Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry Letters
ELSEVIER
Bioorganic & Medicinal Chemistry Letters 14 (2004) 365–368

Covalent binding of the ^{13}C-labeled skin sensitizers 5-chloro-2-methylisothiazol-3-one (MCI) and 2-methylisothiazol-3-one (MI) to a model peptide and glutathione

Rubén Alvarez-Sánchez,[a] David Basketter,[b] Camilla Pease[b] and Jean-Pierre Lepoittevin[a,*]

[a]*Laboratoire de Dermatochimie (UMR 7123), Université Louis Pasteur, Clinique Dermatologique, CHU, Strasbourg, France*
[b]*SEAC, Unilever, Colworth, Sharnbrook, Bedford, UK*

Received 20 August 2003; revised 29 October 2003; accepted 3 November 2003

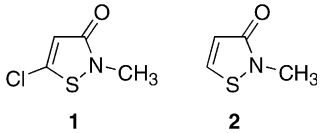
Abstract—The reactivity of 4-[^{13}C]- and 5-[^{13}C]-5-chloro-2-methylisothiazol-3-one (MCI) and 2-methylisothiazol-3-one (MI) towards a model peptide and glutathione was followed by ^{13}C and $^{1}H\{^{13}C\}$ NMR spectroscopy. Both molecules were found to react with GSH but in addition MCI was found to react with histidine and lysine to form adducts of a different nature. Reaction with histidine led to stable substitution adducts through an addition–elimination reaction at position 5 while reaction with lysine led to the formation of open adducts of the thioamide or amide type.

1. Introduction

Allergic contact dermatitis (ACD) is a very common disease resulting from the chemical modification of epidermal proteins by haptens.[1] The processing of such modified proteins by Langerhans cells, the main antigen-presenting cells in the epidermis ensures that the altered peptides are subsequently presented, in association with MHC molecules, to naive T-lymphocytes in the lymph nodes.[2] The process results in the selection and activation of T-lymphocyte sub-populations with T-cell receptors (TcR) specific for the chemical modification.[2] In order to develop predictive strategies based on quantitative structure–activity relationships (QSAR), it is necessary to investigate how a hapten is reacting with protein structures.[3] Haptens are usually low molecular weight molecules, lipophilic enough to penetrate the epidermis through the *stratum corneum*, and with a sufficient degree of chemical reactivity to allow the formation of a covalent link with nucleophilic residues on amino acid side chains.[1] Moreover, it has been hypothesized that the sensitizing potential of a molecule was related to its chemical reactivity toward some specific amino acids.[4] From previous studies and reported data, amino nucleophiles such as lysine and histidine, seemed to play a key role in the induction mechanism of ACD.[5,6]

In order investigate this hypothesis, we have focused on two isothiazolone derivatives widely used as preservatives, 5-chloro-2-methylisothiazol-3-one (MCI) and the 2-methylisothiazol-3-one (MI), a strong and a weak sensitizer, respectively (Scheme 1). Reactivity studies carried out on these molecules toward model nucleophiles such as butylamine, propanethiol, phenol and imidazole have shown differences in reactivity.[7] While MCI was found to react with any model nucleophile to form different adducts, MI reacted exclusively with thiol nucleophiles.[7]

We now report our results on the reactivity of MCI and MI toward a model peptide derived from the N-terminal chain of the globine, containing all potential reactive amino acids except cystein, and glutathione. These studies were carried out using ^{13}C labeled molecules and adducts were characterized by a combination of ^{13}C and $^{1}H\{^{13}C\}$ HSQC and HMBC techniques.



1 2

Scheme. 1. Structure of 5-chloro-2-methylisothiazol-3-one (MCI) **1** and 2-methylisothiazol-3-one (MI) **2**.

* Corresponding author. Tel.: +33-388-350-664; fax: +33-388-140-447; e-mail: jplepoit@chimie.u-strasbg.fr

0960-894X/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2003.11.002

2. Results and discussion

2.1. Reactivity of MCI and MI toward an analogue of the globine N-terminal chain

To test the reactivity of MCI and MI, we used as model an analogue of the N-terminal chain of the globine protein in which Cys-18 has been deleted: H_2N-Val-Leu-Ser-Pro-Ala-Asp-Lys-Thr-Asn-Trp-Gly-His-Glu-Tyr-Arg-Met-Phe-Gln-Ile-Gly-CO_2H. This synthetic peptide including all natural amino acids has already been used in the literature as a model peptide for reactivity studies.[8] This peptide was reacted with 4-[^{13}C]- and 5-[^{13}C]-MCI **1a** and **1b**, respectively, in a semi organic medium (CH_3CN/PBS) at pH 7.7 for 15 days. After separation of the mixture of modified peptides by gel permeation on Sephadex LH-20, $^1H\{^{13}C\}$ HMBC and HSQC experiments were carried out in order to identify new peaks corresponding to potential adducts. After reaction with **1b**, labeled at position 5, three new groups of carbon 13 signals were detected. A first single signal at 151.2 ppm that could correspond to an adduct on histidine, a group of signals around 170 ppm characteristic of amino adducts of the diamide type and a group around 200 ppm that could be associated with amino adducts of the thioamide type were observed. For each new ^{13}C chemical shift, ^{1}H long-range correlations were recorded giving information on other isothiazolone positions and even, in some cases, on the peptide side chain (Fig. 1).

Thus, the ^{13}C NMR signal at 151.2 ppm, assigned to an histidine adduct **3**, was correlated with the ^{1}H NMR signal of the methyl group on the nitrogen atom at position 2 ($\delta = 3.14$ ppm), to the vinylic proton at position 4 ($\delta = 6.26$ ppm) and one of the histidine protons ($\delta = 7.18$ ppm). ^{13}C NMR signals at 169.4 and 170.1 ppm were correlated, respectively, with CH_2 signals at position 4 ($\delta = 3.04$ and 3.18 ppm), and to NH protons (7.75 and 8.04 ppm), characteristics of adducts of the amide type with primary amino groups, ε-NH_2 of Lys-7 **4** and α-NH_2 of Val-1 **5**, respectively. The third group of carbon signals at 196.1 and 199.2 were correlated, respectively, with methylene signals at position 4 (3.46 and 3.63 ppm), characteristics of adducts of the thioamide type with primary amino groups of Lys-7 **6** and Val-1 **7**, respectively. If in this case no correlation were observed with NH protons, a correlation was established between the ^{13}C signal at position 5 and the H_α of Val-1 at 4.55 ppm. These assignments were further confirmed by NMR analysis carried after incubation of the model peptide with MCI **1a** labeled at position 4. ^{13}C NMR chemical shifts for position 4 were in full agreement with proposed structures with $\delta = 43.5$ and 43.8 ppm for amide type adducts on Lys-7 and Val-1, $\delta = 52.5$ and 52.4 ppm for thioamide type adducts on Lys-7 and Val-1, respectively and respectively, and $\delta = 102.8$ ppm for His-12 cyclic adduct. ^{1}H short-range correlations (HSQC) confirmed assignment of methylene signals at position 4 (Table 1).

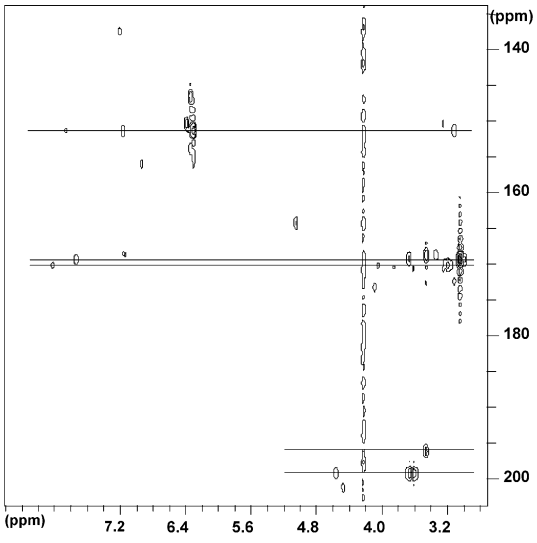


Figure 1. HMBC for the peptide modified by **1a**.

The same reaction carried out under the same conditions (semi-organic medium, PBS pH 7.7) in the presence of MI labeled at position 4 or 5 led to a peptide without any significant modification.

2.2. Reactivity of MCI and MI toward GSH

MCI and MI were reacted with GSH in a semi-organic medium CH_3CN/PBS at pH 7.7 using either an excess of GSH or of isothiazolones in order to mimic a detoxication or a toxication situation. In the presence of 10 molar excess of GHS to mimic a detoxication situation, the reaction was found to be very fast with a complete consumption of MCI **1a** or **1b** to form mainly compound **8** with chemical shifts characteristic of a

Table 1. ^{1}H and ^{13}C NMR data of adducts formed between MCI **1** and the model peptide in a semi-organic medium (CH_3CN/PBS, pH 7.7)

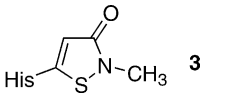
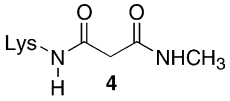
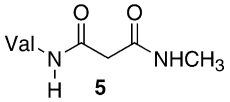
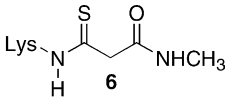
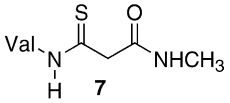
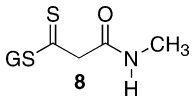
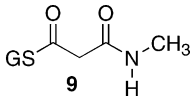
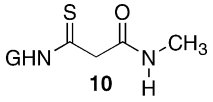
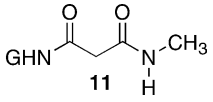
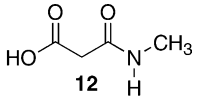
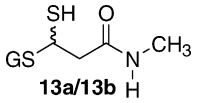
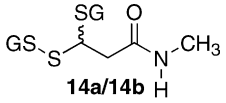
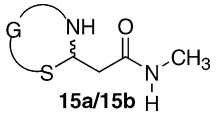
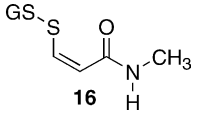
Structure	C_5	C_4/H_4	Other signals
 **3**	151.2	102.8/6.26	N–CH_3, 3.14His, 7.18/7.88
 **4**	169.4	43.8/3.04	NH, 7.75
 **5**	170.1	43.5/3.18	NH, 8.04
 **6**	196.1	52.5/3.46	—
 **7**	199.2	52.4/3.63	Hα, 4.55

Table 2. ^{1}H and ^{13}C NMR data of adducts formed between MCI **1** and GSH in a semi-organic medium (CH_3CN/PBS, pH 7.7)

Structure	C_5	C_4/H_4	Other signals
 **8**	229.8	58.4/3.88	$-CH_2S-$, 3.52/3.83
 **9**	195.4	51.2/3.50	$-CH_2S-$, 3.11/3.35
 **10**	197.5	52.9/3.60	Hα, 4.67 $N-CH_3$, 2.63
 **11**	169.9	43.7/6.22	Hα, 4.48 $N-CH_3$, 2.63
 **12**	175.7	45.9/3.01	

mercaptothioester together with its hydrolysis product **9**. Both adducts then reacted over time through a slow intramolecular nucleophilic attack of the terminal α-NH_2 group to form thioamide and amide type derivatives **10** and **11**. $^{1}H\{^{13}C\}$ HMBC and HSQC experiments gave us access to ^{1}H chemical shifts of H-4 and to either $-CH_2S$ in the case of **8** and **9** or H_α in the case of **10** and **11**. Open structures of the amide type were confirmed by the ^{1}H chemical shift of $N-CH_3$ at 2.63 ppm (Table 2).

Table 3. ^{1}H and ^{13}C NMR data of adducts formed between MI **2** and GSH in a semi-organic medium (CH_3CN/PBS, pH 7.7)

Structure	C_5/H_5	$C_4/H_4/H_{4'}$
 **13a/13b**	42.5/4.28 42.2/4.28	46.4/2.60/2.71 46.6/2.61/2.70
 **14a/14b**	53.4/4.23 53.7/4.23	42.2/2.84/2.56 42.3/2.84/2.56
 **15a/15b**	65.8/4.75 65.9/4.75	42.4/2.69/2.72 42.6/2.69/2.72
 **16**	152.5/6.97	119.3/5.90

In the presence of 40 molar excess of MCI **1b** compared to GSH (mimicking a toxication situation), three new products were formed with ^{13}C chemical shifts at 175.7, 169.7 and 196.9 ppm, respectively, corresponding to the hydrolysis product **12** and adducts **11** and **10**, respectively. Assignments were confirmed by reaction of **1a** under the same conditions.

MI **2**, was also found to be very reactive when treated with a 10 molar excess of GSH to form very different adducts (Table 3). Thus, three pairs of diastereomers **13a**/**13b**, **14a**/**14b** and **15a**/**15b**, respectively, with C_5 chemical shifts around 42, 53 and 65 ppm, respectively, were detected. It was assumed that these products were formed through an initial attack of GSH at the sulfur atom to cleave the S–N bond and subsequent reaction of the formed disulfide intermediate with an other molecule of GSH to give a thioaldehyde which was not detected. The reaction of this later intermediate with GSH led to the formation of a couple of hemithioacetal **13a**/**13b** which can then form **14a**/**14b**. C5 chemical shifts of 65.8 and 65.9, respectively, were in good agreement with a S–C–N bond that could be formed through an intramolecular attack of the N-terminal amino group of the glutathione molecule to form cyclic adducts **15a**/**15b**.

Reaction of an excess of MI **2** with GSH gave only one product **16** with C_5 and C_4 chemical shifts of 152.5 and 119.3 ppm, respectively, characteristic of sp^2 carbon atoms. This adduct confirmed our mechanistic hypothesis of an initial attack of GSH at the sulfur atom with opening of the S–N bond.

2.3. Reactivity of MCI and MI versus sensitizing potential

From these model experiments, it is clear that both molecules have a very different behavior toward nucleophiles potentially present in proteins. Both MCI **1** and MI **2**, a strong and a weak sensitizer, were found to be highly reactive toward GSH used as a model for thiol nucleophile and also to mimic a detoxication process. Adducts formed are of the hemithioacetal, acetal or disulfide nature, that is, labile to some extend. Potential adducts formed on a protein through a cystein amino acid could be cleaved during the processing of this modified protein by antigen presenting cells. On the other hand, the reactivity of both molecules with GSH indicates that both can probably be detoxify through this mechanism. Moreover, in case of competition under equimolar conditions (experiments done with 1 equiv of cystein) MCI was found to be significantly more reactive than MI.

When reacted with a model peptide containing all amino acids except cystein, the difference in reactivity between MCI **1** and MI **2** was striking. While MCI, a strong sensitizer, was found to react with histidine and lysine to form quite stable adducts, MI, a weak sensitizer, was found to be non-reactive under the same reaction conditions.

3. Conclusion

MCI and MI were found to have very different behaviors toward model nucleophiles. While MCI, which is a strong sensitizer,[9] reacted easily and rapidly with most nucleophiles, MI, a weaker sensitizer, reacted only with thiols. The difference in the sensitizing potential could then be explained by the ability of MCI to react with amino nucleophiles such as lysine and histidine. The reactivity toward thiols, which probably plays a major role in the detoxication process, did not seem to correlate with the observed sensitizing potential of these isothiazolone derivatives.

4. Experimental

4.1. Caution

Skin contact with isothiazolone derivatives must be avoided. Since these are sensitizing substances, they must be handled with care.

4.2. NMR experiments

^{13}C NMR spectra were recorded on a Bruker AC200-MHz or Bruker AC300-MHz at 50 and 75 MHz, respectively. $^{1}H\{^{13}C\}$ HSQC and HMBC experiments were recorded on a Bruker AM 400 and Bruker ARX 500. Chemical shifts are reported in ppm (δ) with respect to TMS, and a trace of CH_3CN was used as internal standard (^{1}H, $\delta = 2.06$ ppm; ^{13}C, $\delta = 1.32$ ppm).

4.3. Structure assignment

Structures of the different adducts were assigned using a combination of $\{^{1}H\}$-decoupled ^{13}C NMR and $^{1}H\{^{13}C\}$ HSQC and HMBC. The measured chemical shifts were compared with those calculated using additivity principle (ChemNMR) and NMR data derived from analogous compounds (ACD/CNMR 5.12 and ACD/HNMR 5.12). These assignments are in accordance with the sequence of adduct formation and hydrolysis when observed.

4.3.1. Reaction MCI with GSH. Method A: To a solution of GSH (20.4 mg, 66 μmol, 10 equiv) in phosphate buffer (0.25 mL, 0.1 M, pH 7.7) degassed for 15 min was added MCI (1 mg, 6.6 μmol, 1 equiv) in CD_3CN (0.25 mL). The solution was filtered into an NMR tube, and the reaction was followed by ^{13}C NMR. **Method B:** To a solution of glutathione (0.65 mg, 2.1 μmol, 1 equiv) in phosphate buffer (0.25 mL, 0.1 M, pH 7.7) degassed for 15 min was added MCI (12.8 mg, 86 μmol, 40 equiv) in CD_3CN (0.25 mL). The solution was filtered into a NMR tube, and the reaction followed by ^{13}C NMR.

4.3.2. Reaction MI with glutathione. Method A: To a solution of GSH (26.7 mg, 87 μmol, 10 equiv) in phosphate buffer (0.25 mL, 0.1 M, pH 7.7) degassed for 15 min was added MCI (1 mg, 8.7 μmol, 1 equiv) in CD_3CN (0.25 mL). The solution was filtered into a NMR tube, and the reaction followed by ^{13}C NMR. **Method B:** To a solution of GSH (0.65 mg, 2.1 μmol, 1 equiv) in phosphate buffer (0.25 mL, 0.1 M, pH 7.7) degassed for 15 min was added MCI (9.7 mg, 86 μmol, 40 equiv) in CD_3CN (0.25 mL). The solution was filtered into a NMR tube, and the reaction followed by ^{13}C NMR.

4.3.3. Reaction MCI with the model peptide in a semi-organic medium. To a solution of peptide (5 mg, 2.1 μmol, 1 equiv) in phosphate buffer (0.25 mL, 0.1 M, pH 7.7) degassed for 15 min was added MCI labeled in position 5 or 4 (12.8 mg, 85 μmol, 40 equiv) in degassed CD_3CN (0.25 mL). The reaction mixture was incubated at 25 °C for 15 days and the peptidic fraction recovered by gel permeation on Sephadex LH-20 ($CHCl_3$, MeOH, H_2O, 45/45/10) to give a solid which was dissolved in 0.4 mL (CD_3CN/H_2O, 1/1), filtered into a NMR tube for further analysis.

Acknowledgements

We thank the Centre National de la Recherche Scientifique (CNRS-France) and Unilever Research for a fellowship to R.A.-S.

References and notes

1. Roberts, D. W.; Lepoittevin, J.-P. In *Allergic Contact Dermatitis: The Molecular Basis*; Lepoittevin, J.-P., Basketter, D. A., Goossens, A., Karlberg, A.-T., Eds.; Springer: Berlin, Heidelberg, 1998; p 81.
2. Rustmeyer, T.; von Hoogstraten, M. W.; von Blomberg, M. E.; Scheper, R. J. In *Textbook of Contact Dermatitis*; Rycroft, R. J. G., Menné, T., Frosch, P. J., Lepoittevin, J.-P., Eds.; Springer: Berlin, Heidelberg, 2001; p 13.
3. Barratt, M. D.; Basketter, D. A.; Roberts, D. W. In *Allergic Contact Dermatitis: the Molecular Basis*; Lepoittevin, J.-P., Basketter, D. A., Goossens, A., Karlberg, A.-T., Eds.; Springer: Berlin, Heidelberg, 1998; p 129.
4. Lepoittevin, J.-P.; Leblond, I. *Eur. J. Dermatol.* **1997**, *7*, 151.
5. Meschkat, E.; Barratt, M.; Lepoittevin, J.-P. *Chem. Res. Toxicol.* **2001**, *14*, 118.
6. Kohler, J.; Martin, S.; Weltzien, H. U. *Immunobiol.* **1993**, *189*, 57.
7. Alvarez-Sanchez, R.; Basketter, D.; Pease, C.; Lepoittevin, J.-P. *Chem. Res. Toxicol.* **2003**, *16*, 627.
8. Tracey, B. M.; Shuker, D. E. *Chem. Res. Toxicol.* **1997**, *10*, 1378.
9. Basketter, D. A.; Gilmour, N. J.; Wright, Z. M.; Walters, T.; Boman, A.; Lidén, C. *J. Toxicol: Cut and Ocular Toxicol.*, in press.