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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

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Abstract—To understand the hapten–protein complex formation in the context of skin contact allergy to *p*-amino aromatic derivatives, 2,5-dimethyl-*p*-benzoquinonediimine was used as a model compound to study the reactivity of *p*-benzoquinonediimines, first oxidation intermediates of allergenic *p*-amino aromatic compounds, toward a model peptide containing naturally occurring and potential reactive amino acids. LC–MS analysis, together with electrospray ionization MS/MS, was used for the determination of amino acid selectivity by studying the chemical modifications induced on the peptide due to covalent binding of the *p*-benzoquinonediimine. Results reported in this paper indicated that 2,5-dimethyl-*p*-benzoquinonediimine reacted with the ε-NH_2 group of lysine to first form a covalent adduct of the Schiff's base kind. Besides, an oxido-reduction process started that induced an oxidative deamination of lysine to form a peptidyl α-aminoadipic-δ-semialdehyde, by a mechanism similar to the one known for several enzymatic quinonoid co-factors, followed by an intramolecular cyclization of the peptide. From these results it could be concluded that lysine must be considered as an important amino acid for the hapten–protein complex formation in the case of *p*-benzoquinonediimines and that, in addition to direct covalent binding, further degradation of the peptide can be produced.

1. Introduction

Allergic contact dermatitis is a cutaneous, immunologically based, disease resulting from the chemical modification of epidermal proteins by haptens. The processing of the hapten–protein complex by immunocompetent skin antigen-presenting cells and the transmission of this information to T-cells in the lymphatic nodes lead to erythema and edema, the major clinical aspects of the pathology.[1] The hapten–protein complex formation occurs mainly through the formation of a covalent bond between the hapten, a low molecular weight compound lipophilic enough to penetrate the skin, and nucleophilic groups on proteins. Most skin allergens are, therefore, electrophiles.[2] Besides, initially harmless molecules can be converted into derivatives having electrophilic, and therefore allergenic, properties via metabolic processes, for example, mainly based on oxido-reduction reactions,[3,4] but also via non-enzymatic processes such as reactions with atmospheric oxygen.[5,6] *p*-Amino aromatic compounds, strong skin sensitizers generally related to dyeing products, belong to this category of allergens. The most cited example is *p*-phenylenediamine. The striking growing number of skin allergy cases to temporary henna tattoos with added *p*-phenylenediamine has increased the interest on this allergen worldwide.[7,8] It is also one of the most common active ingredients in oxidative hair dyeing and is known to be a potent contact allergen from human clinical experience as well as from experimental animal tests.[9,10] On complete oxidation, non-electrophilic *p*-phenylenediamine is converted into *p*-benzoquinonediimine and, after hydrolysis, into *p*-benzoquinone, regarded for many years as the electrophilic

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sensitizer.[11] Though, it has also been suggested that *p*-benzoquinone is not the major reactive intermediate and that a spectrum of antigenic structures may result from a mixture of oxidation products.[12]

To understand the skin sensitization process to *p*-amino aromatic compounds, we investigated previously the mechanisms by which electrophilic *p*-benzoquinonediimines can react with nucleophilic residues on amino acids.[13] The reactivity studies were carried out on 2,5-[^{13}C]-dimethyl-*p*-benzoquinonediimine, using ^{13}C and ^{1}H{^{13}C} NMR spectroscopies, associated with liquid chromatography–electrospray ionization–mass spectrometry (LC-ESI-MS). Whereas a classical electrophile–nucleophile mechanism was observed for the direct covalent binding with N_{α}-acetyl-cysteine, an unexpected chemical reactivity was observed in the case of N_{α}-acetyl-tryptophan and N_{α}-acetyl-lysine. The oxidative strength of the *p*-benzoquinonediimine in the reaction media induced at first the chemical modifications on the amino acids themselves by oxido-reduction processes, leading to the reactive intermediates able to covalently bind. N_{α}-acetyl-formylkynurenine resulted from the oxidation of N_{α}-acetyl-tryptophan, and reacted further with the reduced form of the *p*-benzoquinonediimine. Also, a *p*-benzoquinonediimine induced oxidative deamination of the side-chain of N_{α}-acetyl-lysine gave a reactive intermediate for *N*-formylation of the reduced *p*-amino aromatic form.[14] In addition, the formation of an adduct with the amino acid was observed and a similar mechanism was suggested. Still, it might be expected this reactivity to be different with amino acid residues included in a peptide sequence due to the fact that proteins should be regarded as a mixture of nucleophiles having different electronic characteristics, which are also modified by their environment and accessibility, among others. Moreover, numerous studies reported in these last years hypothesize that the allergenicity of a molecule is related to its chemical reactivity toward a few specific amino acids relevant to the sensitization process.[2,15,16] It was therefore of interest to evaluate if there was any selectivity in the *p*-benzoquinonediimines reactivity with nucleophilic amino acids when these are located in a peptide environment.

We now report our studies and results on the reactivity of 2,5-dimethyl-*p*-benzoquinonediimine **1** (Fig. 1) toward a model peptide analogous to the *N*-terminal chain of the globin protein and containing naturally occurring and potential reactive amino acids except for cysteine. Electrospray ionization tandem mass spectrometry (ESI-MS/MS), associated with LC-ESI-MS, was used for the determination of amino acid selectivity by studying the chemical modifications induced on the peptide due to covalent binding of **1**.

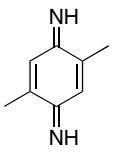

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Figure 1. Chemical structure of 2,5-dimethyl-*p*-benzoquinonediimine **1**.

2. Results and discussion

To test the reactivity of 2,5-dimethyl-*p*-benzoquinonediimine **1**, we used a synthetic peptide with the sequence H_2N-VLSPADKTNWGHEYRMFQIG-CO_2H, analogous to the *N*-terminal chain of globin and already reported in previous hapten–protein binding studies.[16] This peptide included naturally occurring amino acids except cysteine to prevent the formation of a peptide cystine dimer. An additional reason to remove cysteine was to enable the coupling reaction of **1** with other potentially reactive amino acids such as tryptophan and lysine,[13] as it is known that most allergenic compounds, weak or strong, are reactive toward thiol chemical groups.[17]

Very preliminary tests were carried out in a phosphate buffer solution of this synthetic peptide (0.1 M, pH = 7.4) using the derivative of **1** containing two ^{13}C labeled methyl groups at positions C-2 and C-5 (50 equiv). ^{13}C NMR analysis of the modified peptide, isolated from the reaction mixture by gel permeation chromatography, showed a new set of ^{13}C signals in the methyl region between 10 and 20 ppm. It was then deduced that molecules derived from **1** reacted covalently with the peptide. Unfortunately, further ^{1}H{^{13}C} 2D NMR experiments to elucidate the exact structure of the modified peptides as well as to identify which amino acids had been modified and by which mechanism were unsuccessful due to the complexity of the reaction medium.

Electrospray ionization–mass spectrometry (ESI-MS) is today a dominant technique for the structural characterization of peptides and proteins. In particular, the use of ESI-MS/MS has become important for determining the amino acid sequence from specific sequence ions formed during fragmentation of the peptide backbone. In this study, ESI-MS was firstly used to get the best reaction conditions. In addition, the kinetics of the reaction were followed by LC-ESI-MS. Finally, once it was demonstrated that the peptide was chemically modified by reaction with **1**, ESI-MS/MS was performed for the structural determination of the new peptides formed.

2.1. Optimization of the reaction conditions by ESI-MS

To find out the best reaction conditions, the model peptide was reacted with different molar ratios of 2,5-dimethyl-*p*-benzoquinonediimine **1** in deionized water. Best results were obtained using 10 equivalents of **1** (Fig. 2). The ESI-MS spectrum of the reaction mixture showed the formation of two new peptides with quasi-molecular $[M+H]^+$ ions at *m/z* 2467 (Δ_{mass} 118) and *m/z* 2330 (Δ_{mass} −19), besides the $[M+H]^+$ ion of the parent peptide at *m/z* 2349. The same experiment carried out using **1** having two ^{13}C labeled methyl groups at positions C-2 and C-5 showed a shift of two mass units for only one of the two new peptides. Quasimolecular

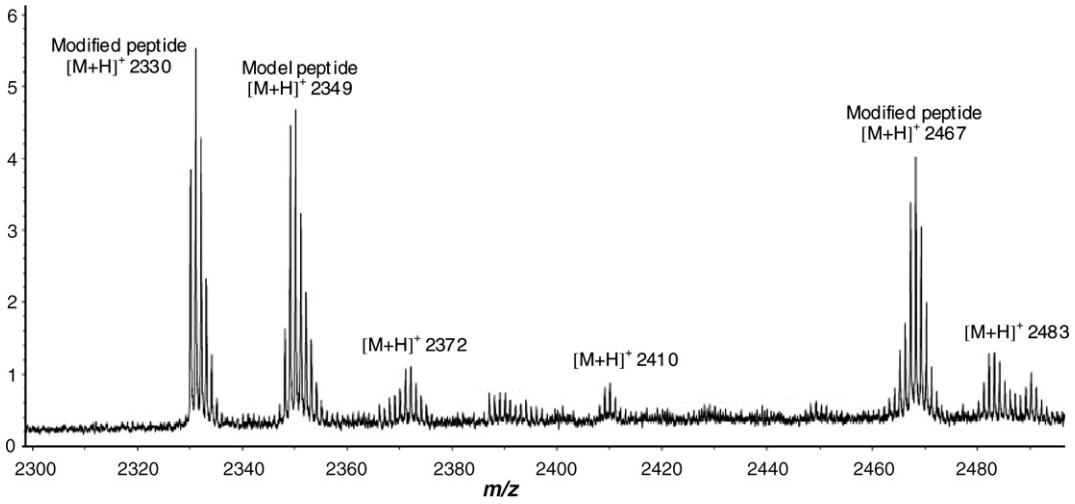


Figure 2. Full scan ESI-MS spectrum of the reaction of **1** (10 equivalents) with the model peptide. The formation of two new peptides with quasimolecular $[M+H]^+$ ions at *m/z* 2467 (Δ_{mass} 118) and *m/z* 2330 (Δ_{mass} −19), besides the $[M+H]^+$ ion of the parent peptide at *m/z* 2349, is shown.

ions at *m/z* 2469 and *m/z* 2330 were observed. It could therefore be assumed that the modified peptide observed at *m/z* 2467 was compatible with a peptide having reacted with a molecule derived from **1**, as an extra difference of two mass units was observed when using the ^{13}C labeled derivative. In contrast, intact *m/z* 2330 should correspond to a peptide modified by other means, without **1** or any of its derivatives covalently bound. Finally, tiny peaks at *m/z* 2365 were detected in all spectra (data not shown) probably resulting from methionine oxidation (Δ_{mass} 16), already observed when studying the reactivity of the *p*-benzoquinonediimine with isolated nucleophilic amino acids.[13]

2.2. Study of the kinetics of the reaction by LC-ESI-MS

Figure 3 shows the evolution of the LC profile of the reaction mixture within five hours (UV detection at 280 nm). For each peak an ESI-MS spectrum was registered. Because of the experimental elution conditions (acetonitrile/water/formic acid), the *m/z* values associated to the LC peaks of the different peptides were observed doubly, triply, and quadruply charged. The model peptide with *m/z* 2349 (equivalent to *m/z* 1175.9^{2+}, 783.8^{3+}, and 588.2^{4+}), the modified peptide with *m/z* 2467 (equivalent to *m/z* 1234.1^{2+}, 823.2^{3+} and 617.9^{4+}), and the modified peptide with *m/z* 2330 (equivalent to *m/z* 1165.9^{2+}, 777.5^{3+}, no signal quadruply charged) were observed at retention times of 7.9, 13, and 13.9 min, respectively. By integrating the UV peaks corresponding to each peptide at different times it was possible to have an analysis of the evolution of the reaction. Approximately 80% of the parent peptide disappeared within five hours of reaction. The peptide with *m/z* 2467 became visible from the beginning of the reaction and the signal raised during the first three hours, to disappear gradually afterward. In parallel, the peptide with *m/z* 2330 appeared after one hour of reaction, and the signal became more intense even after five hours. Clearly, it could be suggested that the peptide reacted initially with **1** at some level and that the new modified peptide formed (*m/z* 2467) was then able to evolve and form the peptide with *m/z* 2330 (Δ_{mass} −137 in respect to *m/z* 2467). It was of interest to notice that *m/z* 2349 and *m/z* 2467 were also observed doubly, triply, and quadruply charged, whereas *m/z* 2330 was only observed doubly and triply charged. This result could suggest that an amino acid side-chain group able to be protonated had been lost in the peptide with *m/z* 2330. Additionally, and also of interest, when searching precisely the HPLC peak matching the *m/z* value of the unmodified peptide (*m/z* 2349), an extra peak with a retention time of 10.8 minutes and having similar *m/z* values was observed (2348^+, 1174.9^{2+}, 783.6^{3+}, although no signal quadruply charged) (Fig. 3a). Finally, even if all the signals were still present after 24 h of reaction, the mass spectra were less resolved with time.

2.3. Sequencing of modified peptides by ESI-MS/MS

ESI-MS/MS was performed in order to find out the chemical structure and chemical modifications of the new peptides (*m/z* 2467 and *m/z* 2330) that were formed after the reaction of **1** with the model peptide. Initially, the fragmentation pattern of the model peptide (*m/z* 2349) in the ESI-MS/MS conditions was studied and the different fragment ions were assigned. Figure 4A shows the observed fragmentation scheme. The b fragment ions happen to extend from the amino *N*-terminus, whereas the y ions happen to extend from the carboxyl C-terminus of the peptide. The sequence of part of the model peptide shown in Figure 4A, derived from the *m/z* 2349 ESI-MS/MS data, was afterward compared with the sequences resulting from the ESI-MS/MS analyses of the quasimolecular ions with associated *m/z* 2467 and *m/z* 2330 values observed in the full scan ESI spectrum of the reaction mixture. Mass differences observed in the y fragment ions were expected to indicate on which amino acid the chemical modifications by reaction with **1** were produced. As an example, at a first sight and as it will be described below, from the fragment ion y_{14} (*m/z* 1768), resulting from the break up between aspartic acid 6 and lysine 7, and up to y_{18} (*m/z* 2138), there was an increment of mass units when analyzing

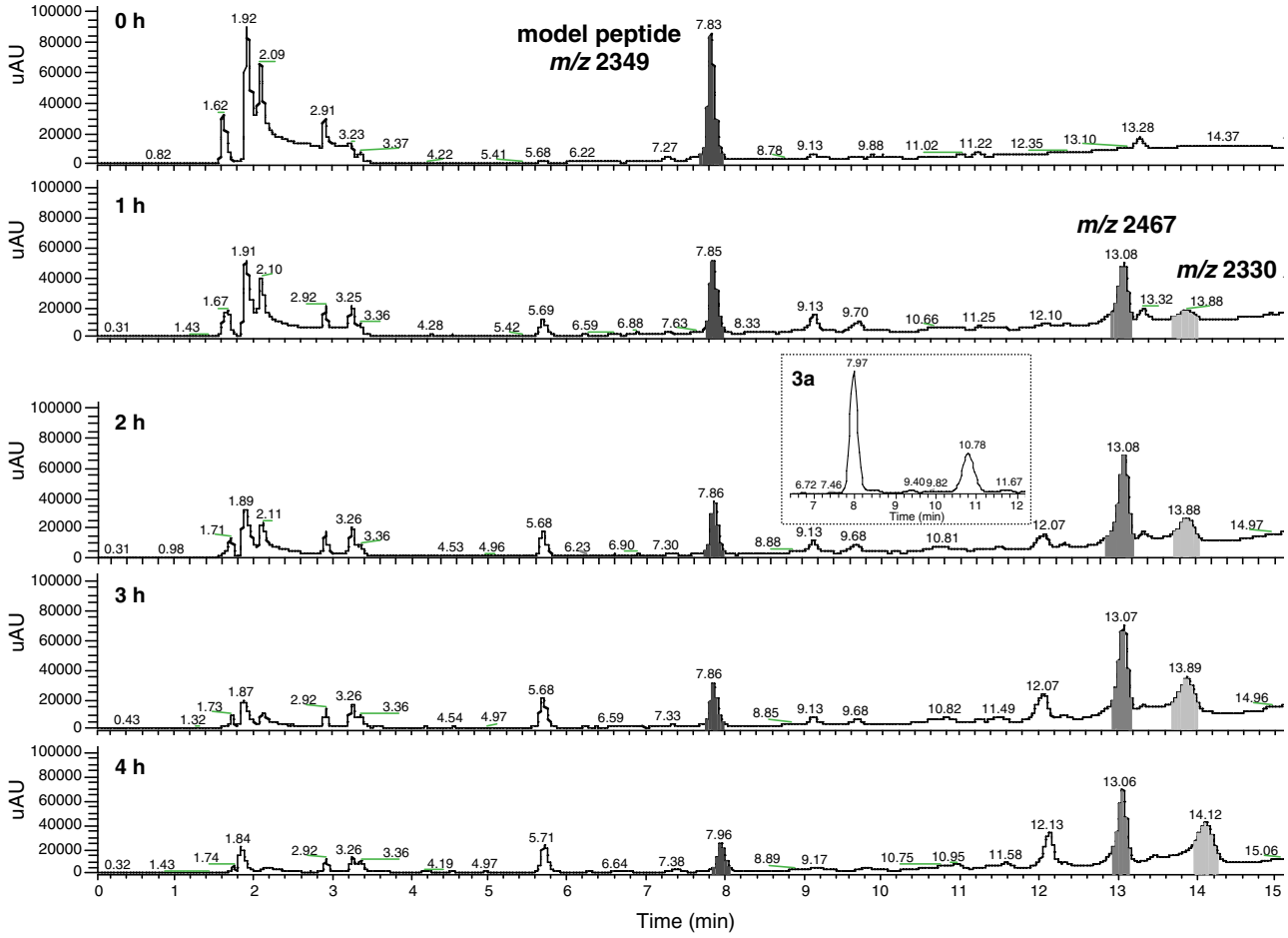


Figure 3. Kinetics of the reaction studied by LC-ESI-MS: evolution of the LC chromatogram of the reaction mixture with time (UV detection at 280 nm). Readily, the model peptide with *m/z* 2349 disappeared to form the two modified peptides with *m/z* 2467 and *m/z* 2330. In Figure 3a are represented the LC peaks exactly matching the *m/z* value of 2349 (extracted from the total ion current chromatogram). Apart from the peak corresponding to the unmodified peptide, another peak with a retention time of 10.8 min was observed.

m/z 2467 (Δ_{mass} 118) and *m/z* 2330 (Δ_{mass} −19). These mass differences were not observed in the case of the y_{10} to y_{13} fragment ions. An implication of the lysine amino acid residue in the reactivity could be, therefore, already underlined.

2.3.1. ESI-MS/MS analysis of *m/z* 2467. The results described in this section are summarized in Scheme 1. As it is shown in Figure 4B, the fragmentation of the *m/z* 2467 parent ion revealed that the shift of 118 mass units (Δ_{mass} 118) was conserved up to the peptide bond between lysine 7 and threonine 8, suggesting that the methylated *p*-benzoquinonediimine had reacted covalently with lysine, the fragment ion y_{14} at *m/z* 1885, resulting from the fragmentation of the peptide bond between aspartic acid 6 and lysine 7, being the last fragment still containing the 118 mass increment. In a second step, y ions were selected from the obtained MS^2 spectra and subjected to further fragmentation. MS^3 fragmentation of *m/z* 1885 (Fig. 5) afforded ions at *m/z* 1639 and *m/z* 1538, corresponding to the break up between lysine 7 and threonine 8, and between threonine 8 and asparagine 9, respectively, together with a fragment ion at *m/z* 1748, which was already observed on the MS/MS spectrum of *m/z* 2467 (Fig. 4B). The difference of 137 mass units existing between fragments *m/z* 1885 and *m/z* 1748 suggested that *m/z* 1748 could result from the fragmentation of an adduct formed between lysine and a molecule derived from **1**, and not from another peptide backbone bond fragmentation. This difference of 137 mass units was also observed in the MS/MS study of *m/z* 2467, as it was shown a molecular ion with an *m/z* 2330 value associated (Fig. 4B). Similarly, *m/z* 2330 could be the product of an *m/z* 2467 fragmentation at an adduct level on lysine. These observations were confirmed by additional MS^3 fragmentation of *m/z* 2330 that afforded once more the ion at *m/z* 1748.

2.3.2. ESI-MS/MS analysis of *m/z* 2330. To get more information, ESI-MS/MS analysis of *m/z* 2330 obtained from the reaction of **1** with the peptide (Fig. 2) was performed. The sequence of part of the modified peptide was identified (Fig. 4C). The decrease of 19 mass units (Δ_{mass} −19) in relation to the unmodified peptide (*m/z* 2349) was conserved up to the peptide bond between lysine 7 and threonine 8, suggesting again that lysine had been chemically modified. The fragment ion y_{14} at *m/z* 1748 resulting this time from the break up between

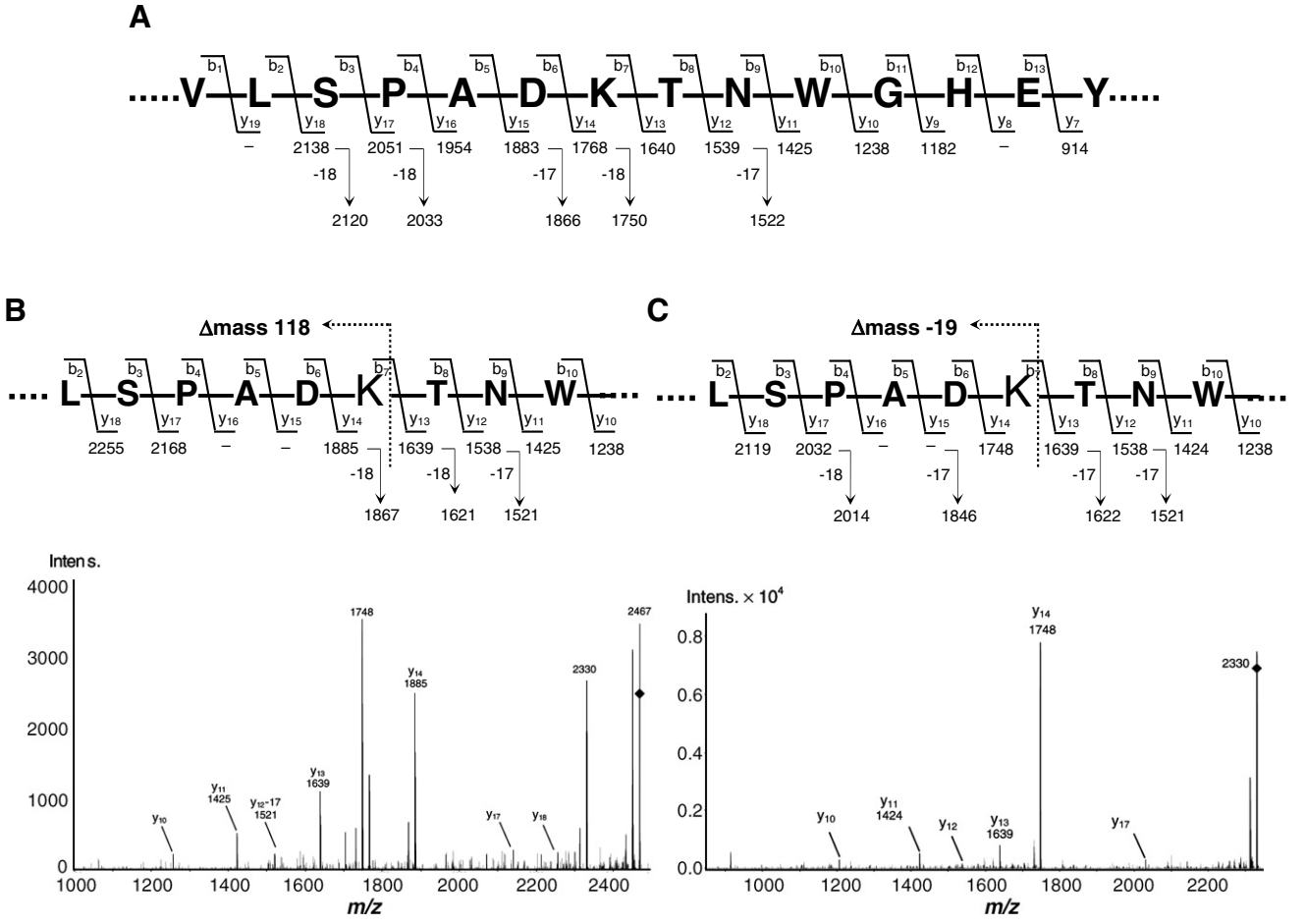
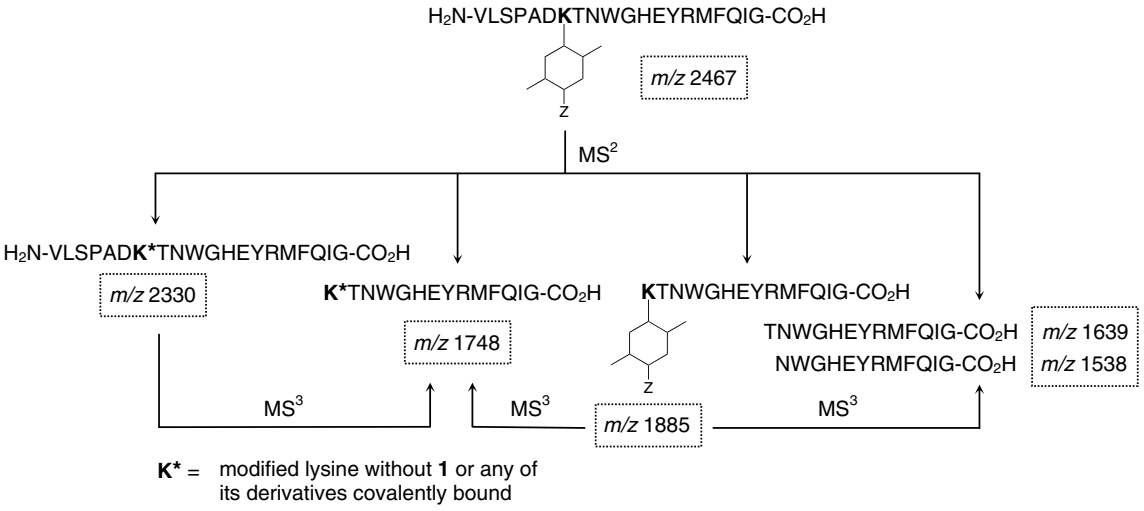


Figure 4. Sequencing by ESI-MS/MS of the modified peptides that resulted from the reaction between **1** and the model peptide. (A) Model peptide sequence derived from the *m/z* 2349 ESI-MS/MS data. (B) ESI-MS/MS spectrum obtained from the analysis of *m/z* 2467 and sequence of the peptide resulting from the data. (C) ESI-MS/MS spectrum obtained from the analysis of *m/z* 2330 and sequence of the peptide derived from the data. Ions resulting from the loss of ammonia or water are also shown.

aspartic acid 6 and modified lysine 7 was once more observed. Further MS^3 of *m/z* 1748 afforded the ion at *m/z* 1639, which is the result of the peptide bond fragmentation between modified lysine 7 and threonine 8.

2.4. Mechanistic interpretations

The different results presented above suggested strongly that 2,5-dimethyl-*p*-benzoquinonediimine **1** reacted with



Scheme 1. Summary of the most important results obtained from ESI-MS/MS analysis of *m/z* 2467. According to the different fragmentations observed, lysine was chemically modified after reaction with **1** via the formation of an adduct. However, the loss of 137 mass units from *m/z* 2467 and from *m/z* 1885 indicated that this adduct could evolve further affording also lysine chemically modified but without **1** covalently bound.

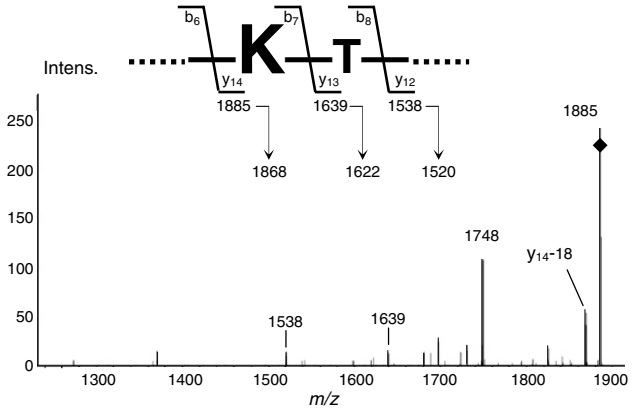


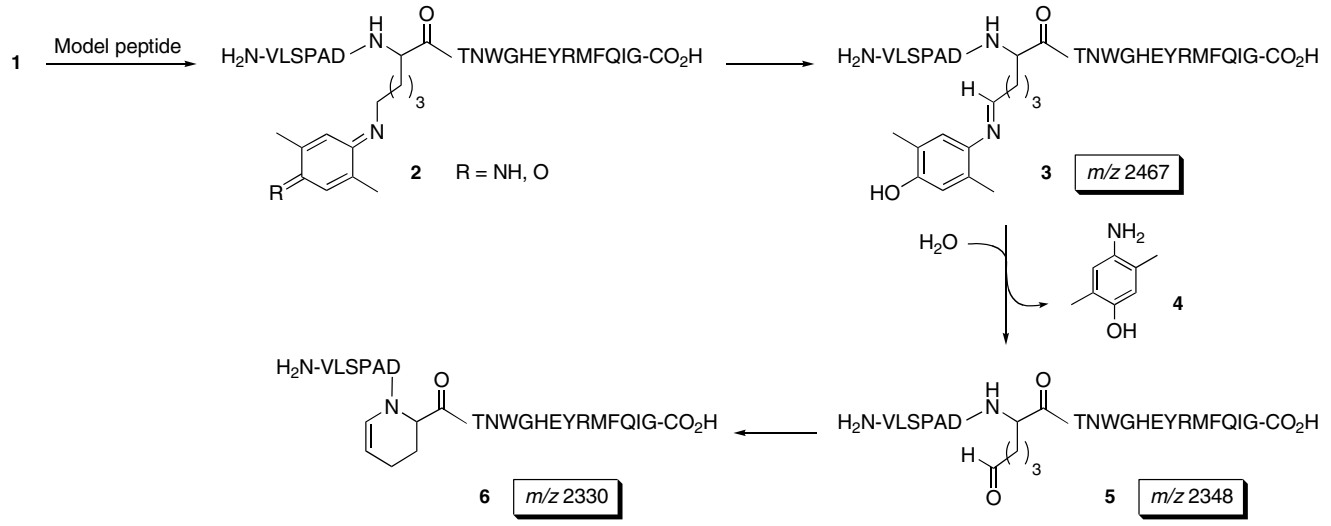
Figure 5. MS^3 analysis of *m*/*z* 1885, itself obtained from ESI-MS/MS of *m*/*z* 2467. The fragment ion y_{14} at *m*/*z* 1885 (Fig. 4B) was the last fragment still containing the 118 mass increment and corresponded to the breakup of the peptide bond between aspartic acid 6 and lysine 7. The fragment ion at *m*/*z* 1748 is shown. The loss of 137 mass units between *m*/*z* 1885 and *m*/*z* 1748 suggested the fragmentation of an adduct formed between lysine and a molecule derived from **1**.

lysine 7 of the model peptide. In the course of our previous studies trying to understand the mechanisms by which *p*-benzoquinonediimines can react with nucleophilic residues on amino acids, we already described the lysine induced *N*-formylation of **1**.[14] The mechanism we proposed was based on the well reported oxidative degradation of α-amino acids to form aldehydes. It was described that a nucleophilic attack of the ε-NH_2 group of lysine on the imine carbon atom of **1**, followed by the release of a NH_3 molecule, gave an intermediate able to eliminate an hydrogen of the α-carbon atom and form, after aromatization of the cycle, a Schiff base type intermediate. Through hydrolysis, *N*-acetyl-α-aminoadipate-δ-semialdehyde could be released from the Schiff base together with the reduced form of **1**. Then, an intramolecular cyclization of *N*-acetyl-α-aminoadipate-δ-semialdehyde yielded *N*-acetyl-1-piperideine-6-carboxylic acid, which on further oxidation could afford *N*-acetyl-*N*-formyl glutamic semialdehyde. Finally, a nucleophilic attack of the released reduced form of **1** on *N*-acetyl-*N*-formyl glutamic semialdehyde explained the formation of the *N*-formylated derivative. An analogous mechanism can be extrapolated for the reaction between **1** and lysine of the model peptide (Scheme 2). The Schiff base like intermediate **3** can be formed succeeding the initial reaction of the ε-NH_2 group of the side-chain of lysine with the imine carbon atom of **1**, the release of a NH_3 molecule, the elimination of a hydrogen of the α-carbon atom and the aromatization of the cycle. However, at some time, a diimine hydrolysis must have been occurred in order to rationalize the exact *m*/*z* value of 2467. No similar hydrolysis was reported when we investigated the adduct formation of **1** with isolated N_α-acetyl-lysine,[13,14] but it was reported when N_α-acetyl-tyrosine or N_α-acetyl-methionine was used affording a *p*-benzoquinone derivative.[13] On the other hand, reactivity studies of 2,5-dimethyl-*p*-benzoquinone, deriving from the hydrolysis of **1**, with nucleophilic amino acids only showed the adduct formation with N_α-acetyl-cysteine (data not shown). Therefore, most probably the initially formed diimine adduct of the peptide must have undergone hydrolysis of the remaining imine group instead of being the starting diimine itself that underwent hydrolysis prior to reaction with the peptide.

After that, and through hydrolysis, peptide **3** can release **4** and be converted into peptide **5**, an intermediate aldehyde of the peptidyl α-aminoadipic-δ-semialdehyde type, with an associated value of *m*/*z* 2348. It is important to remind at this stage that an HPLC peak with a retention time of 10.8 min and with an *m*/*z* associated value of 2348 was indeed observed in the LC-ESI-MS experiments that could correspond to peptide **5** (Fig. 3a). Thus, peptide **5** may result from an oxidative deamination of the side-chain of lysine at position 7, induced by the reactivity with the *p*-benzoquinonediimine, as it has been described for several enzymatic quinonoid co-factors of many amine oxidases.[18–22] Finally, intramolecular cyclization of **5** may well form peptide **6**, with an *m*/*z* associated value of 2330, and corresponding to a peptide in which lysine has been modified but does not contain **1** or any of its derivatives covalently bound. The mechanism presented here explains the order of appearance of *m*/*z* 2467 and *m*/*z* 2330 in the LC-ESI-MS studies, as well as the fact that *m*/*z* 2467 was found doubly, triply and quadruply charged, whereas *m*/*z* 2330 was only found doubly and triply charged, clearly indicating the loss of an amino group.

3. Conclusion

2,5-Dimethyl-*p*-benzoquinonediimine **1**, a model compound used to evaluate potential candidates for the formation of antigenic structures responsible for allergic contact dermatitis to *p*-amino aromatic compounds and their oxidation derivatives, was shown to modify lysine in a model peptide containing naturally occurring and potential reactive amino acids excepted cysteine. Cysteine was not incorporated into the peptide to prevent the formation of a cystine dimer, but also to allow the coupling reaction of **1** with other potentially reactive amino acids, as it is known that thiol chemical groups are highly reactive toward most skin allergens and would hide any other reactivity. From ESI-MS/MS and LC-ESI-MS studies, we were able to demonstrate that 2,5-dimethyl-*p*-benzoquinonediimine **1** reacted specifically with lysine when this amino acid was included in a peptide sequence. Benzoquinonediimine **1** reacted readily with the amino group of the side-chain of lysine to form a covalent adduct of the Schiff base kind. More interestingly, an oxido-reduction process started at this point able to deaminate oxidatively lysine, giving an intermediate aldehyde of the peptidyl α-aminoadipic-δ-semialdehyde type responsible for a further intramolecular cyclization of the peptide. The first covalent adduct formed with lysine was not very stable even if still present in the reaction medium after 24 h of reaction. On the other hand, the important chemical modifications of the peptide at the lysine level induced by the initial reactivity of the *p*-benzoquinonediimine were evidenced. According to our previous studies,[13,14] the chemical behavior and the mechanisms of reaction described for



Scheme 2. Mechanism suggested for the reactivity of **1** with the model peptide. The chemical structures of the peptides modified by the reactivity of lysine and consistent with *m/z* 2467, *m/z* 2348, and *m/z* 2330 are indicated.

1 toward lysine were now confirmed when using a model peptide. Of course, further studies will need to assess this reactivity in the context of bigger proteins as the environment and, therefore, the access to the reactive lysine amino acids will be modified. It can therefore be concluded that, for the formation of antigenic structures, lysine should be considered as a key amino acid for the hapten–protein complex formation in the case of *p*-benzoquinonendiimines. But also, that in parallel to the direct covalent binding, other chemical modifications that are a direct consequence of the reactivity and chemical behavior of the hapten toward the peptide must be considered as potential candidates for the formation of antigenic structures. These chemical modifications will certainly induce conformational changes on the peptide that should also be taken into account for further hapten-protein binding studies.

4. Experimental

Caution: Skin contact with *p*-amino aromatic compounds and oxidation derivatives must be avoided. These are sensitizing substances and must be in consequence handled with care.

4.1. Chemicals

p-Phenylenediamine was kindly supplied by L'Oréal Recherche (Aulnay sous Bois, France). 2,5-Dimethyl-*p*-benzoquinonediimine **1** was synthesized from *p*-phenylenediamine according to a procedure described previously for the synthesis of 2,5-[^{13}C]-dimethyl-*p*-benzoquinonediimine and using methyl iodide instead of [^{13}C]-methyl iodide.[13] The model peptide H_2N-VLSPADKTNWGHEYRMFQIG-CO_2H was synthesized at the Inserm U575 unity (Plateforme Protéines, Centre de Neurochimie, Strasbourg, France) with an automatic Applied Biosystems 433A peptide synthesizer. The purity of the peptide (>99%) was assessed by mass spectrometry. All the reagents and HPLC-grade solvents were obtained from Sigma–Aldrich (Saint Quentin Fallavier, France) and used without further purification. Aqueous solutions were prepared with deionized water.

4.2. Electrospray ionization-mass spectrometry studies (ESI-MS)

To get the best reaction conditions, a solution of model peptide (0.25 mg, 0.1 μmol) in previously degassed water (0.42 mL) was added to 5 or 10 equivalents of 2,5-dimethyl-*p*-benzoquinonediimine **1** (71 μg, 0.5 μmol, 5 equiv; 143 μg, 1 μmol, 10 equiv). The reaction mixture was then stirred at room temperature for 2 h. After this time, 60 μL of the reaction mixture was diluted into 100 μL of water and 1 mL of acetonitrile and the solution was infused by a syringe pump (150 μL/h) on a Bruker micrOTOF™ time of flight mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an ESI source. The spray needle was set at a potential of 350 V, at 40 V for the skimmers, and a nitrogen nebulizing gas at a pressure of 43.5 psi was used to stabilize the spray. Additionally, heated nitrogen (300 °C) drying gas was used at a flow rate of 5 L/min. The ionization method used was ESI in the positive ion mode. Full scan mass spectra were acquired in the profile mode scanning from *m/z* 700 to 2500. A Bruker Daltonics Data Analysis software (version 3.1) was employed for the treatment of the spectra.

4.3. Liquid chromatography-mass spectrometry studies (LC-ESI-MS)

A solution of the model peptide (0.25 mg, 0.1 μmol) in previously degassed water (0.42 mL) was added to 2,5-dimethyl-*p*-benzoquinonediimine **1** (143 μg, 1 μmol, 10 equiv). The reaction mixture was stirred at room temperature and 50 μL was taken at regular times for LC-ESI-MS analysis. LC analyses were performed using a

Surveyor HPLC system (Thermo Finnigan, San José, CA, USA) equipped with a quaternary pump, an automatic sample injector and a diode array absorbance detector scanning from 200 to 700 nm. The peptides were separated by reverse phase chromatography on a C18 column (4.6 × 150 mm; Waters XTerra®; 5 μm particle size) at a flow rate of 0.8 mL/min. The peptides were eluted from the column using a mobile phase B (0.1% formic acid in an acetonitrile/water 9:1 mixture) and a mobile phase A (0.1% formic acid in water) with a three-step linear gradient. The gradient starting at 20% B was increased to 40% B in 15 min, to 50% B in 5 min, and was finally decreased to 20% B in the last 5 min. The injection volume was 10 mL. The eluted peptides (singly, doubly, triply or quadruply charged ions) were analyzed by mass spectrometry. After passing through the diode array absorbance detector, the eluent was directed at a flow rate of 400 μL/min to a connected LCQ Deca XP ion trap mass spectrometer (Thermo Finnigan, San José, CA, USA) with a standard electrospray source. The ionization method used was ESI in the positive ion mode. The heated capillary temperature was 300 °C, the nebulizing gas was nitrogen with sheath gas flow rate of 55 (arbitrary units), and source voltage was at 4 kV. Full scan mass spectra were acquired in the profile mode scanning from *m*/*z* 50 to 2000. All data were processed using Qual Browser (version 1.4), which is part of the Thermo Finnigan Xcalibur™ software.

4.4. Electrospray ionization tandem mass spectrometry studies (ESI-MS/MS)

ESI-MS/MS studies were performed using 10 equivalents of 2,5-dimethyl-*p*-benzoquinonediimine **1** which appeared to be the optimal reaction conditions. A solution of model peptide (0.25 mg, 0.1 μmol) in previously degassed water (0.42 mL) was added to 2,5-dimethyl-*p*-benzoquinonediimine **1** (143 μg, 1 μmol, 10 equiv). After 2 h of stirring at room temperature, 60 μL of the reaction mixture was diluted in 100 μL of water and 1 mL of acetonitrile and infused on a Bruker Esquire3000+ ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with the ESI source used for the ESI-MS studies, described above, and with the same source parameter settings. The ionization method used was ESI in the positive ion mode. Settings for MS^2 were fragmentation amplitude 1.45 and isolation width 3 *m*/*z*. Settings for MS^3 were fragmentation amplitude 1.10 and isolation width 3 *m*/*z*. The mass spectrometer was interfaced to a computer workstation running a Bruker Daltonics Data Analysis software (version 3.1) for data processing. Control ESI-MS/MS analysis of the model peptide was performed using exactly the same experimental conditions.

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