Research article

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Novel glutathione conjugates of phenyl isocyanate identified by ultra-performance liquid chromatography/electrospray ionization mass spectrometry and nuclear magnetic resonance

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Phenyl isocyanate is a highly reactive compound that is used as a reagent in organic synthesis and in the production of polyurethanes. The potential for extensive occupational exposure to this compound makes it important to elucidate its reactivity towards different nucleophiles and potential targets in the body. *In vitro* reactions between glutathione and phenyl isocyanate were studied. Three adducts of glutathione with phenyl isocyanate were identified using ultra-performance liquid chromatography/electrospray ionization mass spectrometry and nuclear magnetic resonance (NMR). Mass spectrometric data for these adducts have not previously been reported. Nucleophilic attack on phenyl isocyanate occurred *via* either the cysteinyl thiol group or the glutamic acid α-amino group of glutathione. In addition, a double adduct was formed by the reaction of both these moieties. NMR analysis confirmed the proposed structure of the double adduct, which has not previously been described. These results suggest that phenyl isocyanate may react with free cysteines, the α-amino group and also with lysine residues whose side chain contains a primary amine. Copyright © 2014 John Wiley & Sons, Ltd.

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Keywords: glutathione; trapping; isocyanate; amino group; thiol group

Introduction

Isocyanates are highly reactive compounds that contain the (−N=C=O) functional group. In general, they are used in organic synthesis and as starting materials for the manufacture of polyurethanes. Polyurethanes are polymers with diverse uses in products such as rubber modifiers, paints, inks, insulating materials, varnishes, foams and lacquers.[1] Methyl isocyanate has been used in the production of carbamate pesticides. Following the Bhopal accident of 1984, in which thousands of people died due to a large-scale release of methyl isocyanate, numerous studies have been performed to investigate the toxicity of this compound.[2]

Phenyl isocyanate is another isocyanate that is used as a reagent in organic synthesis and in the production of polyurethanes.[3] Its chemical structure is shown in Fig. 1. To our knowledge, its toxicity has not been examined in as much detail as that of methyl isocyanate. However, the clinical symptoms of phenyl isocyanate exposure are similar to those described for methyl isocyanate, including respiratory and ocular irritation (with subsequent tissue damage) and delayed death.[4] In rats, phenyl isocyanate has been shown to cause an asthma-like syndrome.[5] The capacity of phenyl isocyanate to cause contact sensitization in mice has been evaluated in the mouse ear swelling test. The results indicated that phenyl isocyanate is a potent inducer of humoral and cellular immune responses.[3] Aniline, the hydrolysis product of phenyl isocyanate, has previously been analyzed in urine and plasma in an effort to measure the exposure of 11 workers to phenyl isocyanate.[6] Studies of reactivity often focus on reactions between a reactive electron-deficient (electrophilic) species and an electron-rich (nucleophilic) species. Glutathione (GSH, γ-L-glutamyl-L-cysteinyl-glycine) is a tripeptide that is both the most abundant cellular thiol and the most abundant low molecular weight peptide in eukaryotic cells. It acts as a nucleophilic scavenger, protecting the cells by functioning as an antioxidant and by detoxifying electrophilic species. Electrophiles may react directly with the nucleophilic thiol group of glutathione and the conjugation may also be catalyzed by glutathione *S*-transferase enzymes. Under certain conditions, the GSH pool in the cells may become depleted, making other endogenous thiol-containing molecules more susceptible to attack by electrophiles. This may lead to the formation of covalent bonds between reactive species and biological macromolecules such as proteins, DNA or enzymes, and can have toxic effects. GSH is widely used as a model nucleophile in studies of reactive molecules.[7,8] For example, in the pharmaceutical industry, GSH is used to trap reactive drug metabolites as their GSH conjugates, which are generally more stable and detectable than the original

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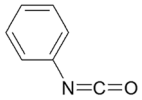

Figure 1. Chemical structure of phenyl isocyanate.

metabolite.[9] The respiratory tract is a major route for isocyanate exposure and isocyanates are known to cause sensitization of the respiratory tract and occupational asthma. The lower respiratory tract contains high levels of glutathione, and it has been shown that methyl isocyanate forms conjugates with glutathione.[10–12] We therefore decided to explore the ability of phenyl isocyanate to form conjugates with glutathione.

The aim of this study was to elucidate the reactivity of the cysteinyl thiol moiety and the glutamic acid α-amino group of glutathione towards phenyl isocyanate *in vitro* and to identify the resulting glutathione conjugates by ultra-performance liquid chromatography/electrospray mass spectrometry (UPLC/ESI-MS) and nuclear magnetic resonance (NMR).

Experimental procedures

Materials

L-glutathione (reduced form), phenyl isocyanate and potassium dihydrogen phosphate were obtained from Sigma–Aldrich (St. Louis, MO, USA). Formic acid was obtained from Honeywell Riedel-de Haën (Seelze, Germany). Dipotassium hydrogen phosphate and acetonitrile were obtained from Merck (Darmstadt, Germany). *S*-acetyl-L-glutathione was obtained from abcr GmbH & Co. KG (Karlsruhe, Germany). All solvents were of analytical grade and the water used in the experiments was obtained from a water purification system (Milli-Q, Merck Millipore, Billerica, MA, USA).

UPLC-ESI/MS method

The samples were analyzed by liquid chromatography (Waters ACQUITY UPLC, Milford, MA, USA), on a 2.1 × 50 mm, 1.7 μm C18-column (Waters Acquity UPLC BEH), at a flow rate of 600 μl/min (the column temperature was set at 60 °C). Mobile phase A consisted of 0.1% (v/v) formic acid in Milli-Q water and mobile phase B consisted of 0.1% (v/v) formic acid in acetonitrile. The solvent gradient was linear, increasing from 5% B to 95% B over 5.5 min. The initial mobile phase composition was then restored over 0.5 min and this condition was maintained for a further 0.5 min. For the time study, the following UPLC conditions were used: 35% B for 0.8 min, increasing to 95% B over 0.1 min and the initial mobile phase conditions were restored over 0.1 min. For the response study, the solvent gradient was linear, increasing from 5% B to 95% B over 0.7 min. The initial mobile phase composition was then restored over 0.1 min and this condition was maintained for a further 0.1 min. The UPLC system was coupled to a triple quadrupole mass spectrometer (Waters Xevo TQ, Milford, MA, USA) interfaced with an electrospray ionization source. A capillary voltage of 3.5 kV was used and the cone voltage was set to 30 V. GSH adducts were detected using full scan analyses acquired in positive ionization mode. For the acquisition of MS/MS spectra, a collision energy (CE) of 15 V was used and argon was employed as collision gas. The injection volume was 5 μl for full scan mode detection and 10 μl for product ion detection. For the time study, the following multiple reaction monitoring transitions were used: 213 → 94 (CE 30 V), 427 → 259 (CE 30 V), 427 → 298 (CE 30 V), 546 → 378 (CE 15 V), the dwell time was set to 10 ms. Data were processed using the MassLynx (version 4.1) and MetaboLynx softwares (Waters, Milford, MA, USA).

Glutathione conjugation reactions

Phosphate buffer (0.1 M, pH 7.4) was prepared from potassium dihydrogen phosphate and dipotassium hydrogen phosphate. A fresh 5 mM glutathione solution was then prepared in the phosphate buffer. Phenyl isocyanate was added to the glutathione solution to a final concentration of 50 μM. The mixture was vortexed for 10 s and then left to react at 37 °C for 2 h. A precipitate formed during the reaction, and so the samples were filtered prior to dilution 1:100 (v/v) with 0.4% formic acid in water. The samples were then injected into the UPLC-ESI/MS system.

Glutathione conjugation time study

A fresh 5 mM glutathione solution was then prepared in the phosphate buffer (0.1 M) and phenyl isocyanate was added to a final concentration of 50 μM. The mixture was vortexed for 10 s, diluted 1:10 (v/v) with 0.1 M phosphate buffer and put in the autosampler (20 °C). Samples were injected every minute during 60 min.

Synthesis of 5-[[2-(carboxymethylamino)-2-oxo-1-(phenylcarbamoylsulfanylmethyl)-ethyl]amino]-5-oxo-2-(phenylcarbamoylamino)pentanoic acid (3) for characterization by NMR

In a 5 ml vial, 2-amino-5-[[1-(carboxymethylamino)-1-oxo-3-sulfanyl-2-propanyl]amino]-5-oxopentanoic acid (GSH) (50 mg, 0.163 mmol) was dissolved in 1 ml DMF and a minimal amount of water. Neat phenyl isocyanate (194 mg, 1.627 mmol, 10 mol.eq.) was then added drop wise at room temperature over a period of two days. The reaction mixture was stirred at room temperature for another 48 h before being quenched with water. The precipitate formed (1,3-diphenyl-urea) was filtered off and the filtrate was concentrated under reduced pressure prior to purification by preparative HPLC. The purest fractions with the desired product were pooled and the solvent removed *in vacuo* to afford **3** as a white solid.

Preparative HPLC

The crude mixture from the synthesis was purified by liquid chromatography (Waters AutoPurification HPLC, Milford, MA, USA), on a 10 × 50 mm, 5 μm C18-column (Waters SunFire), at a flow rate of 25 ml/min. Mobile phase A consisted of 0.1% (v/v) formic acid in water and mobile phase B consisted of 0.1% formic acid in acetonitrile. The solvent gradient was linear, increasing from 5% B to 70% B over 3.33 min and then from 70% B to 95% B over 7 s. This composition was maintained for 3.67 min, after which the initial mobile phase composition was restored over 6 s; the resulting conditions were maintained for 0.87 min. The HPLC system was coupled to a photodiode array detector (Waters 2998 PDA).

NMR

^{1}H NMR (500 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on an Avance 500 instrument from Bruker (Karlsruhe, Germany), using methanol-d_4 as the solvent. Chemical shifts are reported in ppm using the residual solvent peak as an internal standard. The residual ^{1}H peak for methanol occurs at δ_H 3.31 and the corresponding ^{13}C peak occurs at δ_C 49.00.

^{1}H NMR (500 MHz, methanol-d$_4$) δ = 7.47 (dd, J = 1.0, 8.5 Hz, 2 H), 7.35 (dd, J = 1.1, 8.6 Hz, 2 H), 7.30 – 7.19 (m, 4 H), 7.04 (quin, J = 1.0 Hz, 1 H), 6.96 (tt, J = 1.1, 7.4 Hz, 1 H), 4.67 (dd, J = 4.7, 8.6 Hz, 1 H), 4.36 (dd, J = 4.9, 8.2 Hz, 1 H), 3.94 – 3.80 (m, 2 H), 3.53 (dd, J = 4.7, 14.2 Hz, 1 H), 3.24 – 3.13 (m, 1 H), 2.49 – 2.36 (m, 2 H), 2.29 – 2.20 (m, 1 H), 2.07 – 1.95 (m, 1 H); ^{13}C NMR (126 MHz, methanol-d$_4$) d 175.5, 172.8, 157.9, 140.9, 140.2, 130.1, 129.9, 125.1, 123.6, 120.7, 55.1, 48.0, 33.3, 32.3, 29.6, 9.4

Results

In this study, the *in vitro* reaction between phenyl isocyanate and glutathione was investigated. Three putative GSH adducts were detected in the LC-MS full scan analysis: the extracted ion chromatograms are shown in Fig. 2. The first adduct eluted at 1.38 min (**1**, *m/z* 427), the second at 1.58 min (**2**, *m/z* 427) and the third at 2.41 min (**3**, *m/z* 546).

The glutathione adducts detected at *m/z* 427 (**1** and **2**) correspond to an addition of 307 Da to phenyl isocyanate, implying that these adducts were formed by the equimolar reaction of phenyl isocyanate with glutathione.

The product ion spectrum of the first adduct (*m/z* 427, $[M+H]^+$), which eluted after 1.38 min (**1**), is shown in Fig. 3. Proposed precursor ions, neutral losses and fragment ions are shown in Table 1.

The base peak (*m/z* 298) was the y-type ion formed by the characteristic neutral loss of anhydroglutamic acid (129 Da) and

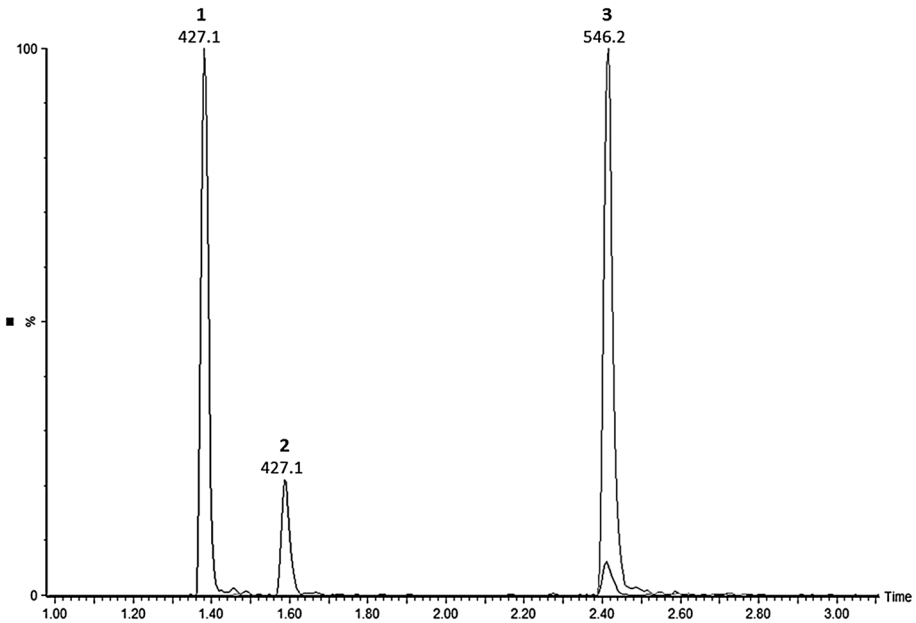

Figure 2. Extracted ion chromatograms of the $[M+H]^+$ ions of **1** (*m/z* 427), **2** (*m/z* 427) and **3** (*m/z* 546).

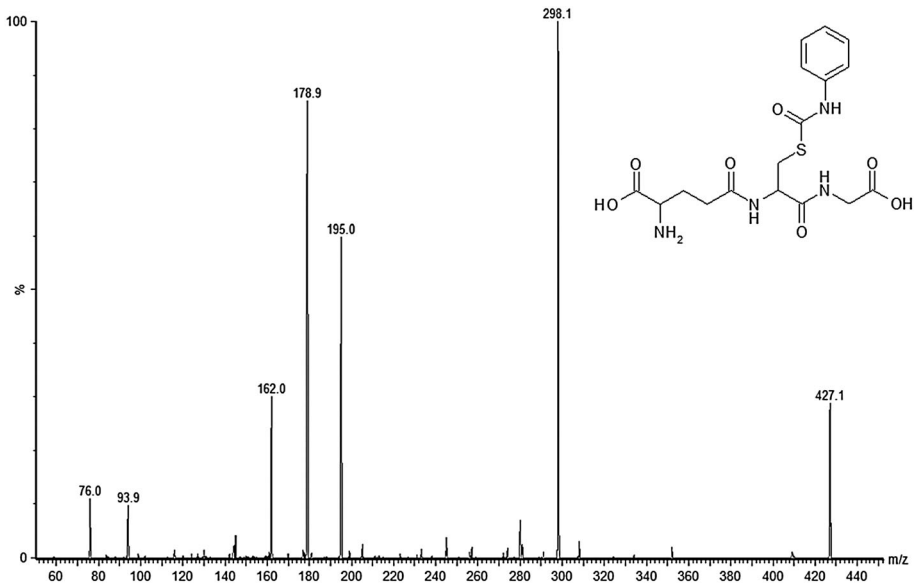

Figure 3. CID (CE 15 V) spectrum of the $[M+H]^+$ ion of the first eluting glutathione adduct **1** (*m/z* 427).

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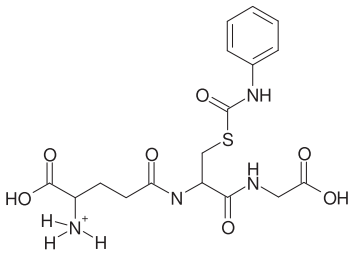
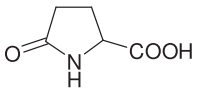
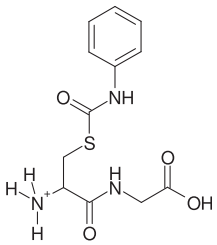
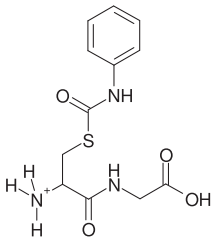
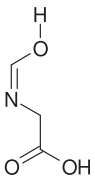
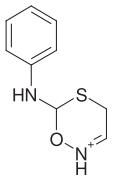
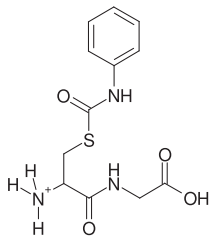
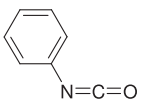
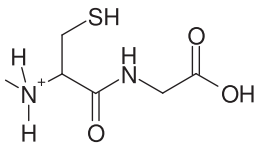
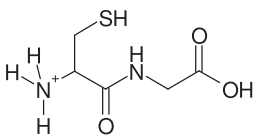
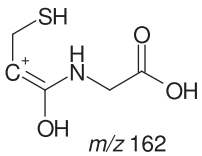
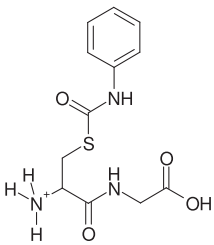
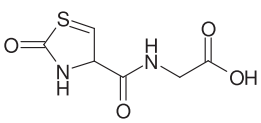
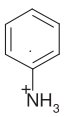
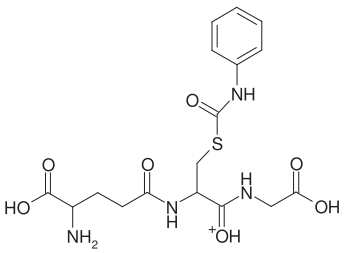
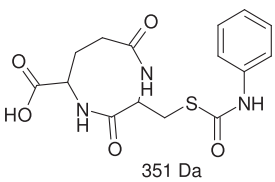
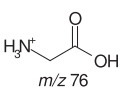
Table 1. Proposed precursor ions, neutral losses and fragment ions for the MS/MS fragmentations of adduct **1** (CE 15 V)

Precursor ion	Neutral loss	Fragment ion
 *m/z* 427	 129 Da	 *m/z* 298
 *m/z* 298	 103 Da	 *m/z* 195
 *m/z* 298	 119 Da	 *m/z* 179
 *m/z* 179	NH_3 17 Da	 *m/z* 162
 *m/z* 298	 204 Da	 *m/z* 94
 *m/z* 427	 351 Da	 *m/z* 76

together with the second y-ion at *m/z* 76 identified adduct **1** as the product arising from a nucleophilic attack of the cysteinyl thiol moiety of glutathione on phenyl isocyanate. The prominent *m/z* 298 ion corresponds to the fragmentation pattern of a short peptide, protonated at the α-amino terminal. In a singly charged, α-linkage peptide, the fragmentation is initiated by the proton transfer from the α-amino group to the C-terminal fragment with the release of a cyclic neutral loss.[13,14] For adduct **1**, the proton transfer to the nearby peptide nitrogen atom producing the *m/z* 298 y-ion will be energetically favourable. From the *m/z* 298 ion, two major fragments ions were formed: the *m/z* 179 ion by the subsequent loss of phenyl isocyanate and the *m/z* 195 fragment ion, presumably produced by a cyclization reaction producing an aldoxime ion (Scheme 1). The *m/z* 195 ion is characteristic of the adducts where phenyl isocyanate has been conjugated to the cysteine moiety of glutathione (adduct **1** and **3**).

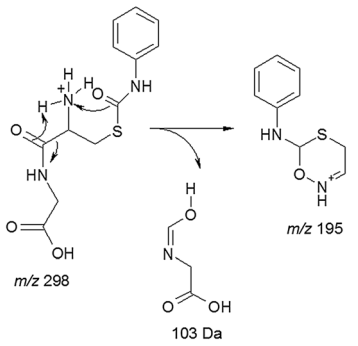
The product ion spectrum of the second adduct (*m/z* 427, $[M+H]^+$), which eluted after 1.58 min (**2**), is shown in Fig. 4. The product ion spectrum of adduct **2** contained more ions than the spectrum of adduct **1**, and y- and b-ion series with about equal abundances were observed. A peptide with multiple sites of equal basicity will produce a heterogeneous population of protonated species upon ionization promoting a high complexity of fragmentation after collision activation. Thus, the adduct was tentatively identified as the product arising from a nucleophilic attack of the basic glutamic acid α-amino moiety of glutathione on phenyl isocyanate to form a urea derivative. In agreement with the 'mobile proton theory', the conversion of the N-terminal to a non-basic urea group promotes the mobility of the proton in the single charged ion with a reduction of the activation energies for a number of fragmentation reactions along the peptide backbone.[15,16]

Proposed precursor ions, neutral losses and fragment ions are shown in Table 2.

The ions at *m/z* 76 and 179 and b-ion series containing the ions of *m/z* 352 and 249 confirmed a modification on the N-terminal of the molecule. The structure of b-ions has been shown to include the interaction of the C-terminal carbonyl with a carbonyl group of the polypeptide backbone creating an oxazolone structure.[17,18] In adduct **2**, the urea carbonyl group offers an alternative reaction site out of the polypeptide chain. The involvement of the urea carbonyl group in the formation of the *m/z* 352 b-ion was confirmed by the subsequent loss of aniline, thereby generating the base peak product ion of *m/z* 259 (Scheme 2). Fragmentation initiated by protonation of the urea group gave rise to the protonated aniline at *m/z* 94, loss of aniline at *m/z* 334 and the loss of phenyl isocyanate to reform charged GSH was detected as the ion *m/z* 308.

The third glutathione adduct (*m/z* 546, $[M+H]^+$), eluting at 2.41 min (**3**), corresponds to the reaction of two phenyl isocyanate molecules with one glutathione molecule and was tentatively identified as the product arising from a nucleophilic attack of both the cysteinyl thiol group and the glutamic α-amino moiety of glutathione. The product ion spectrum of adduct 3 is shown in Fig. 5; proposed precursor ions, neutral losses and fragment ions are shown in Table 3.

The product ion spectrum of the glutathione adduct **3** contained a mixture of specific fragments from both **1** and **2** and a number of new fragments in the high mass region. The major fragmentation reaction is proposed to be the formation of a cyclic b-type fragment ion, involving the carbonyl group of the urea moiety (*m/z* 471) followed by the loss of aniline (*m/z* 378). The mechanism is analogous to the reactions of adduct **2**



Scheme 1. Proposed formation of the fragment ion *m/z* 195.

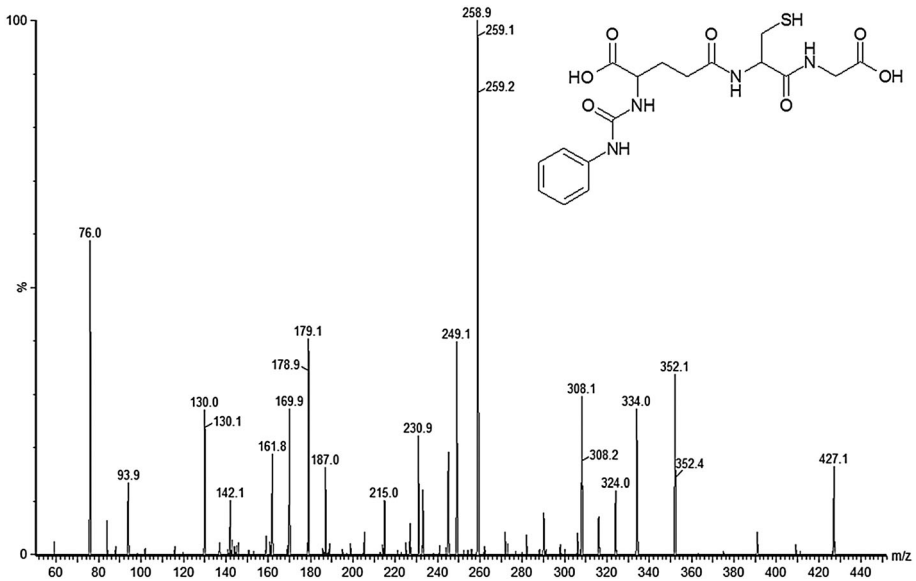


Figure 4. CID (CE 15 V) spectrum of the $[M+H]^+$ ion of the second eluting glutathione adduct **2** (*m/z* 427).

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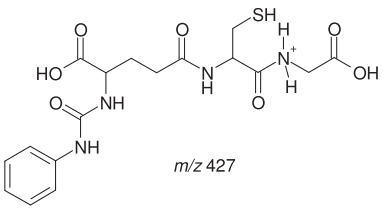
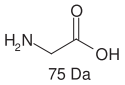
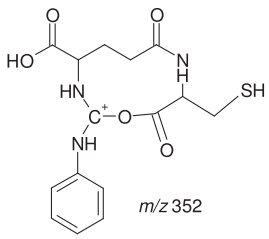
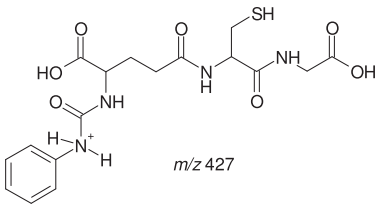
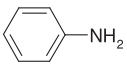
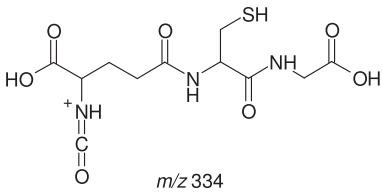
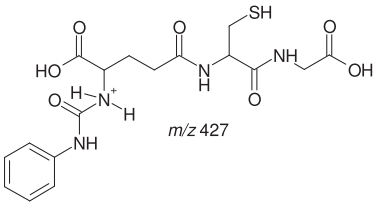
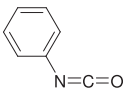
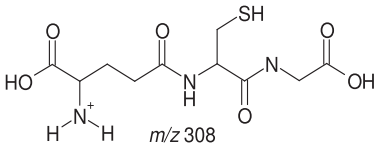
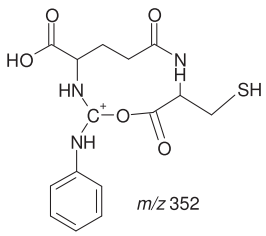
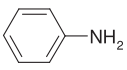
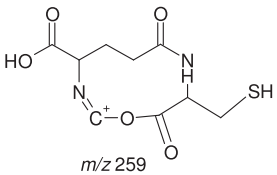
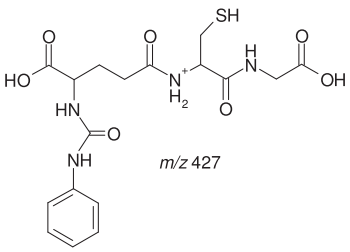
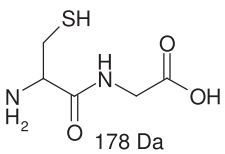
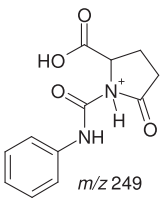
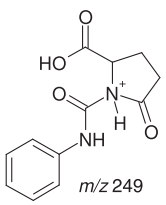
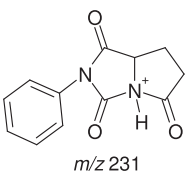
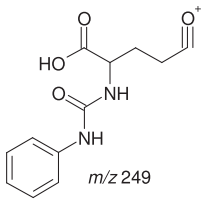
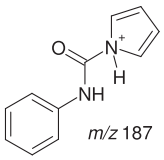
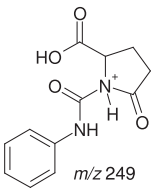
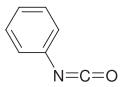
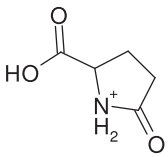
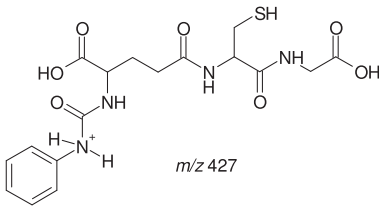
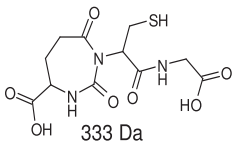
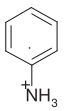
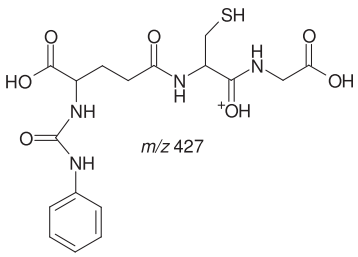
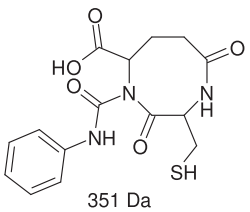
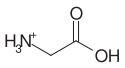
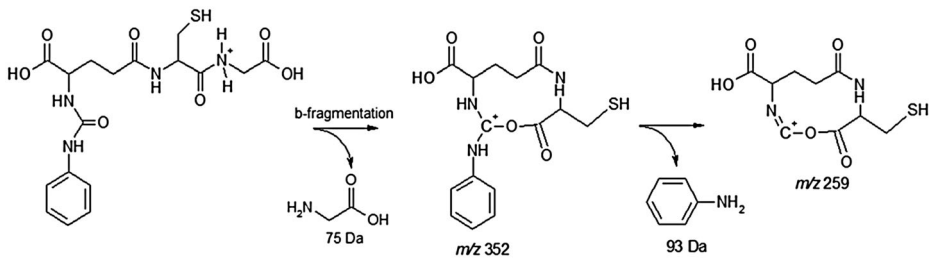
Table 2. Proposed precursor ions, neutral losses and fragment ions for the MS/MS fragmentations of adduct **2** (CE 15 V)

Precursor ion	Neutral loss	Fragment ion
 *m/z* 427	 75 Da	 *m/z* 352
 *m/z* 427	 93 Da	 *m/z* 334
 *m/z* 427	 119 Da	 *m/z* 308
 *m/z* 352	 93 Da	 *m/z* 259
 *m/z* 427	 178 Da	 *m/z* 249
 *m/z* 249	H_2O 18 Da	 *m/z* 231

(Continues)

Table 2. (Continued)

Precursor ion	Neutral loss	Fragment ion
 *m/z* 249	H_2O 18 Da CO_2 44 Da	 *m/z* 187
 *m/z* 249	 119 Da	 *m/z* 130
 *m/z* 427	 333 Da	 *m/z* 94
 *m/z* 427	 351 Da	 *m/z* 76



Scheme 2. Proposed formation of the fragment ions *m/z* 352 and *m/z* 259.

(Scheme 2). The fragmentation pattern of **3** also indicated the formation of an *m/z* 471 b-ion with a proposed oxazolone structure based on the loss of the phenyl thiocarbamide moiety producing the *m/z* 318 product ion. The subsequent loss of aniline from the *m/z* 318 ion, producing the *m/z* 225 ion, was best explained by the *m/z* 318 b-type ion involving the urea carbonyl group. A fragment was detected at *m/z* 427 resulting from loss of one phenyl isocyanate (119 Da) from the adduct. Since this fragment was significant for adduct **2** and not for adduct **1**, the fragment is proposed to be arising from the loss of the phenyl isocyanate that was bound to the glutamic acid α-amino moiety. The proposed structures of the glutathione adducts **1**, **2** and **3** are shown in Fig. 6.

The peak response areas of the extracted ion chromatograms for the glutathione adducts **1** and **3** did not differ significantly. However, the peak response area for adduct **2** was approximately three times smaller than those for **1** and **3**. Adducts **2** and **3**, with the N-terminal converted to phenyl urea groups, are likely to

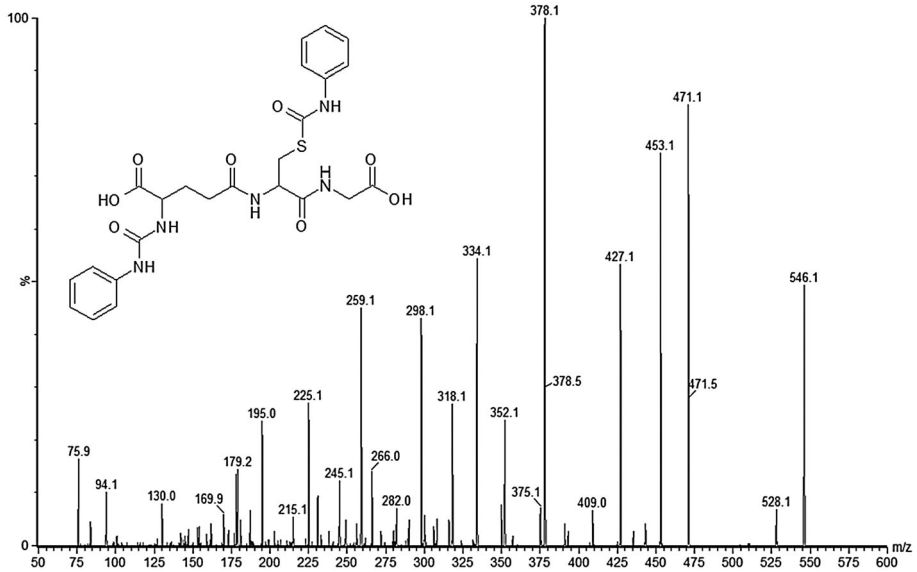


Figure 5. CID (CE 15 V) spectrum of the $[M+H]^+$ ion of the third eluting glutathione adduct **3** (*m/z* 546).

display a lower ionization efficiency based on the lower degree of protonation in solution. The peak response areas of the acetyl analogues of adduct **1** and **2** were determined in a response study and were found to differ by a factor of 2 (Supplemental data). Thus, the levels of adduct **2** and **3** relative to adduct **1** were underestimated based on the peak response.

The chromatogram of the full scan analysis also contained a species that eluted after 2.77 min and had an *m/z* of 213 (data not shown). This species is proposed to be 1,3-diphenyl-urea, formed by the hydrolysis of phenyl isocyanate (with the release of carbon dioxide) to produce aniline, followed by reaction with a second phenyl isocyanate molecule.[19] This suggestion is supported by its product ion spectrum (data not shown) containing fragments at *m/z* 120 (corresponding to a neutral loss of 93 Da to produce phenyl isocyanate), 94 and 77. The peak response area of the extracted ion chromatogram of 1,3-diphenyl-urea is approximately the same as that for glutathione adduct **2**. This is significant because phenyl isocyanate hydrolysis competes with glutathione adduct formation.

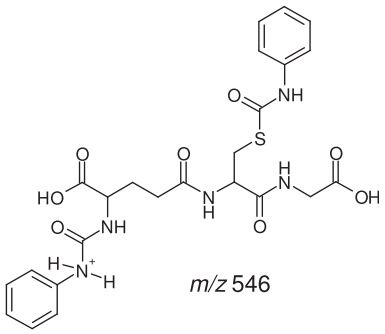
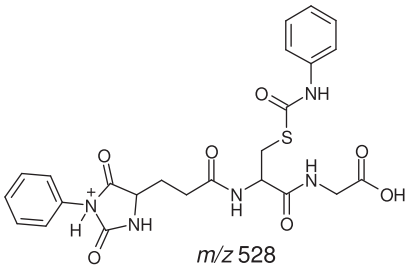
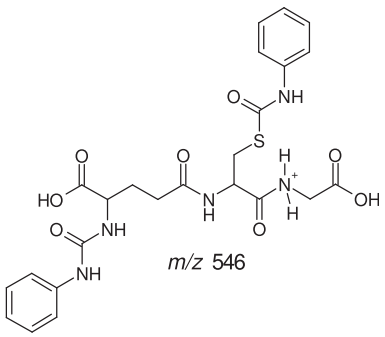
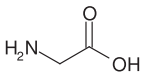
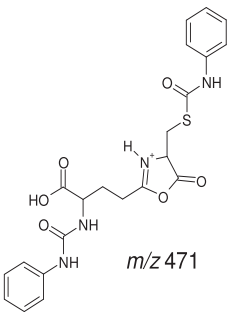
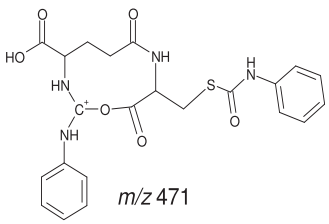
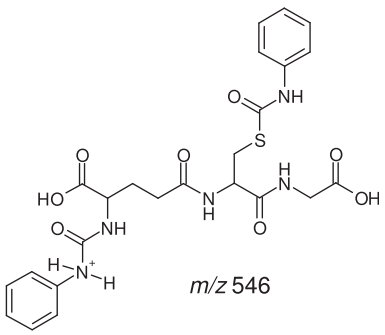
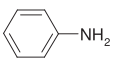
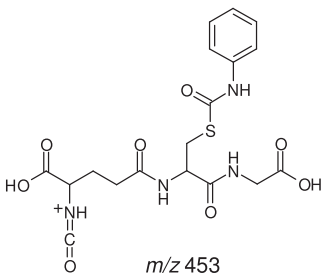
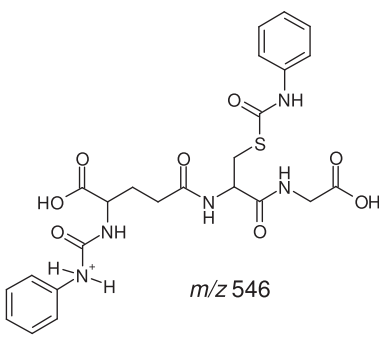
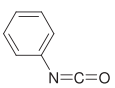
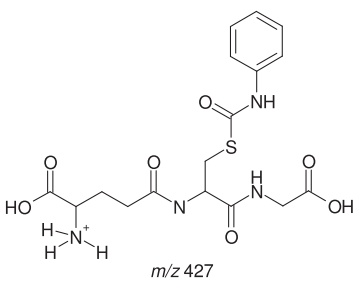
To verify its structure, the proposed glutathione adduct **3** was synthesized by organic synthesis. A higher shift ^{1}H NMR spectrum of the synthetic standard is shown in Fig. 7. The ^{1}H NMR spectrum indicates that the product is pure, having five signals representing a total of ten protons in the aromatic region. This indicates that two nonequivalent phenyl isocyanate groups were added to the GSH backbone. This information, together with an increased number of signals in the aromatic and carbonyl region of ^{13}C NMR, confirmed the proposed structure of the of the reference material. Additional NMR spectra and chemical shifts both for the synthetic standard of **3** and reduced glutathione are available in the supplemental data. The retention time of the glutathione conjugate (**3**) formed *in vitro* matched the data of the synthetic standard (EIC are available in the supplemental data). In addition, the product spectra of the adduct **3** and the synthetic standard were almost identical (supplemental data).

A time study of the reaction between phenyl isocyanate and glutathione was performed. A new UPLC method was developed to separate the three conjugates and the hydrolysis product within 1 min. The response (peak height) of the three glutathione conjugates and the hydrolysis product was measured during 60 min, detected every minute by UPLC-ESI/MS (data not shown). No significant difference in response was observed between the analysis performed after 1 min and the data acquired at the following time points.

Discussion

In a previous study by Fleischel *et al*., two glutathione conjugates resulting from reactions of phenyl isocyanate via the cysteinyl thiol group and the glutamic acid α-amino group were identified by NMR.[20] In this work, we have demonstrated that phenyl isocyanate forms three different adducts with glutathione. Adduct formation occurred via nucleophilic attacks involving either the cysteinyl thiol group or the glutamic acid α-amino group of GSH. In addition, one adduct was formed by reactions involving both of these reactive groups. The adducts were identified by UPLC-ESI/MS and the structure of the double adduct was confirmed by organic synthesis of a reference material with a structure confirmed by NMR. The CID MS/MS fragmentation of the three adduct ions were assessed and the three distinct patterns could be interpreted in terms of protonation sites and ion structures. The CID MS/MS spectra of adduct **1** and **2** were characterized by abundant y- and b-ions, respectively. In addition, a number of fragments produced by reactions in the phenyl urea and the phenyl thiocarbamide groups took place. For adduct **1**, the formation of the cyclic *m/z* 195 product ion provided a diagnostic transition specific for the phenyl thiocarbamide moiety. Another interesting example was the interactions of the urea carbonyl group of adduct **2** in the formation of the *m/z* 352 b-type fragment, stabilized by the high electron density in the urea group. The confirmation of this structure was the subsequent loss of an aniline group which would not take place from the oxazolone b-ion. The CID MS/MS spectrum of adduct **3** was complex and involved the b-type of peptide fragmentation and a high degree of adduct-specific fragments. Analogous to adduct **2**, the b-type fragmentation with a loss of glycine (*m/z* 471),

Table 3. Proposed precursor ions, neutral losses and fragment ions for the MS/MS fragmentations of adduct **3** (CE 15 V)

Precursor ion	Neutral loss	Fragment ion
 *m/z* 546	H_2O 18 Da	 *m/z* 528
 *m/z* 546	 75 Da	 *m/z* 471  *m/z* 471
 *m/z* 546	 93 Da	 *m/z* 453
 *m/z* 546	 119 Da	 *m/z* 427

(Continues)

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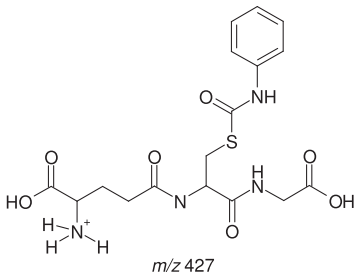
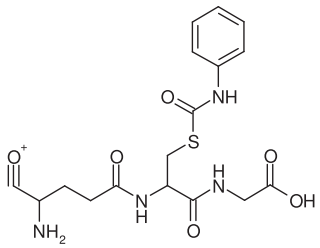
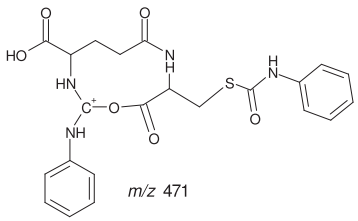
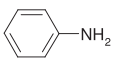
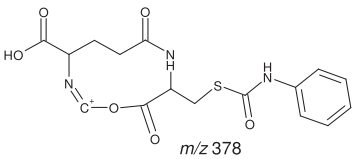
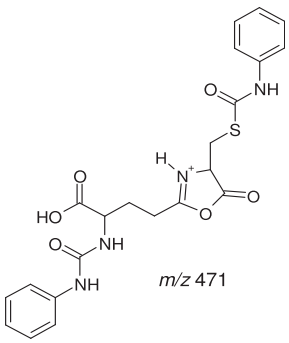
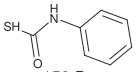
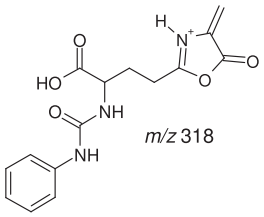
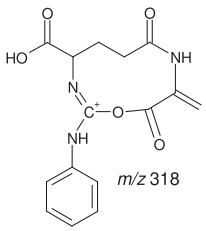
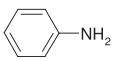
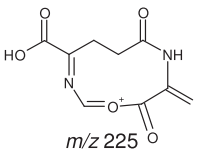
Table 3. (Continued)

Precursor ion	Neutral loss	Fragment ion
 *m/z* 427	H_2O 18 Da	 *m/z* 409
 *m/z* 471	 93 Da	 *m/z* 378
 *m/z* 471	 153 Da	 *m/z* 318
 *m/z* 318	 93 Da	 *m/z* 225

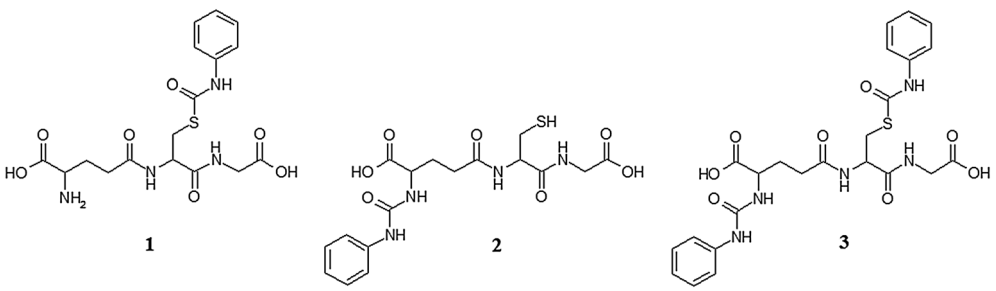


Figure 6. Proposed structures of the glutathione adduct **1** ($C_{17}H_{23}N_4O_7S$), **2** ($C_{17}H_{23}N_4O_7S$) and **3** ($C_{24}H_{28}N_5O_8S$). The protonated elemental compositions are shown in brackets.

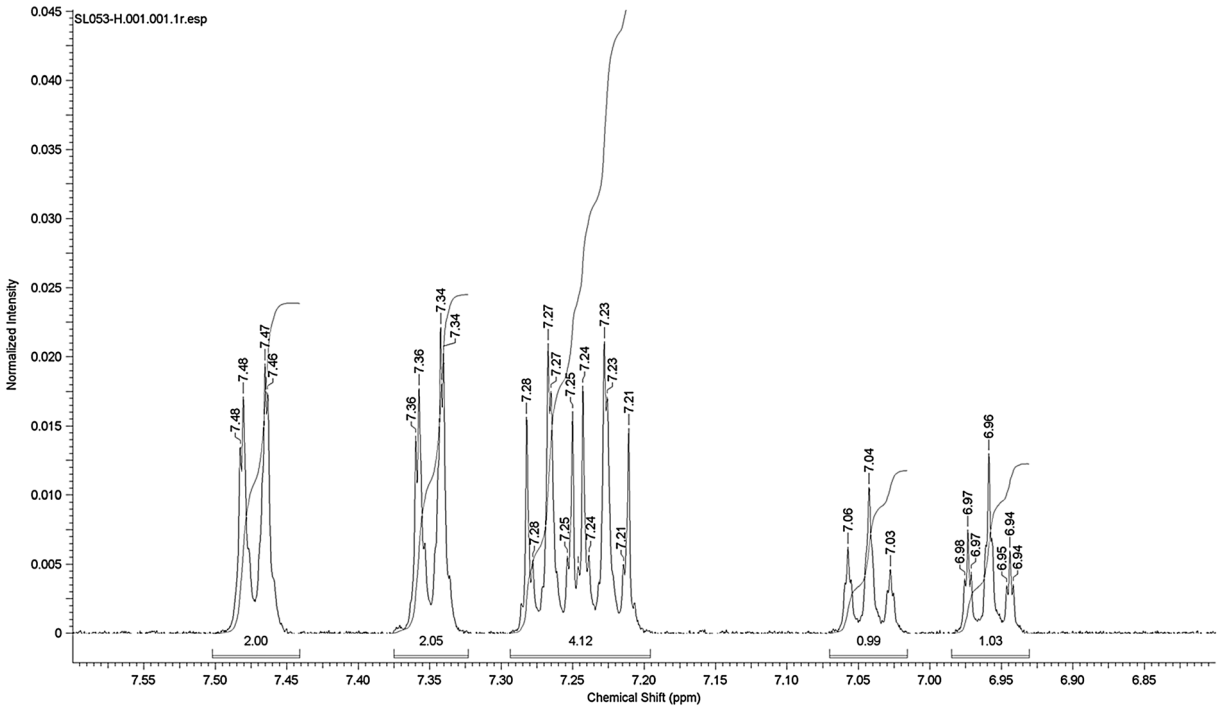


Figure 7. ^{1}H NMR of the synthetic standard of **3** showing the aromatic region. Since the NMR analysis was done with methanol-d_4 as solvent, the heteroatom protons are not visible.

followed by a loss of aniline (*m/z* 378), was detected in the spectra of adduct **3**. However, the formation of the *m/z* 318 fragment ion was proposed to take place by the loss of *N*-phenyl thiocarbamic acid (153 Da) from the proposed *m/z* 471 oxazolone b-ion. The data indicated that both of the two ion isomers are produced and a rapid conversion between the ion structures may occur. The isomerization of the structure element was supported by the subsequent fragmentation of the *m/z* 318 ion. The oxazolone ring is believed to open to allow the loss of CO from the b-ions and a weak *m/z* 290 ion was detected.[17] A recyclization of the open chain *m/z* 318 ion with the urea carbonyl group would finally explain the loss of aniline detected as the *m/z* 225 ion.

To our knowledge, no adducts resulting from conjugation at the glutamic α-amino group of glutathione have been identified using LC-MS. Instead, many publications describe adducts resulting from conjugation at the cysteinyl thiol group of glutathione. In order to detect glutathione conjugates, screening using neutral loss scanning of 129 Da (anhydroglutamic acid/pyroglutamic acid) in positive ion mode and negative precursor ion scanning at *m/z* 272 (deprotonated γ-glutamyl-dehydroalanyl-glycine) is used.[21,22] Once GSH adducts are discovered in the screening, product ion spectra are utilized to elucidate the structures. We conclude that for the adducts identified in this study, the product ion spectrum for the adduct resulting from conjugation at the thiol group of glutathione was less complex, and it was easier to elucidate the fragmentation of this adduct, compared to the product ion spectra for the double adduct and the adduct resulting from conjugation at the α-amino group of glutathione. This may be explained by the fact that adduct **2** turned out to be a peptide with multiple sites of equal basicity and will produce a number of protonated species with about equal abundances upon ionization promoting a high complexity of CID fragmentation. Also, for adduct **2**, the urea carbonyl group offers an alternative reaction site out of the polypeptide chain which increases the complexity of the fragmentation. Regarding the double adduct, the product ion spectrum of this adduct (**3**) contained a mixture of specific fragments from both adduct **1** and adduct **2**, and a number of new fragments in the high mass region.

The peak response areas for the glutathione adducts did not differ significantly, but the peak response area for adduct **2** was approximately three times smaller than those for **1** and **3**. The determination of response factors of the acetylated analogues of adduct **1** and **2** indicated that the difference in concentration probably is below a factor of 1.5. Adduct **3** is expected to have a response factor close to adduct **2** and based on the peak response area, this adduct was produced in the highest levels. Based on the peak response area of **3**, reaction of both the nucleophilic sites of glutathione with phenyl isocyanate is also proposed to occur to a significant extent. Conjugation between glutathione and phenyl isocyanate does not seem to hinder the subsequent nucleophilic attack of the initial adduct on a second phenyl isocyanate molecule. This is presumably because phenyl isocyanate is a highly reactive and relatively small molecule, and the first reaction between glutathione and phenyl isocyanate does not introduce enough steric hindrance to block the reaction with a second phenyl isocyanate molecule.

In the time study, no significant difference was found between the analysis performed after 1 min and the data acquired at following time points. Thus, the reactions of phenyl isocyanate and glutathione seem completed within 1 min and are too rapid to be monitored by the current technique. In an aqueous solution, phenyl isocyanate is prone to hydrolysis with the formation of 1,3-diphenylurea.[19] However, 1,3-diphenylurea was not detected as a dominant component in the conditions used in this study. As discussed in the introduction, phenyl isocyanate is used in the production of various materials. Consequently, there is a significant risk of extensive human exposure to this compound,

making it important to determine its reactivity towards potential targets in the body. This study describes a chemical, non-enzymatic reaction between glutathione and phenyl isocyanate at physiologically relevant conditions. In an *in vivo* situation, glutathione *S*-transferase enzymes will be present and promote the conjugation at the thiol group of glutathione. Therefore, further investigations will be required to determine the relevance of the GSH adducts identified in this work *in vivo*. It has been proposed that the reaction of GSH with isocyanates may be reversible.[23] If so, the electrophilic parent isocyanate could be released from the conjugate at some stage, enabling it to react with alternative nucleophilic functional groups such as those found in proteins and DNA. It would therefore be interesting to study the reactions of phenyl isocyanate with various biologically important macromolecules. Phenyl isocyanate reacted with both the cysteinyl thiol and the glutamic acid α-amine of glutathione. These results suggest that phenyl isocyanate may react with free cysteines, the α-amino group and also with lysine residues whose side chain contains a primary amine.

In conclusion, three different adducts formed by the reaction of glutathione with phenyl isocyanate have been identified. To our knowledge, these compounds have not previously been analyzed by mass spectrometry. Both the cysteinyl thiol group and the glutamic acid α-amino group of glutathione were found to attack phenyl isocyanate. To a significant extent, both of these reactive groups attacked phenyl isocyanate, forming the double adduct **3**. Conjugation occurred to a greater extent on the cysteinyl thiol moiety than on the glutamic acid α-amino group. NMR analysis confirmed the proposed structure of the glutathione adduct formed by conjugation with two phenyl isocyanate molecules. To our knowledge, this double adduct has not previously been described.

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