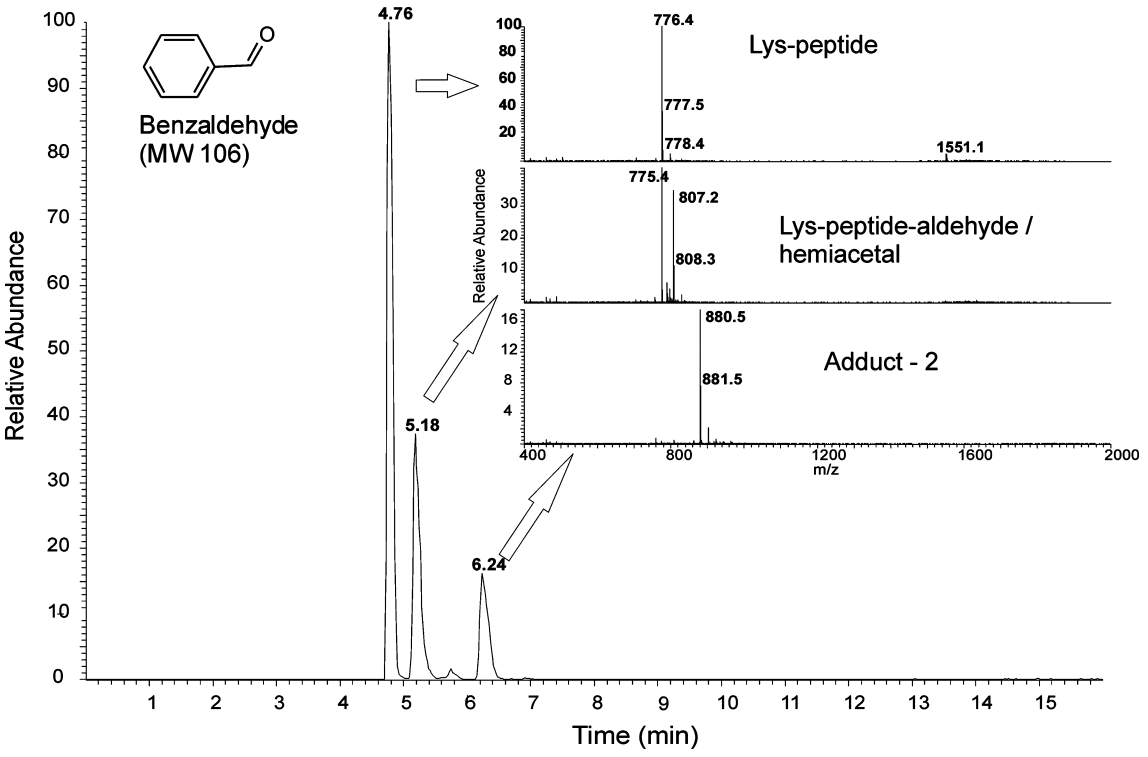


Figure 4. LC-MS analysis of 50 mM BA **1** incubated with 0.5 mM of the Lys-peptide at pH 7.5 for 24 h.

with ACN as eluent. Under these conditions, this peak solely contained the ion at m/z 775.4 (data not shown). This mass would correspond to the Lys-peptide having lost one mass unit. We hypothesized that the ion at m/z 775.4 could correspond to the aldehyde formed at the Lys-residue by oxidative deamination and that the ion at m/z 807.4 would then correspond to the hemiacetal of this aldehyde with methanol formed during chromatography. This hypothesis will be further substantiated below. The second peak observed in incubations with BA **1** has a base ion at m/z 880.4. This ion corresponds to the pseudomolecular ion of a peptide−benzaldehyde reaction product $[M_{Product} + H]^+$ with the formal interpretation of $[(M_{peptide} + M_{Benzaldehyde} - 2) + H]^+$ or $(775.4 + 106 - 2) + 1$ and will be referred to as adduct-2. A similar product (i.e., adduct-2) was observed for a number of other test chemicals, and these results are summarized in Table 4. We wondered whether this unexpected adduct can only be formed from aromatic aldehydes and thus tested the aliphatic skin sensitizing aldehyde hydoxycitronellal under the same conditions. As shown in Supporting Information, Figure S1, this chemical also led to the formation of the peptide-1and the adduct-2 peaks; in this case, the reaction was even quantitative. Hence, this reaction pathway appears to be relevant for the reactivity of different aldehydes when tested under neutral DPRA conditions with the Lys-peptide, and we thus decided to investigate it in greater detail.

Finally, we observed traces of a reaction product with the pseudomolecular ion with the formal interpretation $(775.4 + M_{test\ chemical} - 16) + 1$ for vanillin and ethylvanillin. We currently cannot interpret this result, but it indicates that these molecules still do have a minimal peptide reactivity, but it should be kept in mind that a large excess of test chemical was used and that only very small traces of product were observed.

Structure Elucidation of the Modified Peptide (Peptide-1) and the Peptide Adduct (Adduct-2) in Incubations with BA. To better understand the unexpected adduct-2 between the Lys-peptide and benzaldehyde, we first performed LC-MS^2 analysis (Figure S2 in Supporting Information). The fragments B4 (the largest fragment without the Lys residue) was still observed in the adduct-2 peak, whereas the fragments B5 and B6 containing the Lys residue do carry a mass difference of +104, thus indicating that the peptide does carry the modification at the Lys-residue. In the LC-MS^2 analysis of the peptide-1 peak, the fragments B5 and B6 have lost one mass unit, whereas B4 is unmodified, indicating that this modification also occurred at the Lys residue (data not shown).

We next separated the different modified peaks with preparative HPLC and performed NMR experiments on the purified fractions. Initially, the purified adduct-2 peak was over 90% pure, but it then rapidly reacted further to contain some peptide-1 along with the apparent adduct-2 peak. This mixture then remained stable over >2 weeks (as verified by LC-MS) and was used for the NMR experiments.

Adduct-2 shows two signals in the ^{1}H NMR spectrum, which were key in its structural determination. One of them belongs to a monosubstituted aromatic ring stemming from BA at 7.77 ppm; its doublet-like multiplicity stands for two ortho-protons in an AA'MM'X system. The other one is a triplet (J = 6.8 Hz) at 3.45 ppm. Their respective ^{13}C NMR shifts extracted from a HSQC experiment[20] are 127.2 ppm for the aromatic proton pair and 39.9 ppm for an aliphatic methylenic group. In the HMBC experiment[21] depicted in Figure 5, these two proton groups share $^3J_{C,H}$ couplings to a common carbon atom resonating at 171.1 ppm. These latter results are fully compatible with an aromatic amide of the type Ar-CO-NH-CH_2-R, the R group being the remaining modified lysine residue from the heptapeptide. Furthermore, this HMBC experiment leads to the two next carbons in the lysine chain, both of methylenic type, at 22.7 and 28.2 ppm, still starting

dx.doi.org/10.1021/tx300278t | Chem. Res. Toxicol. XXXX, XXX, XXX−XXX

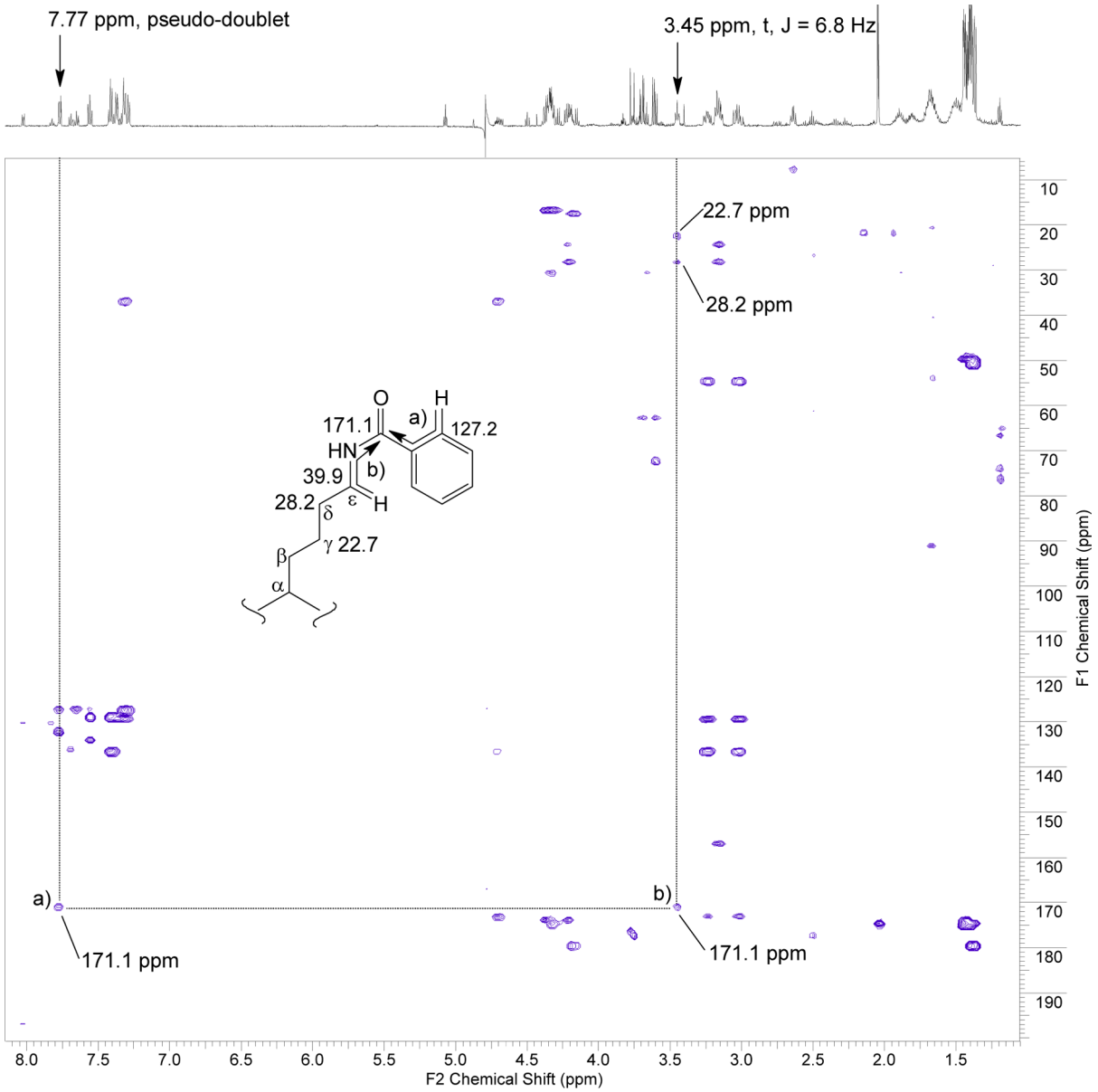


Figure 5. gs-HMBC NMR spectrum of the HPLC purified fraction containing adduct-2. The arrows in the benzamide moiety indicate the two $^{3}J_{C,H}$ coupling pathways corresponding to the cross-peaks a and b; both of them lead to the amide carbonyl. Two further cross-peaks arising from the ε-protons at 3.45 ppm indicate the δ- and γ-neighbor-carbons at, respectively, 28.2 and 22.7 ppm.

from the key signal at 3.45 ppm and also indicated in Figure 5. These two ^{13}C NMR shifts are expected in γ- and δ-positions, respectively, for such a benzamide, as well as 39.9 ppm for the ε-carbon. These values were compared with the values we measured for the commercially available *N*-ε-benzoyl-L-lysine in the same positions, and they were found to be in very good agreement: 22.0 (C_γ), 28.4 (C_δ), and 39.7 ppm (C_ε) (data not shown). However, any modification on the arginine residue can be excluded since it has been found intact in the same HSQC/HMBC experiments. Additionally, a series of 1D-selective TOCSY NMR experiments[22] were performed. The selective excitation offset was set on the key triplet at 3.45 ppm since it is located at the end of the ex-lysine unit, in the ε-position (Figure 6). By increasing the mixing time starting from 20 to 80 ms in four experiments, the δ-, γ-, and β-protons and finally the characteristic α-proton at 4.23 all appear successively on the obtained spectra series. These latter findings as well as the arguments stemming from the two key proton groups demonstrate clearly that the lysine moiety has been transformed into the corresponding benzamide in adduct-2.

Another component present in that mixture is the aldehyde from the formal oxidative deamination of the Lys-peptide, as well as its hydrate. The characteristic aldehyde proton has been found at 9.61 ppm as a triplet (J = 1.5 Hz) in the ^{1}H NMR spectrum, which suggests that the first neighbor (δ-position) is a methylenic pair of protons as would be expected for a lysine-derived aldehyde. The aldehyde carbon (ε-position) resonates at 208.0 ppm according to the HSQC experiment. The HMBC experiment indicates a correlation to a carbon at 42.7 ppm starting from the aldehyde proton; this is in line with a methylene-carbon in δ-position. Further positions in the ex-lysine chain are in important overlapping regions; therefore, the 1D-selective TOCSY NMR experiment was again applied to explore the whole spin-system. This time, a selective excitation

dx.doi.org/10.1021/tx300278t | *Chem. Res. Toxicol.* XXXX, XXX, XXX–XXX

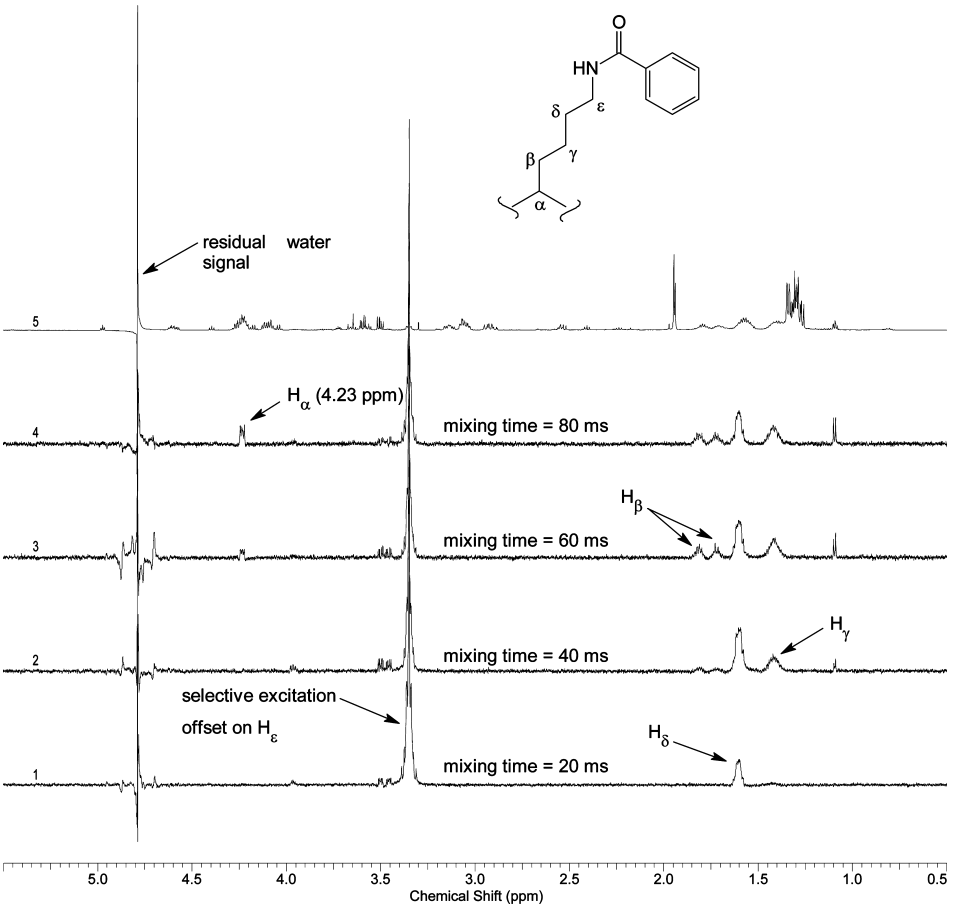


Figure 6. 1D-selective TOCSY NMR series of the HPLC purified fraction containing adduct-2. The selective excitation offset was set on ε-protons at 3.45 ppm for all TOCSY traces (1−4). With a short mixing time of 20 ms as in trace 1, only the first neighbor-protons (H_δ) appear. By increasing the mixing time in 20 ms steps in three further experiments, the γ-, the β-protons, and finally the characteristic α-proton at 4.23 appear all successively. Trace 5 is the corresponding ^{1}H NMR spectrum with presaturation on the residual water in D_2O.

offset on the δ-protons was set; logically, one would expect to start at the beginning of the spin system, at the aldehyde proton, but the very low vicinal coupling constant of this proton (1.5 Hz) will hamper the scalar coupling information transmission along the whole spin-system. A mixing time of 120 ms was necessary to reach the α-proton at 4.24 ppm, as depicted in the middle trace of Figure 7. The aldehyde derived hydrate was also unveiled by a 1D-selective TOCSY experiment; the selective excitation offset on the ε-proton at 4.96 ppm could point out all protons until reaching the α-proton, as shown in the lower trace of Figure 7.

On the basis of these analytical data, we propose that the initially formed Schiff base between BA and the Lys-peptide undergoes spontaneous oxidation leading to the benzamide. In a parallel reaction, the double bond of the Schiff base can migrate and then hydrolyze, which leads to oxidative deamination of the peptide. These reactions are summarized in Scheme 1.

NMR Analysis of the Reaction Products between Salicylaldehyde and the Lys-Peptide or Butylamine. Finally, the formation of the adduct occurring from salicylaldehyde and the Lys-peptide has been followed by ^{1}H NMR over time with a 100-fold excess of the aldehyde, in a phosphate buffered solvent mixture 75% D_2O/25% CD_3CN. After 15 min of reaction time, a singlet at 8.47 ppm as well as a triplet at 3.80 ppm appear. Their relative integral ratio is respectively 1:2. Both of them grow with time until 160 min, conserving their integral ratio. At a first view, these signals match with the imine proton and the α-imine methylene protons of the expected Schiff base. A more extensive analysis with gs-HSQC and gs-HMBC experiments point out a ^{13}C NMR chemical shift of 173.2 ppm for the aromatic carbon bearing the hydroxyl group of the former salicyl aldehyde, which is approximately 10 ppm higher than expected. A control experiment using butylamine instead of the Lys-peptide in CD_3CN shows a shift of 162.1 ppm for the same carbon. Moreover, the α-imine aliphatic methylenic carbon resonates at 51.9 ppm versus 59.5 ppm in this control experiment. This may indicate that we observe an enaminone tautomer in the reaction with the Lys-peptide, which we could confirm by a further control experiment still using butylamine and salicylic aldehyde but with the buffered solvent mixture 75% D_2O/25% CD_3CN. This later experiment showed shifts very similar to those of the Lys-peptide adduct as exposed in Scheme 2.

DISCUSSION

This study reveals a striking difference in the reactivity of differently substituted BA derivatives toward amine nucleophiles, with *o*-hydroxybenzaldehydes having a high propensity to form Schiff-bases which are stable in aqueous conditions. Other benzaldehydes do form Schiff-bases which are labile to hydrolysis, but the Schiff bases can act as an intermediate which is further transformed to stable amide adducts. Thus, Schiff

dx.doi.org/10.1021/tx300278t | *Chem. Res. Toxicol.* XXXX, XXX, XXX−XXX

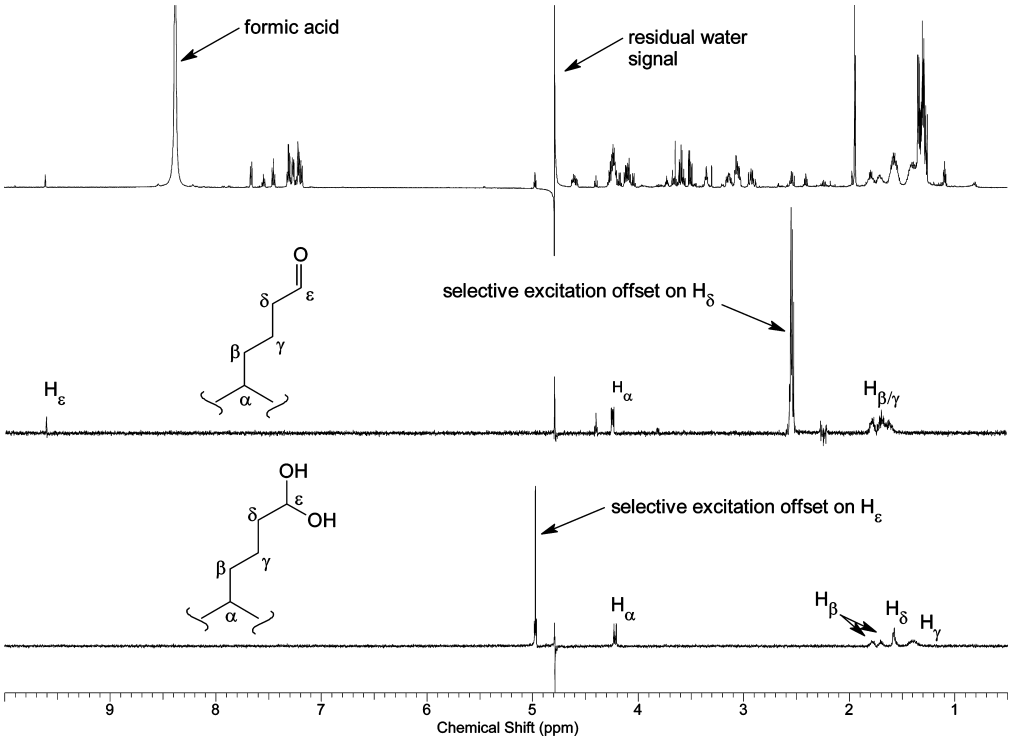
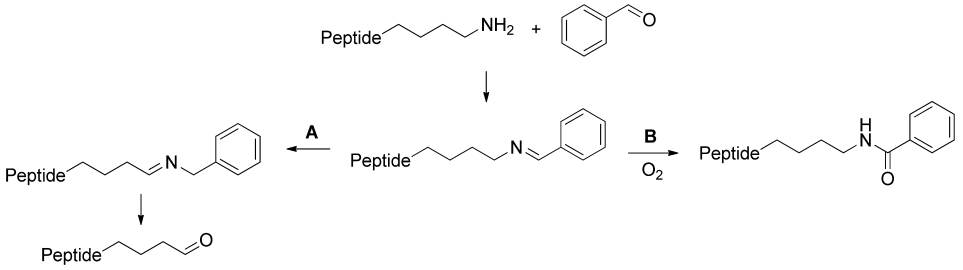
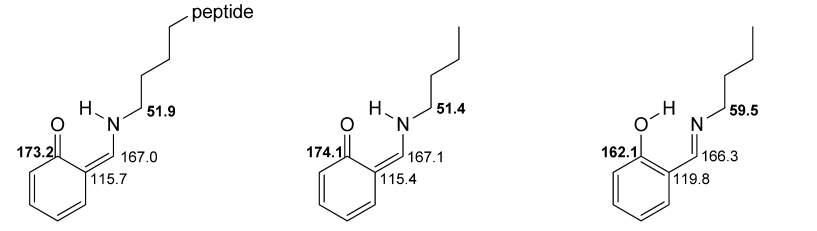


Figure 7. 1D-selective TOCSY NMR series of the HPLC purified fraction containing besides the adduct-2 an aldehyde and its hydrate. In the middle trace, the selective excitation offset was set on the δ-protons at 2.55 ppm. All protons of the aldehyde spin-system appear with a mixing time of 120 ms. With the same mixing time, the lower trace shows the protons H_δ, H_γ, H_β, and finally H_α when the selective excitation offset was set on the ε-proton of the hydrate. The upper trace is the corresponding ^{1}H NMR spectrum with presaturation on the residual water signal in D_2O.

Scheme 1. Possible Pathway of the Oxidative Decomposition of the Schiff Base of Benzaldehyde



Scheme 2. Comparison of Observed ^{13}C-NMR Chemical Shifts at Key Positions in the Enaminone/Imine Tautomers of the Former Salicyl Aldehyde



modified peptide observed as an enaminone tautomer in a phosphate buffered solvent mixture 75 % D_2O / 25 % CD_3CN

butylamine adduct observed as an enaminone tautomer in a phosphate buffered solvent mixture 75 % D_2O / 25 % CD_3CN

butylamine Schiff base in CD_3CN

base formation is an important process to understand skin sensitization by benzaldehydes, but unexpected reactions downstream of the initial Schiff base adduct appear equally important.

SAR for Reactivity with Amines and Relationship to Sensitization Potential. Overall, the reactivity can be summarized in descending order as *o*-hydroxy BAs > unsubstituted BA > *p*-alkyl BAs > *p*-methoxy BAs > *p*-hydroxy BAs. A similar trend is seen in the simple butylamine–acetonitrile system and in the somewhat more biological peptide reactivity conditions with trapping of the formed Schiff bases. These differences can explain some of the experimentally

dx.doi.org/10.1021/tx300278t | *Chem. Res. Toxicol.* XXXX, XXX, XXX–XXX

or clinically reported information on the skin sensitizing potency of BAs. Thus, atranol and chloratranol, which are formed in the classical processing of oak moss absolute, appear to be the most potent chemicals to elicit allergic reactions to fragrances.[16] These two chemicals are also among the most reactive ones in the amine reactivity assay, and they (along with salicylaldehyde **14**) are also the only BAs forming stable Schiff bases in the aqueous conditions of the peptide reactivity assay. Salicylaldehyde finds very little use in commercial fragrances, but positive patch test reactions have been observed by dermatologists.[23] Our reactivity results confirm that the use of this chemical should be limited. The pronounced effect of the *o*-hydroxy group may be explained by the observed stable enaminone tautomer and/or a favorable hydrogen bond between the nitrogen atom and the keto-group of the enaminone. This tautomeric form might render the SB of the *o*-hydroxy BA itself reactive to additional nucleophilic attack. However, in the case of the SB of **14** with the Lys-peptide, addition of a Cys-containing peptide or propylthiol did not change the amount of adduct-18 nor did we observe a new reaction product due to a modification of this adduct (data not shown).

The stable Schiff base formation by *o*-hydroxy BAs is not without precedent. The immunostimulating drug candidate tucaresol (4[2-formyl-3-hydroxyphenoxymethyl] benzoic acid) does contain this structural element and had been reported to form stable Schiff bases with cell surface proteins.[24]

At the other extreme of the above scale of reactivity are the *p*-hydroxy BAs. Schiff base formation in the butylamine–acetonitrile system was detectable for these chemicals in the presence of a high excess of butylamine, but in the dose–response analysis, this group showed no significant reactivity. In addition, only traces of the reduced Schiff bases were detected in the peptide reactivity experiments with $NaBH_3CN$ trapping for *p*-hydroxy BAs. This low reactivity is paralleled by the absence of sensitization potential in animal tests and the low incidence of positive sensitization data from diagnostic patch testing (12 cases in almost 6000 patients tested for vanillin), despite the very broad use of vanillin and ethylvanillin in consumer products.[23,25] The very low reactivity of these agents, as well as of 4-hydroxybenzaldehyde, is in line with their low degree of sensitization.

We did not for all BAs observe a correlation between reported sensitization potential in animals and the ability to form Schiff bases. BA **1**, 4-chloro BA **16**, and 4-nitro BA **15** are negative in the LLNA; **1** has a clear sensitization potential in humans (LOEL in human maximization test of 2760 $\mu g/cm^2$, which would correspond to an LLNA EC3 of around 10%; RIFM, personal communication), and no human data are available for **15** and **16**. These chemicals have a significant reactivity according to our results. Among the *p*-alkyl BAs, **9** was reported to be nonsensitizing at 10%, whereas **10** is almost as strong according to the LLNA as atranol. Compound **10** is only slightly more reactive than **9**, and given the high structural similarity and similar reactivity, this difference in the LLNA might be an overestimation, and it should be kept in mind that the compiled LLNA data in Figure 1 come from different sources which may contribute to the variability of the animal results. A more homogeneous data set would probably only be obtained by retesting all chemicals in one laboratory, which appears not to be warranted for ethical reasons.

The *p*-alkoxy BAs have a slightly higher reactivity as compared to the *p*-hydroxy BAs. Compound **6** is rated as a weak sensitizer (EC3 = 25%), whereas **4** is nonsensitizing in the LLNA (>25%) and a weak sensitizer in human tests (LOEL in human repeat insult patch test of 4724 $\mu g/cm^2$, which would correspond to an LLNA EC3 of ca. 20%; RIFM, personal communication). Hence, we would conclude that *p*-alkoxy BAs do have a low reactivity and only a very weak sensitization potential.

2-Bromo-5-hydroxy-benzaldehyde has a quite unique profile: intermediate reactivity with butylamine and the highest amount of reduced Schiff base with the peptide but no covalent adduct in the absence of *in situ* reduction. Nevertheless, it has a quite low LLNA EC3 value. This may indicate that in this case also the unstable Schiff base is of importance.

Reactivity in Aqueous System. As outlined in the Introduction, Schiff base formation is a reversible process, and the equilibrium is highly dependent on (i) the concentration of water, (ii) the amount of free aldehyde, and (iii) pH/protonation of the amine group. Thus, it is probably not surprising that no evidence for Schiff base formation was found for most aldehydes under the classical peptide reactivity conditions,[11,12] unless reaction conditions were specifically favored to facilitate Schiff base formation.[14] One would anticipate that conditions on the migratory routes of dendritic cells away from the aldehyde-treated skin sites would favor hydrolysis due to neutral pH, presence of water, and absence of free aldehyde. Here, we confirmed that, with the notable exceptions of **7**, **8**, and **14**, Schiff base formation under the aqueous peptide reactivity conditions can only be observed to a significant extent by including a trapping reagent.

Yet the current results indicate that stable peptide modification (aldehyde formation at Lys by oxidative deamination) and stable adduct formation (amide formation) is nevertheless possible under dilute aqueous conditions at physiological pH. Interestingly, these modifications were mostly observed for those aldehydes which also formed higher amounts of Schiff bases in the trapping experiments, probably indicating that Schiff base formation is the first step in the reaction cascade, and we have to invoke oxidation of the Schiff base to explain the results. Indeed, amide formation from Schiff bases of benzaldehyde derivatives in the presence of molecular oxygen had been observed,[26] with the probable involvement of the transient formation of a peroximidic acid. This process was observed in neat preparations of the Schiff base and was accelerated after the addition of a free radical initiator. In a more biological system, the long-lived adduct of 4-oxo-2-nonenal at lysine residues was shown to be a 4-ketoamide[27] formed from an initial Schiff base adduct. However, in this system no oxidation by molecular oxygen had to be invoked, as amide formation was occurring under concomitant reduction of the double bond. In addition, it appears to be a specific process for the 4-oxo-2-enal substructure, and a mechanism with intermediate formation of a pyrrol ring was proposed. Even if the amide is the final stable product, the mechanism thus appears to be significantly different.[27] Amide formation from an initial Schiff base between lysine residues and aliphatic aldehydes had also been reported by Ishino et al.,[28] in that study H_2O_2 was needed to facilitate the reaction. Immunological evidence also indicated that the process occurs *in vivo* under conditions of oxidative stress.

Another surprising result is the formation of the aldehyde functionality at the Lys-residue due to oxidative deamination. This process would require migration of the Schiff base double bond and subsequent hydrolysis. Aldehyde formation at the Lys

residue in reactivity experiments with sensitizers had been reported before upon reaction of a peptide with 2,5-dimethyl-*p*-benzoquinonediimine and subsequent hydrolysis.[29] However, this appeared to be a rather specific case, as the Schiff base-like adduct has a quinoid structure which can tautomerize to the stable hydroquinone form upon migration of the Schiff base double bond (similar to the reaction cascade in the oxidative deamination of primary amines by topaquinone, the cofactor of amine oxidases).[30] Surprisingly, our results indicate that this process also can happen upon reaction of the peptides with simple aldehydes, which do not contain this redox system. The formed peptide/protein aldehyde could in theory then lead to immunogenic novel epitopes by intra- or intermolecular cross-linking, yet these epitopes would not be specific to the sensitizing aldehyde, and we would not expect them to lead to clinically relevant specific allergies.

We have no full explanation of the reaction mechanism leading to the oxidative degradation products of the Schiff base. The most likely explanation is the formation of an intermediate oxaziridine or nitrone (with the same molecular weight as the amide), which further reacts to the amide and the aldehyde. This would explain why the isolated peak with the mass of adduct-2 was not fully stable in the beginning but reacted further to form some of the peptide-1; it might initially contain both the semistable oxaziridine, the nitrone, and the stable amide. Indeed, the addition of oxone ($KHSO_5$), which is known to oxidize Schiff bases to the oxaziridines,[31] to the reaction of BA with the Lys-peptide led to higher yields of adduct-2 and adduct-1 (data not shown).

The stable modifications and adducts occur at a physiological pH. We currently do not know how important the context of the peptide structure is, but we observed that the same reactions do occur but with much lower efficacy for acetyl-lysine (data not shown). We cannot predict whether the same reactions will happen with proteins and in the living body, but if they do, this would describe a mechanism of how aldehydes can form stable immunogenic epitopes since after an equilibration time, the amide-adduct was found to be stable in water in the absence of the free aldehyde for over a week (data not shown). Finally, a similar reaction was observed for the structurally completely different aldehyde hydroxycitronellal, and it is therefore not specific to benzaldehydes. To what extent amide formation correlates to the sensitizing capacity of different aldehydes is a field which warrants further study.

■ ASSOCIATED CONTENT

Supporting Information

Source of the LLNA data; LC-MS analysis of the reaction product between hydroxycitronellal and the Lys-peptide; and LC-MS^2 analysis of adduct-2, i.e., the peak with pseudomolecular ion at 880 formed by incubating BA with the Lys-peptide. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: ++41 44 824 21 05. Fax: ++41 44 824 29 26. E-mail: andreas.natsch@givaudan.com.

Funding

This study was entirely funded by Givaudan Schweiz AG.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Peter Gygax and Felix Flachsmann for interesting discussions and valuable suggestions for structure elucidation, Fabian Kuhn for interpreting GC-MS data, Michael Rothaupt for HPLC purification of peptide adducts, and the Research Institute for Fragrance Materials (RIFM) for providing human and animal data on the sensitization potential.

■ ABBREVIATIONS

ACN, acetonitrile; BA, benzaldehyde; DPRA, direct peptide reactivity assay; ECVAM, European Center for the Validation of Alternative Methods to animal testing; ESI, electrospray ionization; HRRIPT, human repeat insult patch test; LLNA, local lymph node assay; LOEL, lowest observed effect level; MTBE, methyl *tert*-butyl ether; RIFM, Research Institute for Fragrance Materials; SB, Schiff base

■ REFERENCES

(1) Divkovic, M., Pease, C. K., Gerberick, G. F., and Basketter, D. A. (2005) Hapten-protein binding: From theory to practical application in the in vitro prediction of skin sensitization. *Contact Dermatitis 53*, 189−200.

(2) Gerberick, F., Aleksic, M., Basketter, D., Casati, S., Karlberg, A. T., Kern, P., Kimber, I., Lepoittevin, J. P., Natsch, A., Ovigne, J. M., Rovida, C., Sakaguchi, H., and Schultz, T. (2008) Chemical reactivity measurement and the predictive identification of skin sensitisers. *ATLA 36*, 215−242.

(3) Gerberick, G. F., Vassallo, J. D., Bailey, R. E., Chaney, J. G., Morrall, S. W., and Lepoittevin, J. P. (2004) Development of a peptide reactivity assay for screening contact allergens. *Toxicol. Sci. 81*, 332−343.

(4) Chipinda, I., Ajibola, R. O., Morakinyo, M. K., Ruwona, T. B., Simoyi, R. H. Siegel, P. D. Rapid and simple kinetics screening assay for electrophilic dermal sensitizers using nitrobenzenethiol. *Chem. Res. Toxicol. 23*, 918-925.

(5) Schultz, T. W., Rogers, K., and Aptula, A. O. (2009) Read-across to rank skin sensitization potential: subcategories for the Michael acceptor domain. *Contact Dermatitis 60*, 21−31.

(6) Patlewicz, G., Dimitrov, S. D., Low, L. K., Kern, P. S., Dimitrova, G. D., Comber, M. I., Aptula, A. O., Phillips, R. D., Niemela, J., Madsen, C., Wedebye, E. B., Roberts, D. W., Bailey, P. T., and Mekenyan, O. G. (2007) TIMES-SS–a promising tool for the assessment of skin sensitization hazard. A characterization with respect to the OECD validation principles for (Q)SARs and an external evaluation for predictivity. *Regul. Toxicol. Pharmacol. 48*, 225−239.

(7) Roberts, D. W., Patlewicz, G., Kern, P. S., Gerberick, F., Kimber, I., Dearman, R. J., Ryan, C. A., Basketter, D. A., and Aptula, A. O. (2007) Mechanistic applicability domain classification of a local lymph node assay dataset for skin sensitization. *Chem. Res. Toxicol. 20*, 1019−1030.

(8) Roberts, D. W., and Aptula, A. O. (2008) Determinants of skin sensitisation potential. *J. Appl. Toxicol. 28*, 377−387.

(9) Roberts, D. W., Aptula, A. O., and Patlewicz, G. (2006) Mechanistic applicability domains for non-animal based prediction of toxicological endpoints. QSAR analysis of the schiff base applicability domain for skin sensitization. *Chem. Res. Toxicol 19*, 1228−1233.

(10) Aptula, A. O., Patlewicz, G., and Roberts, D. W. (2005) Skin sensitization: Reaction mechanistic applicability domains for structure-activity relationships. *Chem. Res. Toxicol. 18*, 1420−1426.

(11) Gerberick, G. F., Vassallo, J. D., Foertsch, L. M., Price, B. B., Chaney, J. G., and Lepoittevin, J. P. (2007) Quantification of chemical peptide reactivity for screening contact allergens: A classification tree model approach. *Toxicol. Sci. 97*, 417−427.

(12) Natsch, A., and Gfeller, H. (2008) LC-MS-based characterization of the peptide reactivity of chemicals to improve the in vitro prediction of the skin sensitization potential. *Toxicol. Sci. 106*, 464−478.

(13) Reichardt, P., Schreiber, A., Wichmann, G., Metzner, G., Efer, J., and Raabe, F. (2003) Identification and quantification of in vitro adduct formation between protein reactive xenobiotics and a lysine-containing model peptide. *Environ. Toxicol. 18*, 29−36.
(14) Aleksic, M., Thain, E., Roger, D., Saib, O., Davies, M., Li, J., Aptula, A., and Zazzeroni, R. (2009) Reactivity profiling: Covalent modification of single nucleophile peptides for skin sensitization risk assessment. *Toxicol. Sci. 108*, 401−411.
(15) Bernard, G., Gimenez-Arnau, E., Rastogi, S. C., Heydorn, S., Johansen, J. D., Menne, T., Goossens, A., Andersen, K., and Lepoittevin, J. P. (2003) Contact allergy to oak moss: search for sensitizing molecules using combined bioassay-guided chemical fractionation, GC-MS, and structure-activity relationship analysis. *Arch. Dermatol. Res. 295*, 229−235.
(16) Johansen, J. D., Bernard, G., Gimenez-Arnau, E., Lepoittevin, J. P., Bruze, M., and Andersen, K. E. (2006) Comparison of elicitation potential of chloroatranol and atranol–2 allergens in oak moss absolute. *Contact Dermatitis 54*, 192−195.
(17) Patlewicz, G., Basketter, D. A., Smith, C. K., Hotchkiss, S. A., and Roberts, D. W. (2001) Skin-sensitization structure-activity relationships for aldehydes. *Contact Dermatitis 44*, 331−336.
(18) Braun, M. M., and U. Houben, S. (1990) Synthese von (R)-Lasiodiplodin aus Polyhydroxybuttersäure. *Liebigs Ann. Chem.*, 513−517.
(19) Roberts, D. W., and Natsch, A. (2009) High throughput kinetic profiling approach for covalent binding to peptides: Application to skin sensitization potency of michael acceptor electrophiles. *Chem. Res. Toxicol. 22*, 592−603.
(20) Schleucher, J., Schwendinger, M., Sattler, M., Schmidt, P., Schedletzky, O., Glaser, S. J., Sorensen, O. W., and Griesinger, C. (1994) A general enhancement scheme in heteronuclear multidimensional NMR employing pulsed field gradients. *J. Biomol. NMR 4*, 301−306.
(21) Cicero, D. O., Barbato, G., and Bazzo, R. (2001) Sensitivity enhancement of a two-dimensional experiment for the measurement of heteronuclear long-range coupling constants, by a new scheme of coherence selection by gradients. *J. Magn. Reson. 148*, 209−213.
(22) Stott, K., Stonehouse, J., Keeler, J., Hwang, T. L., and Shaka, A. J. (1995) Excitation sculpting in high-resolution nuclear magnetic resonance spectroscopy: Application to selective NOE experiments. *J. Am. Chem. Soc. 117*, 4199−4200.
(23) Uter, W., Geier, J., Frosch, P. Schnuch, A. Contact allergy to fragrances: current patch test results (2005−2008) from the Information Network of Departments of Dermatology. *Contact Dermatitis 63*, 254-261.
(24) Chen, H., and Rhodes, J. (1996) Schiff base forming drugs: mechanisms of immune potentiation and therapeutic potential. *J. Mol. Med. 74*, 497−504.
(25) Hausen, B. M. (2001) Contact allergy to balsam of Peru. II. Patch test results in 102 patients with selected balsam of Peru constituents. *Am. J. Contact Dermatitis 12*, 93−102.
(26) Auret, J. B., Boyd, D. R., and Coulter, P. B. (1984) Autoxidation reactions of imines to form oxaziridines and amides. *J. Chem. Soc., Chem. Commun. 7*, 463−464.
(27) Zhu, X., and Sayre, L. M. (2007) Long-lived 4-oxo-2-enal-derived apparent lysine michael adducts are actually the isomeric 4-ketoamides. *Chem. Res. Toxicol. 20*, 165−170.
(28) Ishino, K., Shibata, T., Ishii, T., Liu, Y. T., Toyokuni, S., Zhu, X., Sayre, L. M., and Uchida, K. (2008) Protein N-acylation: H_2O_2-mediated covalent modification of protein by lipid peroxidation-derived saturated aldehydes. *Chem. Res. Toxicol. 21*, 1261−1270.
(29) Eilstein, J., Gimenez-Arnau, E., Duche, D., Cavusoglu, N., Hussler, G., Rousset, F., and Lepoittevin, J. P. (2008) Sensitization to p-amino aromatic compounds: Study of the covalent binding of 2,5-dimethyl-p-benzoquinonediimine to a model peptide by electrospray ionization tandem mass spectrometry. *Bioorg. Med. Chem. 16*, 5482−5489.
(30) Eilstein, J., Gimenez-Arnau, E., Duche, D., Rousset, F., and Lepoittevin, J. P. (2007) Mechanistic studies on the lysine-induced N-formylation of 2,5-dimethyl-p-benzoquinonediimine. *Chem. Res. Toxicol. 20*, 1155−1161.
(31) Schoumacker, S., Hamelin, O., Teti, S., Pecaut, J., and Fontecave, M. (2005) Activation of oxaziridines by lewis acids: application in enantioselective sulfoxidation. *J. Org. Chem. 70*, 301−308.