

Preservatives in cosmetics: reactivity of allergenic formaldehyde-releasers towards amino acids through breakdown products other than formaldehyde*

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Background: Compounds slowly releasing formaldehyde, the so-called formaldehyde-releasers, are commonly employed as preservatives in cosmetics instead of free formaldehyde, which is a strong skin sensitizer. It has been long accepted that formaldehyde-releaser sensitization is attributable to released formaldehyde. However, clinical studies show the existence of patients allergic to formaldehyde-releasers but not to formaldehyde itself.

Objectives: To prove that, for certain formaldehyde-releasers, reactive intermediates other than formaldehyde could be involved in the formation of the hapten–protein antigenic complex, a key step of the sensitization process, thus explaining their sensitizing potential.

Materials/Methods: DMDM hydantoin, 2-bromo-2-nitropropane-1,3-diol and methenamine were synthesized, ^{13}C -labelled at the position(s) precursor of formaldehyde. Their reactivity towards amino acids was followed by one-dimensional and two-dimensional ^{13}C -nuclear magnetic resonance.

Results: Many adducts formed by reacting formaldehyde-releasers with amino acids resulted from a direct interaction of the releaser or from reaction of a breakdown product, and not from a reaction involving simply released formaldehyde. DMDM hydantoin was reactive *per se*, and 2-bromo-2-nitropropane-1,3-diol and methenamine decomposed in water, producing bromoethanol and diaminomethane, respectively, which were reactive towards some of the amino acids tested.

Conclusion: The reactivity of distinctive formaldehyde-releasers towards amino acids is not limited to formaldehyde release.

Key words: contact allergy; degradation products; DMDM hydantoin; formaldehyde-releasers; methenamine; preservatives; reactivity; 2-bromo-2-nitropropane-1,3-diol. © John Wiley & Sons A/S, 2010.

Conflicts of interest: The authors have declared no conflicts.

Accepted for publication 4 May 2010

Preservatives used in cosmetics constitute an important source of allergic contact dermatitis (1). Some of the most problematic preservatives are formaldehyde and formaldehyde-releasing agents. The use of free formaldehyde, whose bactericidal and fungicidal properties make it a substance of choice

for the preservation of cosmetics, has been reduced because of the bad press that it has received as an irritant, sensitizer and potential carcinogen (2). Formaldehyde is known to be a strong and ubiquitous skin sensitizer, including from non-cosmetic sources of contact. Because of this, exposure to formaldehyde in the EU is subject to restrictions. Free formaldehyde may be used as a preservative in all cosmetic products (maximum authorized concentration 0.2%, except in products for oral

*This work was financially supported by the Afssaps (Agence Française de Sécurité Sanitaire des Produits de Santé), France.

hygiene, where it is 0.1%) except for aerosol cosmetics. Annex VI of the Cosmetics Directive 76/768 EC further stipulates that all finished products containing formaldehyde or substances that release formaldehyde must be labelled with the warning 'contains formaldehyde' when the concentration of free formaldehyde in the finished product exceeds 0.05% (3). As an alternative, chemical compounds that slowly release formaldehyde in the presence of water and under usage conditions, the so-called formaldehyde-releasers, are commonly employed as preservatives in cosmetics (water-based preparations) instead of free formaldehyde. These preservatives have the ability to replace used-up formaldehyde by releasing it in very small amounts over time as needed. If the released formaldehyde is consumed, the formaldehyde-releaser supplies more formaldehyde. The action of formaldehyde-releasing agents is therefore based on the continuous attainment of this equilibrium. In this way, the actual level of free formaldehyde in the product remains low but sufficient enough to prevent or eliminate bacterial growth. Accordingly, the antimicrobial activity of these preservatives most likely results from formaldehyde release, but it has also been postulated that some of them act as biocides on their own, independently of formaldehyde release (4, 5).

Unfortunately, many formaldehyde-releasers used in cosmetics are also skin sensitizers. Once an individual is sensitized to formaldehyde, it is thought that the individual may also react to all of its various releasers. Indeed, patients with allergic patch test reactions to formaldehyde often have concomitant reactions to formaldehyde-releasing agents. However, it has been shown by many reported clinical studies that sensitivity to formaldehyde is not always a requirement for sensitivity to a formaldehyde-releasing preservative. Several years ago, for example, in a review of patch test data for preservative allergy collected from 1982 to 1993 at St John's Institute of Dermatology (London), it appeared that just half of the patients allergic to quaternium-15 also reacted to formaldehyde, and only one-quarter of patients allergic to 2-bromo-2-nitropropane-1,3-diol (bronopol) had concomitant reactions with formaldehyde. Moreover, concomitant reactions between 2-bromo-2-nitropropane-1,3-diol and other formaldehyde-releasers, such as diazolidinyl urea or imidazolidinyl urea, were very rare, regardless of the fact that all compounds release formaldehyde more or less quickly (6). Similarly, in a more recent study including 15 individuals patch tested with formaldehyde and several formaldehyde-releasers, among 8 patients allergic to quaternium-15 only 4 had a concomitant reaction to formaldehyde, and among 6

patients sensitive to 2-bromo-2-nitropropane-1,3-diol, only 1 was positive for formaldehyde (7). Thus, formaldehyde is most probably the common factor when concomitant allergic reactions exist between formaldehyde and formaldehyde-releasers, but it certainly cannot account for patients allergic to certain formaldehyde-releasers and not to formaldehyde itself. In consequence, for a number of formaldehyde-releasers, it may be suggested that either the intact molecule or breakdown/degradation products of the molecule other than formaldehyde could be involved in the formation of the hapten–protein complex, the first key step of any sensitization process. It is therefore of interest to investigate the mechanisms by which formaldehyde-releasers can react with amino acids in proteins, to determine whether this can add some value to our understanding of skin sensitization to these compounds.

In this article, we report the reactivity of three representative formaldehyde-releasers, 1,3-dimethylol-5,5-dimethylhydantoin (DMDM hydantoin), 2-bromo-2-nitropropane-1,3-diol (bronopol) and methenamine (hexamethylenetetramine), towards nucleophilic amino acids. DMDM hydantoin was chosen as a model for the class of compounds known as hydantoins, such as diazolidinyl urea and imidazolidinyl urea, all of which contain a heterocycle part derived from imidazole and urea-like subunits. For these compounds, similar chemical reactivity could be expected, as the chemical functions that are potentially reactive towards the amino acids were initially the same. Following the same idea, methenamine was chosen as a model of its chloroallyl derivative quaternium-15. Finally, 2-bromo-2-nitropropane-1,3-diol was selected, as it is the formaldehyde-releasing agent for which there is the largest number of reported cases of clinically positive reactions to the releaser and not to formaldehyde. The compounds were ^{13}C -labelled at the position(s) precursor of the release of formaldehyde (Fig. 1), and the studies were carried out by using associated ^{13}C -nuclear magnetic resonance (NMR) and $^1\text{H}\{^{13}\text{C}\}$ -NMR spectroscopy. Except for the expected reactivity of released formaldehyde, the results of the experiments also showed, in some cases, direct reactivity of the formaldehyde-releaser, and in other cases reactivity of degradation products other than released formaldehyde. We therefore suggest that the reactivity of distinctive formaldehyde-releasers towards nucleophilic amino acids and proteins is not limited to the release of formaldehyde. Many antigenic structures could derive from the reactivity observed, and could be at the origin of the differences described in the clinically reported reactions to these compounds.

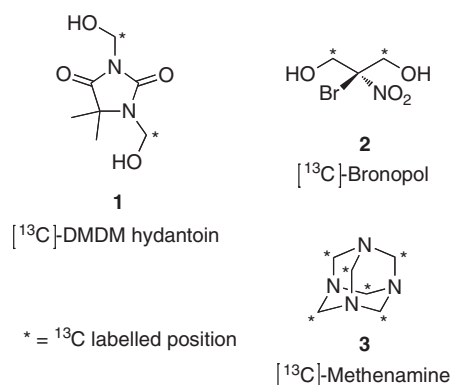


Fig. 1. Chemical structures of ^{13}C DMDM hydantoin (1) (1,3- ^{13}C dimethylol-5,5-dimethylhydantoin), ^{13}C 2-bromo-2-nitropropane-1,3-diol (bronopol) (2) (2-bromo-2-nitro-1,3- ^{13}C propane-1,3-diol) and ^{13}C -methenamine (3) (^{13}C hexamethylenetetramine), ^{13}C -labelled at the position(s) precursor of the release of formaldehyde.

Materials and Methods

Chemicals

^{13}C DMDM hydantoin (1) was synthesized from dimethyl hydantoin and ^{13}C formaldehyde, using a previously reported procedure (8). Non-labelled DMDM hydantoin was obtained by using the same procedure and non-labelled formaldehyde. ^{13}C 2-Bromo-2-nitropropane-1,3-diol (2) was synthesized according to a procedure described for the synthesis of 2-bromo-2-nitropropane-1,3-diol, and employing bromonitromethane and ^{13}C formaldehyde as reagents (9). Commercial non-labelled 2-bromo-2-nitropropane-1,3-diol was purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). ^{13}C Methenamine (3) was prepared by the reaction of aqueous ammonia (four equivalents, 30% solution) and ^{13}C formaldehyde (six equivalents). The pH of the solution was adjusted to 8–9 with a solution of hydrochloric acid (0.1 M). The reaction mixture was then stirred for 3 hr at room temperature and for 1 hr at 70°C. The solvent was removed under reduced pressure, and the crude material obtained was recrystallized in acetone to give ^{13}C methenamine (3). Commercial non-labelled methenamine was purchased from Fluka Biochemika (Saint Quentin Fallavier, France). ^{13}C Formaldehyde (20% in aqueous solution) and deuterated solvents were obtained from Euriso-Top (Saint Aubin, France). Formaldehyde (37% in aqueous solution, stabilized with 10% methanol) was purchased from Merck (Darmstadt, Germany). Sodium phosphate monobasic and sodium phosphate dibasic, for the preparation of buffers, were purchased from Merck. *t*-Butanol, used as an NMR internal standard, was acquired from Acros Organics (Illkirch, France). *N*-Acetylated amino acids were purchased from Sigma-Aldrich (Saint Quentin

Fallavier, France) and were used as delivered. All other chemicals and solvents were also purchased from Sigma-Aldrich.

Quantitative determination of formaldehyde release by ^{13}C -NMR

Initially, a calibration curve was prepared with 26 solutions of formaldehyde of known concentration (0–0.37 M) prepared in NMR tubes as follows: x μl of an aqueous solution of formaldehyde (37%), used without determining its absolute concentration by titration, (450– x) μl of phosphate buffer (0.13 M, pH 7.8), and 150 μl of a solution of *t*-butanol in deuterated water as internal standard (0.1 M). The final buffer solution in the NMR tube was 0.1 M at pH 7.4. A one-dimensional ^{13}C NMR spectrum of each solution was recorded on a Bruker Avance spectrometer at 75 MHz. For each spectrum, the area of the formaldehyde signal at 82.0 ppm (hydrated form) was compared with that of the *t*-butanol signal at 30.3 ppm (internal standard technique), using NMRNOTEBOOK software (version 2.0; NMRtec®, Illkirch Graffenstaden, France). This was done to correct for any variation or loss of formaldehyde during sample preparation or sample inlet. For each solution, the calculated area of the formaldehyde ^{13}C -NMR signal was correlated with the known formaldehyde concentration. The calibration curve obtained in this way corresponded to a straight line (correlation coefficient $r^2 = 0.989$) of the following equation: $\text{Area}_{(\text{HCHO}_{\text{peak}})} = 2 \times 10^9 [\text{HCHO}]$.

Quantification of formaldehyde released by DMDM hydantoin, 2-bromo-2-nitropropane-1,3-diol and methenamine, in a phosphate buffer solution (pH 7.4), was performed by extrapolation into the calibration curve of the area of the ^{13}C -NMR signal related to released formaldehyde. NMR samples were prepared as follows: x mg of formaldehyde-releaser, 450 μl of phosphate buffer (0.13 M, pH 7.8), and 150 μl of a solution of *t*-butanol in deuterated water as internal standard (0.1 M). The final buffer solution in the NMR tube was 0.1 M at pH 7.4. A one-dimensional ^{13}C -NMR spectrum of each solution was recorded on a Bruker Avance spectrometer at 75 MHz. The area of the ^{13}C -NMR signal corresponding to formaldehyde released was calculated by the internal standard technique, using NMRNOTEBOOK software.

Reaction of formaldehyde-releasers 1–3 with nucleophilic amino acids: general procedure

A phosphate buffer solution (450 μl , 0.1 M, pH 7.4) containing the amino acid in its *N*-acetylated form (10 equivalents) was added to a solution of

labelled formaldehyde-releaser (7 μmol) in deuterated water (150 μl). The final solution (600 μl) was filtered into an NMR tube. The reaction was followed by one-dimensional ^{13}C -NMR carried out on a Bruker Avance 300 spectrometer at 75 MHz. The amino acids tested were *N*-Ac-cysteine (*N*-Ac-Cys), *N*-Ac-lysine (*N*-Ac-Lys), *N*-Ac-histidine (*N*-Ac-His), *N*-Ac-arginine (*N*-Ac-Arg), *N*-Ac-tryptophan (*N*-Ac-Trp), *N*-Ac-asparagine (*N*-Ac-Asn), *N*-Ac-serine (*N*-Ac-Ser), *N*-Ac-tyrosine (*N*-Ac-Tyr), *N*-Ac-threonine (*N*-Ac-Thr), *N*-Ac-methionine (*N*-Ac-Met) and *N*-Ac-glutamine (*N*-Ac-Gln).

Structural characterization of the products

In order to elucidate the structure of the products formed by the reaction of releasers **1–3** with the amino acids, two-dimensional heteronuclear [^1H – ^{13}C]-NMR experiments were carried out at each important step of the reaction. Structures of the products were assigned using a combination of heteronuclear single-quantum correlation and heteronuclear multiple-bond correlation data, and by comparing the measured chemical shifts (δ) with those calculated by ACD/CNMR and ACD/HNMR PREDICTOR (version 6.0; ACD/Labs, Toronto, Canada) and OFFICE CHEM DRAW ULTRA (version 11.0; CambridgeSoft, Cambridge, MA, USA) software. ^1H -NMR and ^{13}C -NMR data were recorded on a Bruker Avance 400 spectrometer (^1H , 400 MHz; ^{13}C , 100 MHz). Chemical shifts (δ) are reported in ppm with respect to tetramethylsilane, and acetonitrile was the internal standard ($\delta^1\text{H} = 2.06$, $\delta^{13}\text{C} = 119.7$). Spectra were treated using NMRNOTEBOOK (version 2.0; NMRtec®). The chemical structures of adducts resulting from the reaction with released formaldehyde were also corroborated by comparison of the chemical shifts with those of reference compounds produced by the reaction of [^{13}C]formaldehyde with the amino acids.

Results

Quantitative determination of formaldehyde release by ^{13}C -NMR

Before studying the reactivity of [^{13}C]formaldehyde-releasers **1–3**, we decided to evaluate the release of formaldehyde by these compounds in the same experimental conditions used for the reactions with the amino acids.

Many different methods are available for the qualitative and quantitative analysis of formaldehyde in the presence of formaldehyde donors. The most well known is probably the one adopted within the EU since 1990 as the official method for the determination of free formaldehyde content in cosmetic

products (10). In this method, the quantification of free formaldehyde is based on its reaction with acetylacetone and ammonia, with subsequent photometric determination of the diacetyldihydrolutidine formed. Chromatographic separation with post-column derivation is used to separate free formaldehyde from the donor (11). Other methods based on chromatographic, photometric and titrimetric procedures have also been reported (12–14). However, a major drawback of these analytical methods, which are based on the chemical modification of free formaldehyde by derivation, is that they have an effect on the equilibrium set up between formaldehyde and the formaldehyde-releaser. Therefore, misleading results on the real amount of formaldehyde released can be obtained. This problem can be overcome by using ^{13}C -NMR spectroscopy. ^{13}C -NMR spectroscopy is merely a physical method, with sufficient sensitivity, that does not affect the equilibrium between formaldehyde and the formaldehyde-releaser during the measurement. As has been demonstrated by comparing several *O*-formal-based and *N*-formal-based formaldehyde-releasers used in consumer products, it can be used as a non-invasive method for the determination in finished products of formaldehyde concentrations much lower than the legally required duty of declaration of 0.05% (15).

We used ^{13}C -NMR spectroscopy to quantitatively measure the release of formaldehyde in phosphate buffer solutions at physiological pH (0.1 M, pH 7.4) from DMDM hydantoin, 2-bromo-2-nitropropane-1,3-diol and methenamine. It was important to be sure of the pH value of the samples analysed, as it is known that the release of formaldehyde from different formaldehyde-releasers can be dependent on the pH. 2-Bromo-2-nitropropane-1,3-diol, for example, releases more formaldehyde in alkaline buffer than in acidic buffer (16). On the other hand, it is known that the antibacterial effect of methenamine derives from the slow release of formaldehyde by hydrolysis at acidic pH (17, 18). The concentration of free formaldehyde was measured by extrapolating the area of the ^{13}C -NMR signal related to released formaldehyde into a formerly established calibration curve. This calibration curve was set up with 26 formaldehyde solutions of known concentration (0–0.37 M) in phosphate buffer, using *t*-butanol as internal standard, and by correlating the calculated area of the formaldehyde ^{13}C -NMR signal with the known formaldehyde concentration of each solution.

Table 1 shows, for each formaldehyde donor, the amounts of released formaldehyde in the case of three solutions of different concentration. Measurements were initially made 24 hr after the preparation of the solutions. DMDM hydantoin released formaldehyde quickly. About 25% of the theoretical amount of formaldehyde that could be released

Table 1. Quantitative release of formaldehyde in the experimental conditions used for the reactivity studies

Chemicals	Concentration of chemical (M) ^a	Concentration of free formaldehyde (M) ^b
HCHO ^c	0.049	0.049
HCHO	0.098	0.098
HCHO	0.146	0.146
DMDM hydantoin	0.096	0.060
	0.193	0.092
	0.289	0.121
2-Bromo-2-nitropropane-1,3-diol	0.091	0
	0.182	0
	0.273	0.017
		0.020 ^d
Methenamine		0.104 ^e
	0.130	0
	0.259	0
	0.389	0

^aConcentration of chemical at time 0 in the phosphate buffer solution (0.1 M, pH 7.4).

^bConcentration of free formaldehyde in the solution 1 day after the preparation of the samples.

^cControl experiment.

^dConcentration of free formaldehyde in the solution 7 days after the preparation of the sample.

^eConcentration of free formaldehyde in the solution 40 days after the preparation of the sample.

was measured in the solutions, no matter what the initial concentration of DMDM hydantoin. In contrast, it was not possible to detect free formaldehyde released from 2-bromo-2-nitropropane-1,3-diol at physiological pH after 24 hr. Extra measurements on the more concentrated solution (0.273), after 7 and 40 days, indicated, however, the presence of free formaldehyde at 0.02 M and at 0.104 M, respectively. Thus, nearly 6% of the theoretical amount of formaldehyde that could be released was observed after 40 days. These results suggested that 2-bromo-2-nitropropane-1,3-diol was releasing formaldehyde very slowly at physiological pH. Also, it could not be excluded that the small amount released could be reacting with some other intermediates present in the solution and was thus not being detected. In the case of methenamine, no release of formaldehyde was detected even after 40 days.

Reactivity of [¹³C]DMDM hydantoin (**1**)

[¹³C]DMDM hydantoin (**1**) reacted with several amino acids. Some adducts resulted from the reaction with released formaldehyde, as expected. However, some other adducts resulted from the direct reaction of **1** or from the reaction of a degradation product obtained after release of formaldehyde (Fig. 2).

Reaction with *N*-Ac-Cys gave, after only a few hours, a first adduct **4** by reaction of the thiol

group of *N*-Ac-Cys with the electrophilic carbon atom of released formaldehyde. After 5 days, compound **5** appeared in the spectra, resulting from the intramolecular cyclization of **4**. This kind of cyclization has been already described in the literature for the N_α nucleophilic attack on the hydroxymethyl group (19). The characteristic signal of formaldehyde at 82.0 ppm (hydrated form) was not observed in the spectra, indicating that released formaldehyde possibly reacted immediately with *N*-Ac-Cys. After 25 days of reaction, a tiny signal appeared and, interestingly, corresponded to adduct **6**. It is essential to note here that the reaction times given throughout this article did not correspond with kinetic studies of the formation of the reaction products but with the time needed to detect them distinctly by NMR. Thus, it could not be excluded that the formation of the NMR-identified reaction products happened before the time needed to detect them. Only the ¹³C-NMR signal of **1** at 60.5 ppm (Fig. 2) disappeared almost completely in the spectra, suggesting that the ¹³C hydroxymethyl group at 60.5 ppm was most likely the precursor of released formaldehyde, and that **6** was probably obtained by reaction of the thiol group of *N*-Ac-Cys with the ¹³C hydroxymethyl group at 62.5 ppm. The dimer of *N*-Ac-Cys was also observed, indicating that oxidation–reduction processes were occurring in the reaction mixture.

In the case of the reaction with *N*-Ac-Lys, released formaldehyde at 82.0 ppm was observed. In contrast to what happened with *N*-Ac-Cys, there was a clear decrease in the intensity of the ¹³C-NMR signal of **1** at 62.5 ppm, as compared with that at 60.5 ppm, suggesting that, in the presence of a basic amino acid, the hydroxymethyl position of **1** at 62.5 ppm was favoured for the release of formaldehyde. However, released formaldehyde seemed to have no further reactivity with the amino acid. In addition, a tiny signal at 52.6 ppm appeared on the first day of the reaction, its intensity increasing poorly with time. It was correlated via ¹J(C, H) coupling to protons at 4.29 ppm. The chemical shifts 52.6/4.29 ppm corresponded well with **7**, obtained by reaction of the ε-NH₂ group of lysine with the hydroxymethyl group at 62.5 ppm of remaining **1**. However, a doubt remains about the exact structure of this adduct, as **1** could follow a nucleophilic attack by the ε-NH₂ group of *N*-Ac-Lys on both hydroxymethyl groups. Nevertheless, the great loss of intensity of the signal at 62.5 ppm suggested that this attack occurred on the hydroxymethyl group located between the carbonyl chemical functions.

For *N*-Ac-His, *N*-Ac-Trp and *N*-Ac-Arg, released formaldehyde was observed in the spectra on the first day of the reaction, and only

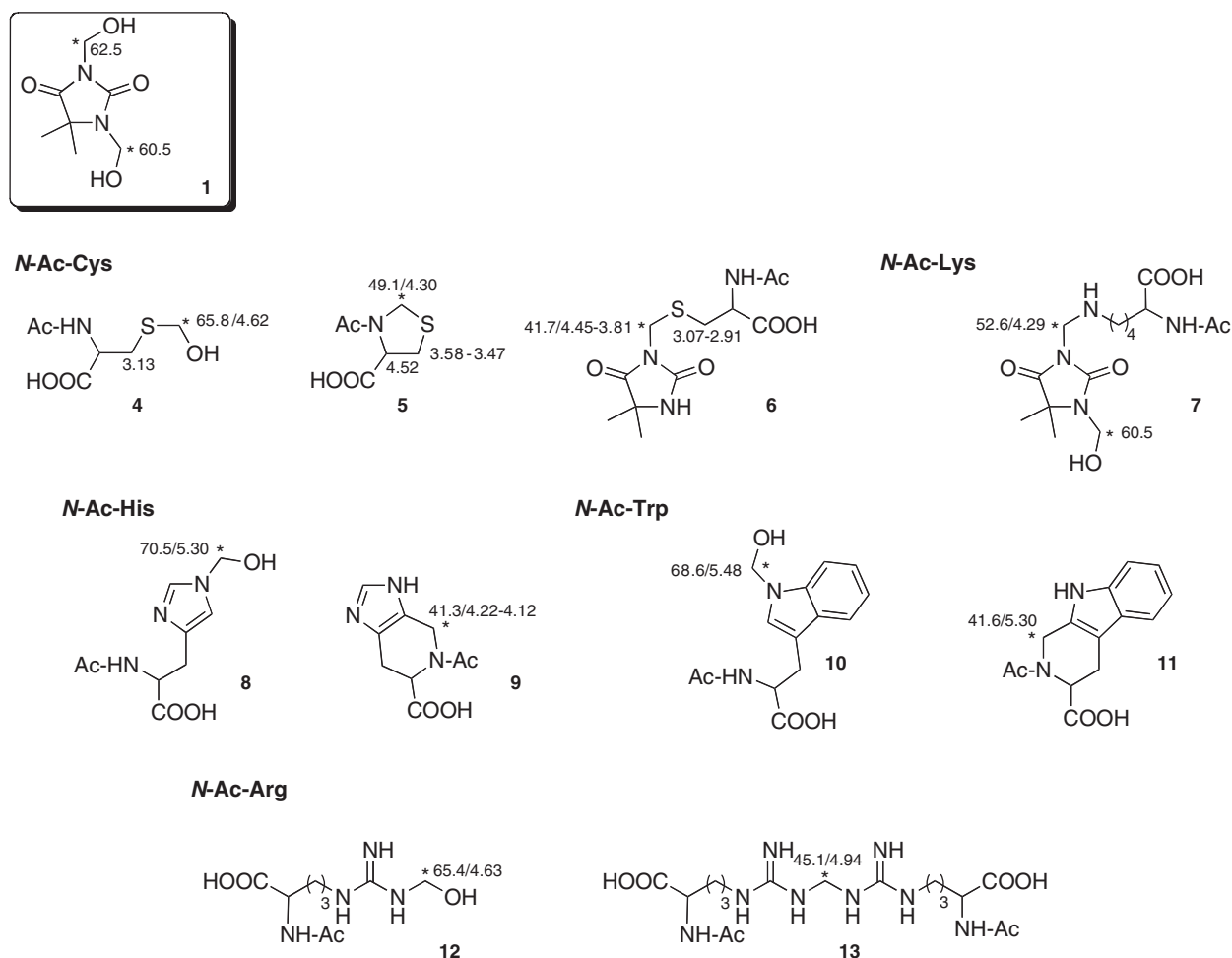


Fig. 2. Compounds formed from the reaction of [^{13}C]DMDM hydantoin (**1**) with nucleophilic amino acids, and characteristic ^1H -NMR and ^{13}C -NMR data (* = ^{13}C -labelled position).

adducts resulting from its reaction with the amino acids could be identified. The $\text{N}_\epsilon\text{H}$ of *N*-Ac-His reacted easily, on the first day of the reaction, with formaldehyde to give **8**. After 1 week, **9** was detected. From a mechanistic point of view, *N*-Ac-His was also able to react with formaldehyde via nucleophilic addition of the C_γ of the imidazole part. Then, further cyclization of the N_α of modified *N*-Ac-His with the new hydroxymethyl group formed on the C_γ of the imidazole part could give **9**. The formation of **9** was, indeed, corroborated by previous studies reported in the literature (20, 21). By similar mechanisms, adducts **10** and **11** were obtained with *N*-Ac-Trp. In the case of *N*-Ac-Arg, reaction of the free NH_2 group of the guanidine part of the lateral chain with formaldehyde gave **12** initially. A second reaction of another molecule of *N*-Ac-Arg with the hydroxymethyl group formed in this way then gave adduct **13**.

No adducts were observed with the other amino acids tested, but only the release of formaldehyde.

Reactivity of [^{13}C]2-bromo-2-nitropropane-1,3-diol (**2**)

It was observed in all the spectra, from the first day of every reaction, that [^{13}C]2-bromo-2-nitropropane-1,3-diol (**2**) underwent degradation in aqueous media, giving several products (Fig. 3), as previously reported (22). Initially, **2** produced 2-bromo-2-nitro- ^{13}C ethanol (**14**), with characteristic chemical shifts at 65.4/4.28 ppm, by releasing one unit of formaldehyde. Then, **14** could lose the nitro group, forming 2-bromo- ^{13}C ethanol (**15**), observed at 63.6/3.44 ppm. Theoretically, **14** could also still lose another molecule of formaldehyde, giving bromonitromethane (**16**). However, as **16** did not contain any ^{13}C label, it could not be identified in the ^{13}C -NMR spectra. Finally, [^{13}C]tris-(hydroxymethyl)nitromethane (**17**), with typical chemical shifts at 59.8/3.84 ppm, was the most important compound detected, and resulted from the reaction of **2** with released formaldehyde.

Some of these degradation products were potentially reactive towards nucleophilic amino acids.

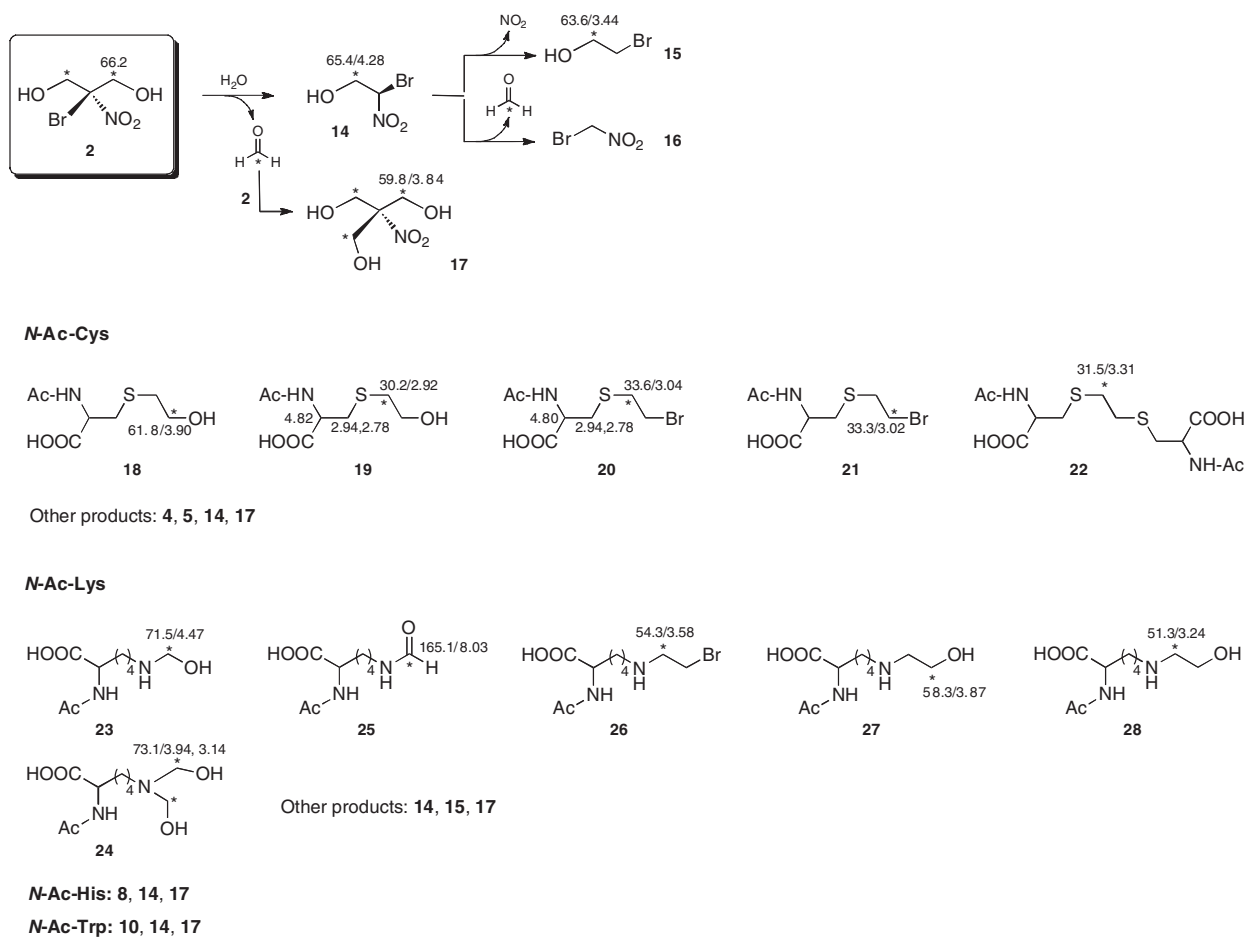


Fig. 3. Compounds and adducts from the reaction of [^{13}C]2-bromo-2-nitropropane-1,3-diol (bronopol) (**2**) with nucleophilic amino acids, and characteristic ^1H -NMR and ^{13}C -NMR data (* = ^{13}C -labelled position). Decomposition products in aqueous medium are also shown.

This was indeed reflected in the various adducts formed after reaction of **2** with certain amino acids that did not result from reactivity with released formaldehyde. Interesting reactivity was particularly observed with *N*-Ac-Cys and *N*-Ac-Lys (Fig. 3).

Reactivity of **2** with *N*-Ac-Cys was fast, strong and diverse. On the first day of the reaction, the expected reactivity with released formaldehyde was observed through the formation of the previously described adducts **4** and **5**. Signals corresponding to the dimer of *N*-Ac-Cys were also detected, being representative of the involvement of oxidation–reduction processes in the reactions. Moreover, some studies reported in the literature suggest that the formation of (*N*-Ac-Cys) $_2$ can occur by the formation in the reaction mixture of radical anion intermediates derived from 2-bromo-2-nitropropane-1,3-diol (**5**). In addition, unexpected new adducts, **18**–**20**, were observed (Fig. 4). Signals at 61.8/3.90 and 30.2/2.92 ppm corresponded well with methylene groups included in a hydroxyethylthio chemical function, fitting with the structures of **18** and **19**. Also, the signal at 33.6/3.04 ppm

was compatible with **20**. Most probably, from the mechanistic point of view, nucleophilic attack of the thiol group of *N*-Ac-Cys on the carbon atom of **15**, bearing bromine, gave **18**. The very reactive degradation intermediate **15** was not observed in the spectra, so it was assumed that it reacted extremely rapidly with the amino acid. Its formation was indirectly proved, as precursor **14** was identified. On another hand, it is well known that ethylthio derivatives such as **18** can undergo cyclization and form ethylenesulfonium ion intermediates (**23**). A second nucleophilic attack on this ion intermediate by water in the medium or by bromine ions could then give **19** and **20**. After 40 days of reaction, adducts **18**–**20** were much more abundant in the reaction mixture, together with **21** (already observed at day 7, results not shown) and **22**. The identification of **22** suggested that *N*-Ac-Cys could also carry out a second nucleophilic attack on the ethylsulfonium intermediate.

In the case of reactivity studies with *N*-Ac-Lys, the presence of **15** in the spectra led to the confirmation of this kind of mechanism(s) for

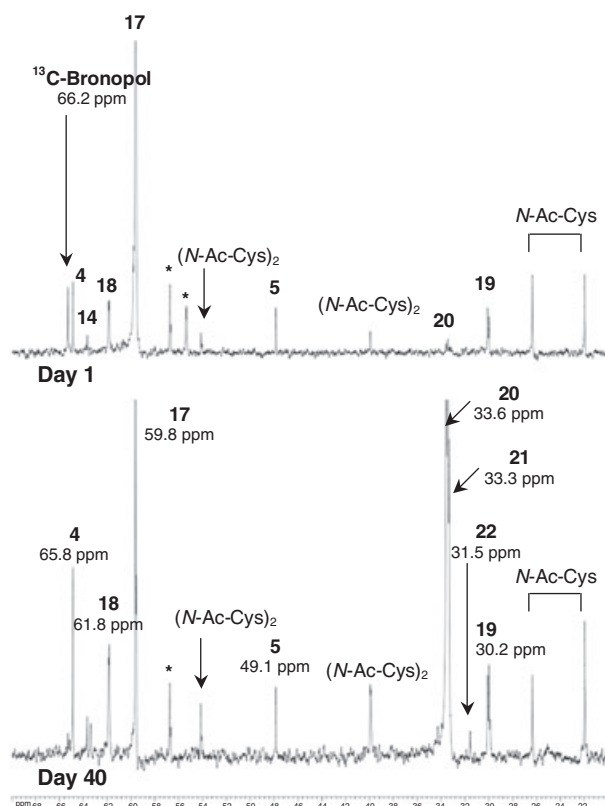
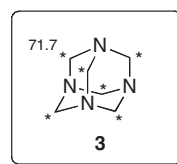


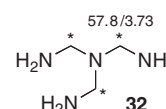
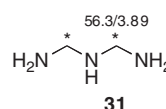
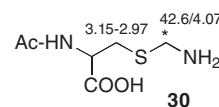
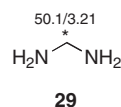
Fig. 4. Reaction of [^{13}C]2-bromo-2-nitropropane-1,3-diol (bronopol) (**2**) with *N*-Ac-Cys. One-dimensional ^{13}C -NMR spectra after 1 and 40 days of reaction (* = non-identified).

the formation of some of the products observed. According to the computer predictions, the chemical shifts of 54.3/3.58, 58.3/3.87 and 51.3/3.24 ppm matched well with adducts **26**, **27** and **28**, respectively, observed on the first day of the reaction (Fig. 3). From the mechanistic point of view, it was not surprising that the ε - NH_2 group of *N*-Ac-Lys reacted with the electrophilic sites of **15**, as described for the thiol group of *N*-Ac-Cys. Compound **26** could then undergo an intramolecular cyclization, this time forming an electrophilic intermediate of the aziridinium kind (24). Reaction of this intermediate with the diverse nucleophile species present in the reaction mixture could explain the production of the other adducts detected. In parallel with this unexpected reactivity, two adducts, **23** and **24**, resulting from the reaction of the ε - NH_2 group of *N*-Ac-Lys with released formaldehyde, appeared quickly (day 1) at 71.5/4.47 and 73.1/3.94–3.14 ppm, respectively. With time, **23** was oxidized to give **25** (day 7).

Finally, with *N*-Ac-His and *N*-Ac-Trp, we only saw very tiny signals corresponding to the earlier described adducts **8** and **10**, resulting from the reaction of the side chain of the amino acid with released formaldehyde.

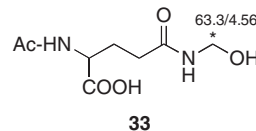


N-Ac-Cys

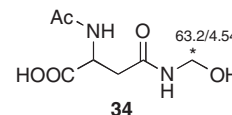


Other products: **4**

N-Ac-Gln



N-Ac-Asn



Other products: **29**

N-Ac-His: **8**, **9**, **29**

N-Ac-Trp: **10**, **11**

Fig. 5. Reactivity of [^{13}C]methenamine (**3**) with nucleophilic amino acids, and characteristic ^1H -NMR and ^{13}C -NMR data (* = ^{13}C -labelled position).

No adducts were observed with the other amino acids tested.

Reactivity of [^{13}C]methenamine (**3**)

In contrast to the other formaldehyde-releasers under study, **3** in the presence of *N*-Ac-Lys did not show any reactivity at all. However, interesting reactivity was again observed with *N*-Ac-Cys (Fig. 5). On the first day of the reaction, the known compound **4**, resulting from the reaction with released formaldehyde, showed in the spectra, but a very tiny signal at 42.6/4.07 ppm, corresponding to **30**, was also detected. Then, after 1 week, diaminomethane (**29**) clearly appeared in the spectra, and the amount of **30** formed increased, and after approximately 1 month, this was the case for **31** and **32**. Once more, obvious reactivity with a derivative other than released formaldehyde was shown. From the mechanistic point of view, **3** decomposed, giving the reactive **29**. Nucleophilic attack of the thiol group of *N*-Ac-Cys on **29** gave adduct **30**, containing a thiomethylamine substituent. With time, self-reaction of diaminomethane produced **31** and **32**.

With the other amino acids, the reactivity was totally attributable to released formaldehyde. This

was, for example, the case for **33** and **34**, obtained by reaction with *N*-Ac-Gln and *N*-Ac-Asn, respectively, and for the already described **8** and **9**, with *N*-Ac-His, and **10** and **11**, with *N*-Ac-Trp.

Discussion

Monitoring of the prevalence of sensitivity to preservatives in Europe showed, in the early 2000s, that formaldehyde sensitivity levels remained high, mostly between 2% and 2.5% (25, 26). This was thought to be a result of the common use of cosmetics containing formaldehyde-releasers as preservatives (27). Concomitant allergic reactions are indeed often observed when patients are tested with formaldehyde and one or more formaldehyde-releasers. Moreover, in some cases, more than one releaser reacts in formaldehyde-sensitive patients, even if they are not structurally related. However, sensitization to some formaldehyde-releasers can exist *per se*, independently of formaldehyde release. It has been recently reported that, in general, 40–60% of the reactions to formaldehyde-releasers are caused by reaction to formaldehyde, and that for 2-bromo-2-nitropropane-1,3-diol, the relationship with formaldehyde contact allergy is even lower, at only 15% (28). In this article, we considered DMDM hydantoin, 2-bromo-2-nitropropane-1,3-diol and methenamine. For these releasers, it is suspected that the sensitizing potential could also be attributable to the entire molecule or to a breakdown product, and not necessarily to released formaldehyde alone.

DMDM hydantoin is a preservative that is mostly used in cosmetics, where it is permitted in the EU at a maximum concentration of 0.6%. DMDM hydantoin was chosen for our studies as a representative of the class of compounds known as hydantoins, which are heterocyclic compounds derived from imidazoles and containing urea-like subunits. Diazolidinyl urea and imidazolidinyl urea belong to this category of formaldehyde-releasers. De Groot et al. demonstrated that 57% of 35 formaldehyde-sensitive patients also reacted to DMDM hydantoin (29). Later, another study conducted by the Information Network of Departments of Dermatology and the German Contact Dermatitis Research Group found that 488 patients were positive to formaldehyde, but not to DMDM hydantoin, with the opposite being observed in 95 cases (30). Also, for diazolidinyl urea, which belongs to the same category of molecules, it has been proposed that allergy may develop to the molecule itself or to free formaldehyde (31), and for imidazolidinyl urea, for which allergic sensitization is less frequent, to the entire molecule and, much less often, to free formaldehyde (32,

33). Methenamine was chosen as a representative of compounds with formaldehyde-releasing ability, such as quaternium-15. Methenamine, if used as a preservative in cosmetics, must not exceed a maximum authorized concentration of 0.15%. Quaternium-15 is a chloroallyl derivative of methenamine. Studies on simultaneous allergic reactions to quaternium-15 and methenamine confirmed that these could not be explained only by the release of formaldehyde. In a study conducted in Helsinki, 32 patients positive to quaternium-15 were tested with methenamine (34). Simultaneous allergy occurred in 21 patients. Twenty of 32 patients were positive to formaldehyde, and among these, 7 were positive to methenamine. However, 4 of the 12 formaldehyde-negative patients were also positive for methenamine. In addition, in the same clinic, they had 4 patients positive for methenamine but negative for quaternium-15 and for formaldehyde. The authors suggested that simultaneous reactions to quaternium-15 and methenamine might be more related to the similarity in their chemical structures than to their formaldehyde-releasing capacity. The same idea was considered by a Spanish study on allergic contact dermatitis caused by methenamine in an antiperspirant spray (35). Finally, 2-bromo-2-nitropropane-1,3-diol, the perfect example, was chosen, as it is the formaldehyde-releasing compound for which the largest number of studies have been reported showing positive reactions to the releaser in patients negative for formaldehyde (28). In 1983, Storrs and Bell described 7 patients sensitized to Eucerin® cream preserved with 2-bromo-2-nitropropane-1,3-diol. All patients were positive to the cream and to 2-bromo-2-nitropropane-1,3-diol, but none of them was allergic to formaldehyde or to any other preservative known to be a formaldehyde donor (36). These authors also pointed out that the decomposition of 2-bromo-2-nitropropane-1,3-diol gives formaldehyde and also bromonitroethanol and tris-(hydroxymethyl)nitromethane as breakdown products. Heat and alkaline conditions speed up this process. Today, 2-bromo-2-nitropropane-1,3-diol is permitted in cosmetics in the EU up to a concentration of 0.1%.

The results that we present in this article fully support the belief that, for the representative formaldehyde-releasers that we chose for the reactivity studies, many reactive species, in addition to formaldehyde, could be involved in the formation of antigenic structures. By using ¹³C-labelled compounds and following up their reactivity towards nucleophilic amino acids by ¹³C-NMR, we proved that direct reactivity of the entire molecule is possible in some cases, and that, in other cases, degradation products can also be reactive (Fig. 6). The times of reaction indicated do not refer to

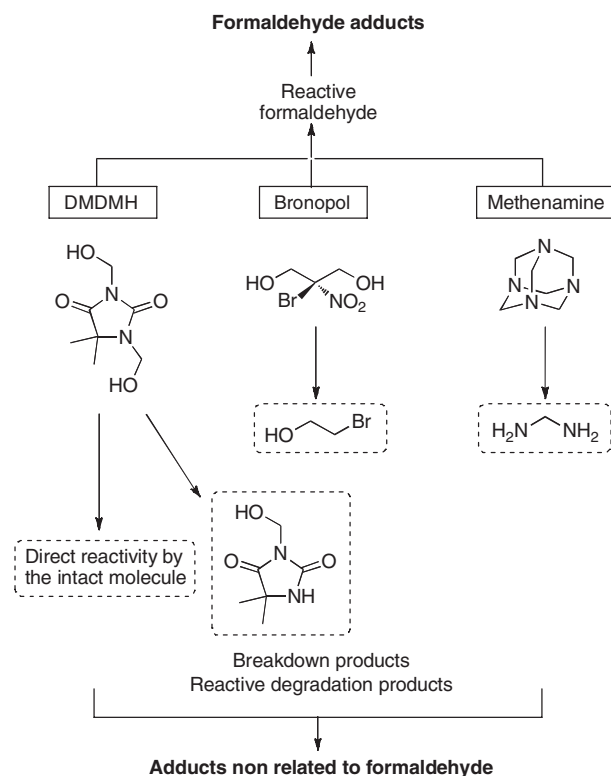


Fig. 6. Breakdown products of DMDM hydantoin (DMDMH), 2-bromo-2-nitropropane-1,3-diol (bronopol) and methenamine that are reactive towards amino acids.

the kinetics of the reactions, but to the instant at which the signals relevant to the products formed were detected in the NMR spectra. It is important to stress here that the observed unexpected chemistry can occur independently of the related kinetics, which can be different from one medium to another, if we consider, for example, the skin and a phosphate buffer solution. This diverse reactivity could explain why some patients are allergic to the formaldehyde-releaser and not to formaldehyde itself.

The most striking case was 2-bromo-2-nitropropane-1,3-diol. It is known that it releases formaldehyde at alkaline pH, and that the main by-product resulting from this release is bromonitroethanol. In our studies, reactivity was examined at a physiological pH of 7.4, and the quantitative determination of formaldehyde release by ¹³C-NMR at this pH confirmed the actual slow release of formaldehyde under these pH conditions. However, we clearly saw the decomposition of 2-bromo-2-nitropropane-1,3-diol in the phosphate aqueous buffer medium employed, giving many products. Not only could bromonitroethanol be detected, but also bromoethanol, itself obtained by release of the nitro group from bromonitroethanol. Interestingly, bromoethanol was shown to be the degradation product that was most reactive towards

the nucleophilic amino acids tested, giving, more precisely, adducts **18–21**, through a mechanism involving a cyclic ethylsulfonium intermediate in the case of reactivity with *N*-Ac-Cys, and adducts **26–28**, through a mechanism involving a cyclic aziridinium type of intermediate in the case of reactivity with *N*-Ac-Lys. The same behaviour was observed for methenamine, in the sense that it decomposed in the aqueous buffer medium, affording diaminomethane, which is reactive towards *N*-Ac-Cys though the formation of adduct **30**. Again, clear reactivity with a derivative other than possible released formaldehyde was seen. For both 2-bromo-2-nitropropane-1,3-diol and methenamine, in addition, some reactivity related to formaldehyde was also observed, but to a minor extent. Finally, among the compounds chosen for the study, DMDM hydantoin was the real archetype of a true formaldehyde-releaser. It is stable in an acidic environment (low pH), and when the pH is increased, the amount of free formaldehyde also increases (15). In our experimental conditions, we observed approximately 25% of the theoretical amount of formaldehyde that could be released. Therefore, the expected reactivity of released formaldehyde towards the amino acids was substantial this time. Nevertheless, some adducts resulting from other reactive species were also formed. Actually, it has been reported that in aqueous solutions, depending on the dilution and pH, different structures derived from DMDM hydantoin can be found in different ratios (15). These structures correspond essentially to DMDM hydantoin and monomethylol dimethylhydantoin, and other dimethylhydantoin derivatives complete the balance. We have shown here that DMDM hydantoin can react directly with *N*-Ac-Lys and form adduct **7**, but also that monomethylol dimethylhydantoin, present in the solution after release of one unit of formaldehyde, can react with *N*-Ac-Cys, forming **6**. Albeit to a minor extent, DMDM hydantoin can thus also react with some amino acids through the entire molecule or through a degradation product obtained after release of formaldehyde.

The reactivity of distinct formaldehyde-releasers towards amino acids and proteins is not limited to the release of formaldehyde. As a consequence, any attempt to predict the sensitizing potential of formaldehyde-releasers by, for example, alternative methods based on chemical reactivity cannot refer only to the release of formaldehyde. From one releaser to another, multiple different adducts can be formed by reactions with amino acids, and thus many potential antigenic structures can be derived. Formaldehyde-releasers cannot be classified as a unique family of chemical compounds.

Acknowledgements

The authors would like to acknowledge the Agence Française de Sécurité Sanitaire des Produits de Santé (Afssaps) for funding to M.K.

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