Research Article

Journal of Physical Organic Chemistry

Received: 3 February 2014, Revised: 1 April 2014, Accepted: 18 April 2014, Published online in Wiley Online Library: 19 May 2014

(wileyonlinelibrary.com) DOI: 10.1002/poc.3316

Kinetic study on the aromatic nucleophilic substitution reaction of 3,6-dichloro-1,2,4,5-tetrazine by biothiols

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The aromatic nucleophilic substitution reaction of 3,6-dichloro-1,2,4,5-tetrazine (DCT) with a series of biothiols RSH: (cysteine, homocysteine, cysteinyl–glycine, N-acetylcysteine, and glutathione) is subjected to a kinetic investigation. The reactions are studied by following spectrophotometrically the disappearance of DCT at 370 nm. In the case of an excess of N-acetylcysteine and glutathione, clean pseudo first-order rate constants (k_{obs1}) are found. However, for cysteine, homocysteine and cysteinyl–glycine, two consecutive reactions are observed. The first one is the nucleophilic aromatic substitution of the chlorine by the sulfhydryl group of these biothiols (RSH) and the second one is the intramolecular and intermolecular nucleophilic aromatic substitutions of their alkylthio with the amine group of RSH to give the di-substituted compound. Therefore, in these cases, two pseudo first-order rate constants (k_{obs1} and k_{obs2}, respectively) are found under biothiol excess. Plots of k_{obs1} versus free thiol concentration at constant pH are linear, with the slope (k_N) independent of pH (from 6.8 to 7.4). The kinetic data analysis (Brønsted-type plot and activation parameters) is consistent with an addition–elimination mechanism with the nucleophilic attack as the rate-determining step. Copyright © 2014 John Wiley & Sons, Ltd.

Keywords: aromatic nucleophilic substitution; biothiolysis; inter/intramolecular displacement; kinetic study; tetrazine

INTRODUCTION

The *s*-tetrazines are relatively old molecules, their discovery dated back to the end of the 19th century. The first synthesis was reported by Pinner who performed the reaction of equimolar quantities of hydrazine and benzonitrile. Upon further oxidation of the dihydrotetrazine intermediate, the deep red 3,6-diphenyl-*s*-tetrazine was isolated.[1,2]

Tetrazines are unique heterocyclic compounds containing the maximum number of nitrogen atoms in the aromatic ring. Because of the presence of four electron-withdrawing heteroatoms in the tetrazine core,[3] these compounds exhibit particular properties among which highlights a high electrophilicity.

Regarding the latter, the introduction of fragments at positions 3 and 6 capable of leaving as anions, opens a possible pathway for the modification of 1,2,4,5-tetrazines using *aromatic nucleophilic substitution*. These reactions enable the preparation of new symmetrically and asymmetrically substituted derivatives, including products containing heteroatoms. The most common groups in tetrazines capable of leaving as anions are halogen atoms,[4] the methylsulfanyl group,[5,6] and the 3,5-dimethylpyrazolyl fragment.[3]

One excellent example of what is mentioned earlier is the modification of 3,6-dichloro-1,2,4,5-tetrazine (DCT) by the action of N-nucleophiles[4,7] and O-nucleophiles,[8–12], which are often used to obtain tetrazine derivatives symmetrically and asymmetrically substituted with heteroatoms. In addition, reactions involving nucleophilic substitution of the halogen atoms in this tetrazine by the action of isobutyl mercaptan have also been reported.[4] However, there are only a few works related to the ability of DCT to react with sulfhydryl groups of amino acids.[13] In this context, it is important to note its reaction with cysteine, because it may be used for introducing a tetrazine group capable of undergoing photofragmentation into a protein structure with the aim of subsequent conformational analysis of peptides.[13,14] On the other hand, the development of selective thiol-reactive linkers and the reactions of biothiols with alternative probes is a subject of much current interest.[15–22]

Therefore, with the aim to shed some light on the mechanisms of these reactions and to propose a strategy for thiol detection based on nucleophilic aromatic substitution, this work undertakes a kinetic investigation on the reactions of biothiols with DCT. The studied biothiols are as follows: cysteine (Cys), homocysteine (Hcy), cysteinyl–glycine (Cys–Gly), N-acetylcysteine (NAC) and glutathione (GSH), and the conventional thiols 2-mercaptoethanol (2-ME) and 1-propanethiol (1-PT).

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EXPERIMENTAL

Materials

The reagents were purchased from Sigma-Aldrich (St. Louis, MO) and used as received. Unless indicated, all solutions employed in this study were prepared in Chelex-100-treated HEPES buffer (20 mM; pH 7.4).

The compound DCT was prepared as reported by Hiskey *et al.*[23,24] This modified Pinner synthesis involves four steps (see Scheme 1). First, triaminoguanidine monohydrochloride (**2**) was obtained by mixing (**1**) with hydrazine in dioxane. Compound **2** was then condensed with two equivalents of 2,4-pentanedione to yield dihydrotetrazine (**3**), which was oxidized using $NaNO_2$ to form dipirazol tetrazine (**4**). Finally, the latter compound was treated with hydrazine and chlorine gas to obtain DCT.

This compound was identified by its spectroscopic properties. ^{13}C-NMR (100 MHz, $CDCl_3$) δ (ppm): 167.42 and by MS (low resolution), *m/z* (I %) = 150.13 (30), 87.09 (85), 61.03 (100), (Fig. S1).

Kinetic measurements

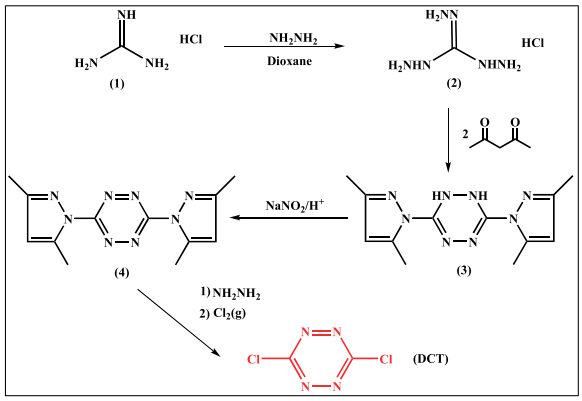
These measurements were carried out by either an HP-8453 diode array spectrophotometer or an Applied Photophysics DX17MV stopped-flow spectrophotometer in aqueous solution at different temperatures (10–40 ± 0.1 °C) under an ionic strength of 0.2 M (KCl). HEPES buffers were used in all reactions. The reactions studied in this work, under excess of the biothiols over the substrate, were initiated with an injection of a substrate stock solution in DMSO (10 μL) into the biothiols aqueous solution (2.5 mL in the spectrophotometric cell). The initial DCT concentration was about 2×10^{-4} M.

The reactions in the stopped-flow spectrophotometer were carried out with unequal mixing. The DCT dissolved in dry DMSO was placed in a small syringe (0.1 mL), and a larger syringe (2.5 mL) was filled with the thiol aqueous solution. Pseudo first-order rate constants (k_{obs1}) were found for all reactions. These were determined through the spectrophotometer kinetic software, by least-squares fitting of the experimental absorbance data to the single-exponential curve $A_t = A_0 \exp(-k_{obs1}\, t) + C$, where A_t and A_0 are the absorbances at time *t* and 0, respectively. The experimental conditions of the reactions and the k_{obs1} values are shown in Table S1.

From temperature-dependent rate constants, the Arrhenius and Eyring equations were plotted as follows: log (k_N) versus 1/T and log (k_N/T) versus 1/T, respectively. From these plots, energy (E_a) and the enthalpy ($\Delta H^{\neq}$) and entropy ($\Delta S^{\neq}$) of activation were determined for the reaction of DCT with biothiols.

Product studies

For the reaction between DCT and RSH, it was possible to identify the final product of each reaction and also to compare them with others using the model thiol 2-mercaptoethanol in the same experimental conditions. In the case of high-performance liquid chromatography–mass spectrometry (HPLC-MS), the analysis was carried out by direct insertion of the sample in an electrospray ionization (ESI) source. The low mass EI-MS experiments were performed on a MAT 95XP Thermo-Finnigann spectrometer by direct inlet probe (Thermo Fisher Scientific). HPLC-MS experiments were performed on an Exactive Plus Orbitrap MS Thermo Scientific (Thermo Fisher Scientific). The accurate mass measurements were performed at a resolution of 140.000. On the other hand, ^{1}H-NMR spectra were acquired on a Bruker AM-400 instrument using tetramethylsilane as internal reference.



Scheme 1. Synthetic route to 3,6-dichloro-1,2,4,5-tetrazine (DCT)

Computational methods

To ensure the consistence of our theoretical predictions, the molecular geometries of the ground state structures were optimized using the hybrid PBE0 and the range-separated hybrid ωB97X-D functionals. Both functionals have been recently proposed as adequate to model reaction profiles and their stationary points (reagents, transition state, and product) in thiol-Michael additions.[25]

The calculations employed a triple-ζ (6-311++G(d,p))[26–28a] and double-ζ (6-31 + G(d)).[28b,28c] Solvent effects (in water) were included using the polarizable continuum model.[29] The nature of the stationary points was checked by means of frequency calculations. All calculations were performed using the Gaussian 09, Revision C.01, suite of programs.[30]

RESULTS AND DISCUSSION

Spectrophotometric study

The compound DCT provides an important optical signal (compound highly colored). Figure 1 shows the UV/visible spectrum of DCT, which exhibits two characteristic bands (λ_1 and λ_2). As reported previously,[7] the first transition is attributed to that of $n \rightarrow \pi^*$ transition of the *s*-tetrazine with a small molar extinction coefficient. The second band is attributed to a $\pi \rightarrow \pi^*$ transition and is more intense. In the presence of 10 equiv. of GSH, both absorption bands decrease (Fig. 1). Similar spectral behaviors were observed when other biothiols including Cys (Fig. S2), Hcy, Cys–Gly, and NAC were used. Thus, the reaction between DCT and the series of biothiols was followed spectrophotometrically at 370 nm. The insert in Fig. 1 depicts the kinetic profile at this wavelength obtained for the reaction between DCT and GSH. For this reaction, only a fast decrease of absorbance due to the disappearance of DCT was observed. Similar results were

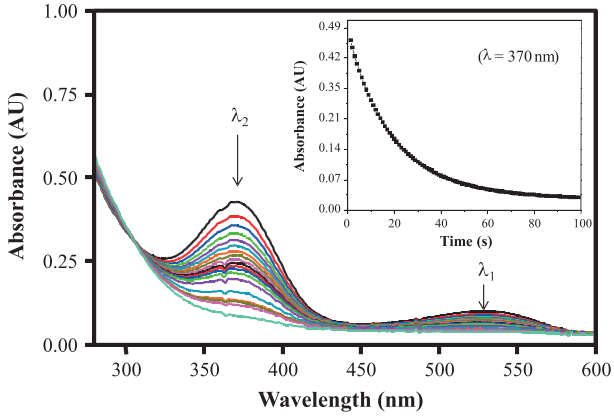


Figure 1. Absorption spectra of DCT (200 μM) in the presence of glutathione (GSH, 10 equiv.) in aqueous solution (20 mM HEPES buffer, pH 7.4, 1% DMSO) at different times. Insert shows the kinetic profile, at 370 nm, for this reaction

obtained for NAC and other conventional thiols, such as 2-mercaptoethanol (2-ME) and 1-propanethiol (1-PT), (Fig. S3). However, in some cases (i.e., Cys, Hcy, and Cys–Gly), a fast absorbance decrease, followed by a slower decrease at 370 nm was also observed (for Cys, see insert in Fig. S2).

Kinetic results

For the reactions of DCT with GSH, NAC, 2-ME, and 1-PT, pseudo first-order rate constants (k_{obs1}) were obtained under nucleophile (thiol) excess. These values were determined by means of the spectrophotometer kinetic software for first-order reactions. The values of k_{obs1} for these reactions are shown in Table S1. The values of k_{obs1} versus free nucleophile concentration obey Eqn. (1), where k_0 and k_{N1} are the rate constants for solvolysis and thiolysis of the substrate (DCT). The k_0 and k_{N1} values were obtained as the intercept and slope, respectively, from linear plots of k_{obs1} against free nucleophile concentration at constant pH. The values of k_0 and k_{N1} show no dependence on pH within the pH range employed (from 6.8 to 7.4).

$$k_{obs1} = k_0 + k_{N1}[\text{free nucleophile}] \quad (1)$$

For these reactions, the k_0 values were much smaller than the k_{N1} [free nucleophile] term in Eqn. (1). Table 1 summarizes the values of k_{N1} found, together with those for the pK_a of the series of nucleophiles employed. As shown, all reactions are associated with large rate constant values ($>37\ s^{-1}\ M^{-1}$). Considering the high values of k_{N1} obtained in this study, DCT could be proposed as an efficient electrophile toward these biothiols. In fact, as suggested by Gao *et al.*,[31] DCT is a powerful electrophile, similar in reactivity toward other nucleophiles, as picryl fluoride.

In contrast, the biothiols Cys, Hcy, and Cys–Gly showed toward DCT a consecutive reaction model. This behavior can be explained considering the recently reported ability of Cys and Hcy to react via nucleophilic substitution by its sulfhydryl group to form an intermediate thioether.[32–34] Subsequently, this intermediate undergoes further intramolecular aromatic substitution reaction that causes the displacement of the thio group by the amine group present in the thiol.

Therefore, we propose that the first decrease observed at 370 nm (Fig. S2) is attributed to the thiol-halogen nucleophilic aromatic substitution of DCT by thiolate of biothiol (kinetic values for the fast process, k_{obs1}) and the second slow decrease could be due to the intramolecular nucleophilic aromatic substitution of alkylthio with the amine group of RSH (k_{obs2}). This latter reaction could also be an intermolecular process (see succeeding text). The kinetic values for k_{obs1} and k_{obs2} are summarized in Table S1. The second-order rate constants (k_{N1} and k_{N2}) were obtained as the slopes of plots (k_{obs1}) and (k_{obs2}) against free thiol concentration or free amine concentration, respectively. Figure S4 shows an example of such plots.

The absence of a consecutive reaction for 1-PT (Fig. S3) and NAC is due to the lack of an amine group responsible to undergo the aforementioned second reaction, and thus, only the first reaction is observed. However, in the case of GSH, the apparent absence of the second reaction (insert in Fig. 1) is in line with the recently reported inability of the amine group present in GSH to undergo an intramolecular re-arrangement.[33]

The values of k_{N1} found in this work, together with the pK_a values of the sulfhydryl group of the tested thiols,[15,35,36] are summarized in Table 1 showing the following reactivity order for the series of the studied thiols toward DCT: Cys–Gly < Cys < Hcy < GSH <2-ME < NAC < 1-PT. Consequently, the reactivity increases with the basicity of the sulfhydryl group of the corresponding thiol. With the pK_a and k_{N1} values in Table 1, the Brønsted type plot for the thiolysis studied was obtained. As shown in Fig. 2, this plot is linear with a slope (β) of 0.33. The value of β found for the reactions of biothiols with DCT is in agreement with the β values found for stepwise reactions when the formation of the intermediate is the rate-limiting step.[36]

To gain further understanding on the mechanism of the reaction of DCT with biothiols, the activation parameters were calculated from the rate constants measured at five different temperatures (10–40 °C). These thermodynamic activation parameters are summarized in Table 2. The large negative $\Delta S^{\#}$ values obtained indicate that the transition states are highly ordered compared with reactants; this is consistent with the fact that the translational degree of freedom are restricted because of a condensation reaction or great participation of water molecules in the activated complex. We find it interesting that the values obtained for $\Delta H^{\neq}$ and $\Delta S^{\neq}$ are in line with a bimolecular reaction similar to that commonly accepted for aromatic bimolecular two-stage process in which the formation of a Meisenheimer complex (intermediate MC) is the rate-determining step, with a rapid decomposition of this intermediate into product.[37]

On the basis of the kinetic results and by comparing the reactions under investigation between each other and with other similar processes, we propose a bimolecular reaction type:

Table 1. Values of pK_a for the sulfhydryl group of tested thiols (RSH) and k_{N1} values for the reactions of the RSH with DCT

Thiols (RSH)	pK_a[a]	$k_{N1}/s^{-1}\ M^{-1}$
Cys–Gly	7.00	37.7 ± 0.9
Cys	8.10	98 ± 3
Hcy	8.25	102 ± 6
GSH	8.72	167 ± 5
2-ME	9.70	193 ± 8
NAC	9.90	207 ± 12
1-PT	10.7	1160 ± 30

[a]pK_a data in water were taken from[15,35,36]

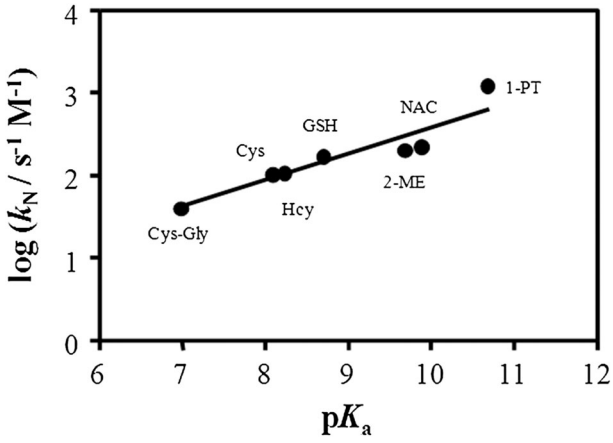


Figure 2. Brønsted-type plot for the thiolysis of DCT, in aqueous solution, at 25.0 °C and ionic strength 0.2 M (KCl)

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Table 2. Thermodynamic activation parameters for the reactions of biothiols (RSH) with DCT

	DCT		
Biothiols	E_a/kJ mol^{-1}	$\Delta H^{\neq}$/kJ mol^{-1}	$\Delta S^{\neq}$/J mol^{-1} K^{-1}
Cys–Gly	56 ± 3	54 ± 9	−33 ± 2
Cys	54 ± 4	51 ± 2	−34 ± 1
Hcy	47 ± 3	45 ± 2	−53 ± 4
GSH	38 ± 2	36 ± 3	−83 ± 5
NAC	21 ± 2	18 ± 3	−138 ± 4

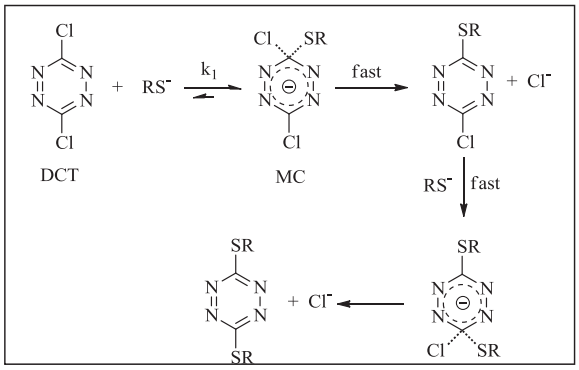
addition–elimination (S_NAr) for the nucleophilic attack of the series of thiols to DCT (as suggested in Scheme 2).

On the other hand, it is known that when a series of structurally related substrates undergo the same general reaction or when the reaction conditions for a single substrate are changed in a systematic way, the enthalpies and entropies sometimes satisfy a linear isokinetic relationship, as written in Eqn. (2).

$$\Delta H_i^{\neq} = \alpha + \beta \Delta S_i^{\neq} \quad (2)$$

In this equation, α and β are constants, where β is the so-called 'compensation temperature' and α has energy dimension. Figure 3 shows that a plot of $\Delta H^{\neq}$ versus $\Delta S^{\neq}$ for the tested reactions gives a straight line. This behavior is related to the enthalpy–entropy compensation effect,[38,39] also known as the isokinetic relationship,[40] which suggests that all k_{N1} values for the studied reactions correspond to the same rate-limiting step.

Also, it is interesting to mention that the rate constants (k_{obs2}, Fig. S4) obtained for the decomposition of the intermediate product (**P1** in Scheme 3) show a linear dependence on the corresponding free amine (N) concentration. Therefore, we do not only suggest an intramolecular substitution reaction but also an intermolecular one, as shown in Scheme 3.



Scheme 2. Proposed mechanism for the reaction between DCT and biothiols (RSH)

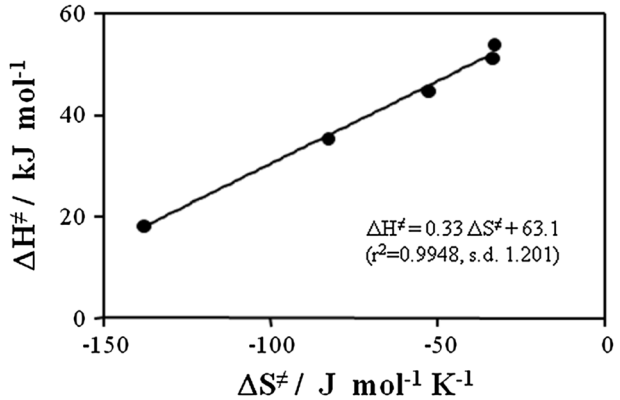


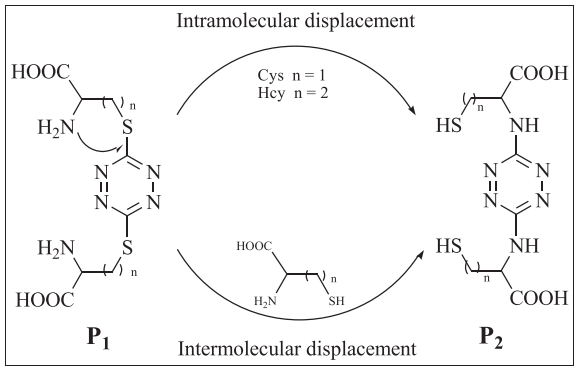
Figure 3. Correlation between activation enthalpy ($\Delta H^{\neq}$) and activation entropy ($\Delta S^{\neq}$) for the reaction of biothiols with DCT

Theoretical calculations

The present study is complemented with theoretical calculations of the Gibbs free energy (ΔG) of the two possible products proposed (**P1** and **P2**, see Scheme 3).

The calculation of the Gibbs free energies, allow us to predict that the product P2, obtained from the nucleophilic aromatic substitution of DCT by the amine group of Cys, is thermodynamically preferred by ~10 kcal mol^{-1} in comparison with the formation of P1, as shown in Fig. 4.

Similar results were obtained when Hcy was used instead of Cys. It is important to remark that different structural possibilities have been tested as starting geometries for the minimization of each isomer. The most stable geometry at ωB97X-D/6-311++G** level for the reaction of DCT with Cys ($n = 1$) is presented in Fig. 4. The optimized structural parameters (distances and angles) are



Scheme 3. Proposed mechanism for the reactions of DCT with Cys ($n = 1$) and Hcy ($n = 2$)

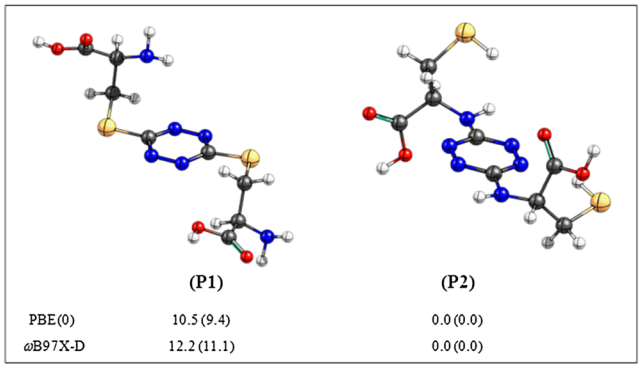


Figure 4. Computed relative Gibbs free energies (ΔG) in kcal mol^{-1} at the PBE(0) and ωB97X-D with the 6-311++G** basis set level of theory. The values without and in parenthesis are those that include solvent effects (read the computational details) and in vacuum, respectively. P1 and P2 represent the products for the reaction of DCT with cysteine as nucleophile

almost the same when PBE(0) or ωB97X-D is used in the calculations. In addition, it is observed that the use of a small base set (6-31 + G*) in vacuum is enough to provide similar description about the thermodynamic preference of the P2 against P1 (see Tables S2–S3).

Therefore, considering the kinetic profiles and theoretical results presented, we propose that for the biothiols Cys, Hcy, and Cys–Gly, the kinetic product P1 is formed at short times. However, under long reaction times, the thermodynamic product P2 is favored.

Product analysis

Finally, with the objective to assess the products formed by interaction between each biothiol and DCT, we performed a product analysis using mass spectrometry (HPLC-ESI-MS) and ^{1}H-NMR.

The mass spectrum obtained after the addition of an excess of Cys on DCT (Fig. 5) depicts a series of mass fragments with different intensities. The more important signals correspond to $m/z = 200$, which was assigned as $[\text{M-Cys-CO}_2 + \text{H}]^+$, $m/z = 157$ is attributed to $[\text{M-Cys}]^+$, and the molecular ion with $m/z = 319$ corresponds to $[\text{M-H}]^+$. It is noteworthy that the latter involves the formation of a di-substituted compound. However, it is not possible to decide if this compound corresponds at P1 or P2, due to their identical molecular ions. A similar result was also attained for Hcy (Fig. S5) and 2-ME (Fig. S6), using the same technique.

In the ^{1}H-NMR spectrum, obtained after the addition of DCT (in DMSO-d_6) on Cys (in D_2O, Figs. 6 and S7), we assigned a broad signal at ~7.8 ppm and a signal at 2.4 ppm to the exchangeable protons of the amine group and SH group, respectively. With the aim to confirm this exchange, we compared the spectra obtained by reacting DCT with an authentic amino-substituted compound, such as n-butylamine (BA) and a model compound of amino-free thiol, such as NAC. For the former (Fig. S8), we first observed that the resonance signals of the protons of amine group of BA were shifted to lower field due to the nucleophilic substitution of chlorine atoms in DCT by the amine group. Second, it is possible to observe that the signal at 2.7 ppm, corresponding to the proton of alpha carbon of the BA, is shifted to 3.3 ppm for the product between DCT and BA. A similar effect was observed for the alpha carbon of Cys after its reaction with DCT (shifted by ~0.2 ppm). In the case of NAC, we observed the disappearance of the signal at 2.4 ppm associated with the proton of its sulfhydryl group. We find it interesting that Fig. 6(A) shows the existence of the aforementioned signal at 2.4 ppm.

Thus, results obtained from NMR spectra suggest that Cys is attached to DCT through its amine group and this is in concordance with those described in other study for a process based on a similar intramolecular displacement.[33] Therefore, this information is crucial to corroborate the final product P2 previously proposed in Scheme 3, as the product thermodynamically favored, and the P1 as the product kinetically favored.

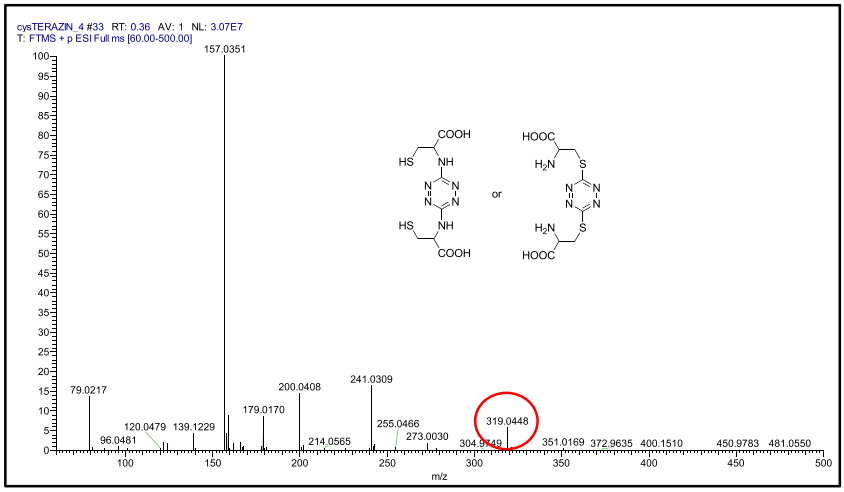


Figure 5. Mass spectrum obtained after the addition of DCT to cysteine

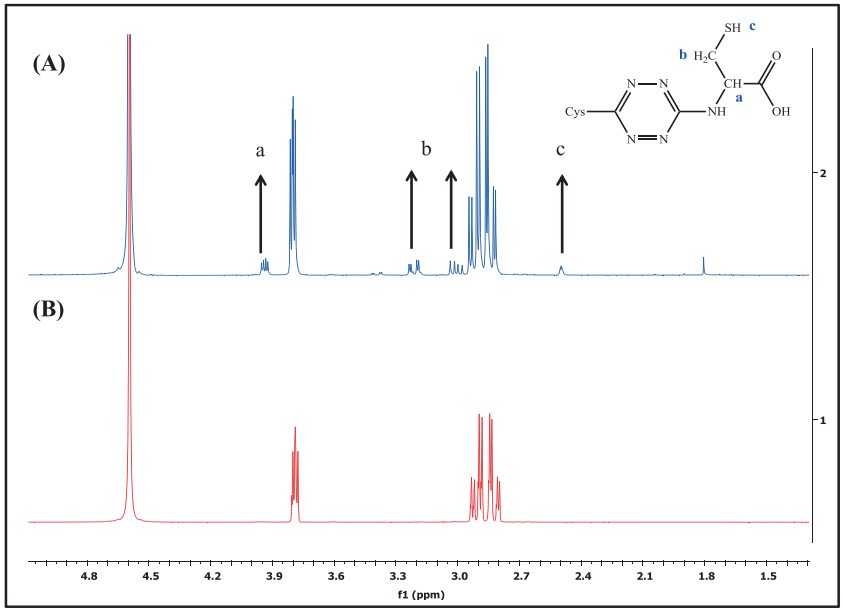


Figure 6. Changes in the Cys ^{1}H-NMR spectrum induced by the addition of DCT. Spectra of Cys in the presence of DCT (A) or in the absence of DCT (B)

CONCLUSIONS

On the basis of the overall evidence available, we have proposed that the nucleophilic reactivity of the tested sulfhydryl groups toward DCT increases in the sequence Cys–Gly < Cys < Hcy < GSH <2-ME NAC <1-PT. By comparing the reactions under investigation between each other and with other similar processes, we propose a bimolecular reaction type: addition–elimination (S_NAr) for the nucleophilic attack of the series of thiols to DCT, in which the formation of the Meisenheimer-type complex (intermediate MC) is the rate-determining step of the reaction, followed by a fast substitution of the

second chlorine atom in DCT. Subsequently, in the case of Cys, Hcy, and Cys–Gly, a second intramolecular and intermolecular nucleophilic aromatic substitutions of alkylthio with the amine group was evidenced, being the latter compound the thermodynamically favored product.

Acknowledgements

This work was supported by FONDECYT with grant #1130062 and FONDEQUIP EQM120163. The authors are grateful to Prof. Luis García-Río for the use of spectroscopic facilities (stopped-flow spectrophotometer) in the Centro Singular de Investigación en Química Biológica y Materiales Moleculares (CIQUS), Universidade de Santiago de Compostela, Santiago de Compostela, España.

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