Synthesis, Characterization, and Solvolysis of Mono- and Bis-*S*-(glutathionyl) Adducts of Methylene-bis-(phenylisocyanate) (MDI)

Martin Reisser, Brigitte F. Schmidt, and William E. Brown*

Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, Pennsylvania 15213

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Bifunctional isocyanates are highly reactive compounds that undergo nucleophilic attack by a variety of functional groups available in the biological system. While the etiology of the respiratory disease caused by diisocyanates is not fully understood, a great deal of research has been performed to elucidate the chemical mechanisms involved in the direct and indirect effects of these compounds. Since adducts of isocyanates are found not only to proteins along the entire respiratory tree but also to proteins in the circulatory system, it is likely that a transport mechanism for the isocyanate from the respiratory to the circulatory system exists. The initial reaction of isocyanates with cellular thiols to form thiocarbamates, which are known to release the isocyanate under physiological conditions, is believed to provide a possible carrier mechanism for the isocyanate functional group. Previous work with aliphatic mono-isocyanates and the aromatic diisocyanate toluene diisocyanate has demonstrated the feasibility of this mechanism. Adding to this database, the products of the reaction of the highly water-insoluble, low vapor pressure, methylene-bis-(phenylisocyanate) (MDI) with glutathione were synthesized, and their chemical stability under various pH and buffer conditions was tested. Novel synthetic routes were developed for both the mono- and bis-*S*-(glutathionyl) adducts with MDI that yielded each compound in analytically pure form. Both compounds were found to be unstable under mild basic conditions (phosphate-buffered saline, pH 7.4, and $NaHCO_3$, pH 8.2), however to a different degree. Furthermore, a significant influence of the pH value (the rate of degradation increases with pH) and the concentration of free glutathione (increasing thiol stabilizes the adduct) on the stability was observed, indicating a base-catalyzed mechanism of the degradation/formation of the thiocarbamate bond. Unlike the monoadduct, which forms almost exclusively the polyurea upon degradation, a variety of products were formed upon degradation of the bis adduct. Though the disappearance of the bis adduct was complete as measured by HPLC, ^{1}H NMR spectra showed the existence of residual thiocarbamate bonds in the final mixture. In both cases, no evidence of the free methylene-bis-phenylamine (MDA) could be detected under the applicable conditions.

Introduction

Diisocyanates are highly reactive, bifunctional compounds used in the production of foams, coatings, and various plastic polymers. The isocyanate functional group is susceptible to nucleophilic attack at the carbonyl carbon by a number of nucleophiles among which are the hydroxyl, the amino, and the sulfhydryl groups that are reactive in biological systems at the physiological pH of 7.4 (*1*, *2*). The reaction of the isocyanate group with these functional groups also competes with its hydrolysis to the corresponding amine and the release of carbon dioxide. Of the types of bonds formed with the hydroxyl (a carbamate), the amino (a urea), and the sulfhydryl (a thiocarbamate) groups, the former two are essentially irreversible under physiological conditions while the latter thiocarbamate bond has been shown to be reversible under similar conditions (*3*−*6*).

Exposure to diisocyanates has been linked to a range of clinical outcomes such as nonspecific bronchial hyperresponsiveness (NSBH), reactive airways dysfunction syndrome (RADS), asthma, and alveolitis (hypersensitivity pneumonitis) (*7*). The mechanism for induction of these various outcomes has been attributed to immunological and pharmacological mechanisms; however, currently the precise mechanism or mechanisms are not known. What is known is that the highly reactive nature of the isocyanate functional group results in the modification of a variety of proteins accessible either directly or indirectly from the airways. These include albumin (*8*), laminin (*9*), keratin (*8*), tubulin (*10*), hemoglobin (*11*), and glutathione (*6*, *12*, *13*). Glutathione, a tripeptide of γ-glutamyl-cysteinyl-glycine, has been hypothesized as a molecule capable of 'capturing' the isocyanate through formation of a reversible thioester on the cysteine side chain and transporting the residue of isocyanate in an inert form to a distal location where it can reverse through the isocyanate functional group, which can subsequently react with a nucleophile in the vicinity (*13*). Formation of such exchangeable thioester conjugates between cysteine and methyl isocyanate (MIC) (*13*) and toluene diisocyanate (TDI) (*6*) has been demonstrated.

* Correspondence should be addressed to this author at the Department of Biological Sciences, Carnegie Mellon University, 4400 Fifth Ave., Pittsburgh, PA 15213.

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Each of the thioester conjugates thus far studied between glutathione and either MIC or TDI has demonstrated variable lifetimes under physiological conditions. To undergo significant exchange between the thioester conjugates and some distal 'target' molecule, the thioester must persist long enough to reach the 'target' molecule intact while passing through the environment of the lung and blood. The present study was undertaken to synthesize and characterize the conjugate between glutathione and another of the commercially used diisocyanates, 4,4′-methylene- bis-(phenylisocyanate) (MDI). Stability studies of the bis- and mono-adducts of glutathione with MDI were performed and their lifetimes compared to those reported for the TDI bis-glutathione adduct (*6*).

Materials

Chemical Synthesis. All reactions, except those involving water, were carried out in dried glassware under an argon atmosphere. Dimethyl sulfoxide (DMSO), dimethylformamide (DMF), and acetone (water content <0.005%) were purchased from Aldrich Chemical Co. Inc. (Milwaukee, WI). THF was distilled from Na/benzophenone ketyl prior to use. Column chromatography was performed using reversed phase material (STM Bulk-C18, Separation Methods Technologies Inc., Moorestown, NJ). NMR spectra were recorded on a Bruker AC 300 operating at 300.1 MHz for ^{1}H and at 75.5 MHz for ^{13}C. Chemical shifts are given relative to trimethylsilane (TMS) as an internal standard unless otherwise noted ($\delta_{TMS} = 0$ ppm). IR spectra were obtained on a ATI Mattson Infinity Series FTIR. MS were carried out on a Finnigan Mat LCQ. Melting points were determined on an Electrothermal Melting Point Apparatus and are uncorrected. Elemental analyses were carried out by Atlantic Microlab Inc. (Norcross, GA). MDI was a gift of Bayer Corp. (Pittsburgh, PA).

Methylene-bis-(*S*-{[(4-phenyl)amino]carbonyl}glutathione) (3). GSH (1.23 g; 4 mmol) (dried 24 h at 100 °C/0.1 mbar prior to use) was dissolved in 25 mL of DMSO. The solution was diluted with 25 mL of DMF and 25 mL of THF, and then brought to −25 °C in a methanol/dry ice bath. At this temperature, a solution of 0.25 g (1 mmol) of MDI in 2 mL of THF was added under vigorous stirring. The mixture was held at this temperature for 1 h, and then allowed to come to room temperature within 6 h. After most of the THF was evaporated at 15 mbar, the remaining solution was poured into 1.5 L of water (pH 3, HCl) and the product allowed to precipitate. The precipitate was collected on a Büchner funnel, washed with water, and dried at 0.1 mbar. A total of 675 mg (0.78 mmol, 78%) of compound **3** was obtained as an amorphous colorless solid in 90−95% purity. Further purification was achieved by column chromatography [RP-18, 250 g, solvent: H_2O/CH_3CN/trifluoroacetic acid (TFA) 80:20:0.1, 200 mL, then 70:30:0.1, 500 mL]. IR (KBr): 3365, 3349 (s, br), 3061 (w), 1678 (s), 1658 (s), 1649 (s), 1517 (s), 1410 (m), 1307 (m), 1233 (m) cm^{-1}. ^{1}H NMR (DMSO-d_6, 300.13 MHz): 2.05 (m_c, 4 H, CH_2−CH_2−CH), 2.35 (m_c, 4 H, CH$_2$−CH_2−CH), 3.07 (dd, 2 H, ^2J = 13.9 Hz, ^3J = 9.5 Hz, S−C*H*H), 3.35 (dd, 2 H, ^2J = 13.9 Hz, ^3J = 5.1 Hz, S−C*H*H), 3.77 (d, 4 H, ^3J = 5.9 Hz, CO−CH_2−NH), 3.83 (s, 2 H, Ar−CH_2−Ar), 3.93 (m_c, 2 H, CH$_2$−CH$_2$−C*H*), 4.51 (m_c, 2 H, S−CH$_2$−C*H*). ^{13}C NMR (DMSO-d_6): 26.6, 31.4, 31.9, 40.7, 41.5, 52.5, 53.4 (13 C-aliphatic), 120.8, 129.6, 137.3, 137.7 (12 C-aryl), 165.0 (2 NH−*C*O−S), 171.1, 171.6, 171.7, 172.1 (4 NH−*C*O, 4 *C*OOH) ppm. MS (EIS) *m/z* (%) = (865.9, MH$^+$). $C_{35}H_{44}N_8O_{14}S_2$ calcd for $C_{35}H_{44}N_8O_{14}S_2 \cdot H_2O$: C 46.66, H 5.37, N12.44 (864.90); found C 47.00, H 5.26, N 12.44.

4-{4′-[(*tert*-Butyloxycarbonyl)amino]benzyl}benzoic Acid (5). 4-(4′-Aminobenzyl)benzoic acid (**4**) (23.82 g; 0.1 mol) was dissolved in 800 mL of a water/dioxane (1:1) mixture which contained 4.5 g of NaOH. To this solution was added 25 g/26.5 mL (0.115 mol) of di-*tert*-butyl dicarbonate (Boc_2O) at room temperature. After stirring for 5 h, additional Boc_2O (4 mL) was added, and stirring was continued for 12 h. The solution was finally acidified with HCl to pH 2, and, after most of the dioxane was evaporated, the product was collected on a Büchner funnel. Recrystallization from EtOH yielded 25.66 g (0.078 mol, 78%) of 4-[4′-(*tert*-butyloxycarbonyl)aminobenzyl]benzoic acid (**5**) as a pale yellow powder; mp decomposition from 180 °C on. IR (KBr): 3327 (s), 2978 (w), 1699 (vs), 1526 (s), 1412 (m), 1161 (s) cm^{-1}. ^{1}H NMR (DMSO-d_6, 300.13 MHz): 1.46 (s, 9 H, C(CH_3)$_3$), 3.93 (s, 2 H, CH$_2$), 7.11 (d, 2 H, ^3J = 8.1 Hz), 7.30 (d, 2 H, ^3J = 8.1 Hz), 7.38 (d, 2 H, ^3J = 8.1 Hz), 7.86 (d, 2 H, ^3J = 8.1 Hz), 9.21 (s, 1 H, Ar−N*H*−Boc) ppm. $C_{19}H_{21}NO_4$: calcd C 69.71, H 6.47, N 4.28 (327.38); found C 69.99, H 6.63, N 4.35.

4-{4′-[(*tert*-Butyloxycarbonyl)amino]benzyl}phenylisocyanate (6). A solution from 24.32 g (0.074 mol) of compound **5** and 8.27 g/11.4 mL (0.081 mol) of triethylamine in 300 mL of dry acetone was brought to 0 °C. At this temperature, 8.87 g/7.8 mL (0.081 mol) of ethyl chloroformate in 30 mL of acetone was slowly added. The solution was kept at this temperature for 2 h; then 6.5 g (0.1 mol) of sodium azide in 20 mL of water was added. Stirring was continued for 1 h at 0 °C. After pouring the solution into 800 mL of ice/water, the formed azide was extracted with toluene (3 × 150 mL), and the toluene layer was washed with water (3 × 100 mL) and dried over $MgSO_4$ at −10 °C for 48 h. The drying agent was removed, and the solution of the azide was slowly heated to 100 °C in a water bath. After 1 h at this temperature, the evolution of nitrogen was finished, and the solvent was evaporated at 15 mbar. The residue was recrystallized from hexane/toluene (3:1); yield 17.3 g (0.053 mol, 72%) of isocyanate **6** obtained as pale yellow crystals; mp 122−123 °C. IR (KBr): 3387 (s), 2979 (w), 2279 (vs, br), 1594 (s), 1519 (s), 1412 (m), 1234 (m), 1157 (s) cm^{-1}. ^{1}H NMR (DMSO-d_6, 300.13 MHz): 1.47 (s, 9 H, C(CH_3)$_3$), 3.83 (s, 2 H, CH$_2$), 7.0−7.4 (m, 8 H-aryl), 9.18 (s, 1 H, N*H*-Boc) ppm. ^{13}C NMR ($CDCl_3$, 125.77 MHz, ($CDCl_3$) = 77.0): 28.3 (C−(*C*H$_3$)$_3$), 40.6 (*C*−(CH$_3$)$_3$), 80.4 (CH$_2$), 118.8, 124.6, 129.3, 129.9, 131.2, 135.2, 136.6, 139.0 (12 C-aryl), 152.8 (C═O) ppm. The NCO signal was not visible. $C_{19}H_{20}N_2O_3$: calcd C 70.35, H 6.21, N 8.64 (324.38); found C 70.45, H 6.22, N 8.64.

***N*-(*tert*-Butyloxycarbonyl)-*S*-{[({4-[4′-(*tert*-butyloxycarbonyl)-amino]benzyl}phenyl)amino]carbonyl}glutathione (8).** To a suspension of 1.02 g (2.5 mmol) of *N*-*tert*-butyloxycarbonyl (Boc)-protected GSH **7** (dried 24 h at 100 °C/0.1 mbar prior to use) in 20 mL of acetone was added 0.81 g (2.5 mmol) of the Boc-protected MDI derivative **6** at room temperature. After 30 min, a clear solution was obtained. Stirring was continued for 12 h after which TLC (silica gel, solvent: *n*-BuOH/H_2O/CH_3-COOH 3:1:1) showed complete consumption of the starting material. The acetone was then removed at 15 mbar, and the resulting colorless solid was purified by column chromatography (RP-18, 250 g, H_2O/CH_3CN/TFA 60:40:0.01, 200 mL, then 50:50:0.01, 500 mL). Yield: 1.83 g (1.72 mmol, 69%) of compound **8**; mp 130 °C (dec). IR (KBr): 3500−3200 (m, br), 1695 (s), 1681 (s), 1667 (s), 1522 (s), 1516 (s), 1411 (m), 1245 (m), 1162 (s) cm^{-1}. ^{1}H NMR (DMSO-d_6, 300.13 MHz): 1.37 (s, 9 H, C−(CH_3)$_3$), 1.46 (s, 9 H, ArNH(CO)OC−(*CH*$_3$)$_3$), 1.81, 1.93 (m_c, 2 H, CH$_2$−*CH*$_2$−CH), 2.22 (m_c, 2 H, *CH*$_2$−CH$_2$−CH), 3.01 (dd, 1 H, ^2J = 13.9 Hz, ^3J = 8.8 Hz), 3.34 (dd, 1 H, ^2J = 13.9 Hz, ^3J = 5.1 Hz), 3.76 (d, 2 H, ^3J = 6.6 Hz, CO−*CH*$_2$−NH), 3.80 (s, 2 H, Ar−*CH*$_2$−Ar), 3.90 (m_c, 1 H, CH$_2$−CH$_2$−*CH*), 4.48 (m_c, 1 H, S−CH$_2$−*CH*), 7.06 (d, 2 H$_{Aryl}$, ^3J = 8.1 Hz), 7.10 (d, 2 H$_{Aryl}$, ^3J = 8.1 Hz), 7.34 (d, 2 H$_{Aryl}$, ^3J = 8.1 Hz), 7.39 (d, 2 H$_{Aryl}$, ^3J = 8.1 Hz), 6.9−7.1 (1 H, *NH*−Boc), 8.09 (d, 1 H, ^3J = 8.1 Hz, SCH$_2$CH−N*H*), 8.16 (t, 1 H, ^3J = 6.6 Hz, HOOCCH$_2$−N*H*), 9.14 (s, 1 H, Ar−N*H*−Boc), 10.17 (s, 1 H, N*H*−CO−S) ppm. ^{13}C NMR (DMSO-d_6): 26.7, 28.0 (C(*C*H$_3$)$_3$), 28.1 (C(*C*H$_3$)$_3$), 31.2, 31.8, 40.7, 52.2, 53.0, 78.0 (*C*(CH$_3$)$_3$), 78.7 (*C*(CH$_3$)$_3$), 118.2, 119.0, 128.7, 128.8, 134.8, 136.6, 136.7, 137.4 (12 C-aryl), 152.7 (*C*O-*t*Bu), 155.5 (*C*O-*t*Bu), 164.0 (NH-*C*O−S), 170.2, 170.8, 171.6, 173.7 (2 *C*OOH, 2 NH-*C*O) ppm, Ar−*C*H$_2$−Ar overlapped by DMSO-d_6. $C_{34}H_{45}N_5O_{11}S$ calcd for $C_{34}H_{45}N_5O_{11}S \cdot H_2O$: C 54.46, H 6.32, N 9.34 (731.81); found C 54.65, H 6.29, N 9.32.

***S*-({[4-(4′-Aminobenzyl)phenyl]amino}carbonyl)-glutathione (9).** Bis-Boc protected monoadduct **8** (50 mg) was dissolved in 0.5 mL of TFA at room temperature. After 15 min, the excess TFA was removed at 0.1 mbar. Compound **9** was used as obtained. IR (KBr): 3380, 3354 (m, br), 3069 (w), 2958 (w), 1652 (sh, vs), 1529 (s), 1513 (s), 1411 (m), 1308 (m), 1234 (m), 1200 (s), 1141 (m) cm^{-1}. ^1H NMR (DMSO-d_6, 300.13 MHz): 2.14 (m_c, 2 H, CH_2−CH_2−CH), 2.46 (m_c, 2 H, CH_2−CH_2−CH), 3.12 (dd, 1 H, ^2J = 13.9 Hz, ^3J = 8.8 Hz, S−C*H*H), 3.45 (dd, 1 H, ^2J = 13.9 Hz, ^3J = 5.0 Hz, S−C*H*H), 3.85 (d, 2 H, ^3J = 5.7 Hz, CO−CH_2−NH), 3.94 (s, 2 H, Ar−CH_2−Ar), 3.98 (m_c, 1 H, CH_2−CH_2−*CH*), 4.61 (m_c, 1 H, S−CH_2−*CH*), 7.18 (d, 2 H-aryl, ^3J = 8.5 Hz), 7.34 (s, 4 H-aryl), 7.49 (d, 2 H-aryl, ^3J = 8.5 Hz), 8.2−8.4 (m, 2 H, N*H*−CO), 10.28 (s, 1 H, N*H*−CO−S) ppm. ^{13}C NMR (DMSO-d_6): 26.6, 31.4, 31.9, 40.6, 41.5, 52.5, 53.5 (7 C-aliphatic), 120.1, 124.0, 129.7, 130.0, 130.5, 136.6, 137.9, 142.7 (12 C-aryl), 165.1 (NH−*C*O−S), 171.1, 171.6, 171.7, 172.2 (2 *C*OOH, 2 NH−*C*O) ppm. MS (EIS) *m*/*z* (%) = (532.1, MH^+). $C_{24}H_{29}N_5O_7S$ (531.5).

Bis-4-{[4′-(*tert*-butyloxycarbonyl)amino]benzyl}phenyl Urea (10). To a solution of 1.62 g (5 mmol) of the isocyanate **6** in 20 mL of acetonitrile was given 1 mL of water. Stirring was continued for 12 h at 20 °C. Then the solution was diluted with 20 mL of water and the precipitate isolated on a Büchner funnel. The product was recrystallized from methanol. Yield: 1.39 g (2.2 mmol, 86%) of a colorless powder; mp: decomposition from 190 °C on. IR (KBr): 3325 (s), 2978 (m), 1698 (s), 1594 (m), 1505 (s), 1411 (m), 1313 (m), 1237 (s), 1162 (s) cm^{-1}. ^1H NMR (DMSO-d_6, 300.13 MHz): 1.46 (s, 18 H, $C(CH_3)_3$), 3.78 (s, 4 H, CH_2), 7.0−7.1 (m, 8 H-aryl), 7.3−7.4 (m, 8 H-aryl), 8.47 (s, 2 H, N*H*−Boc), 9.14 (2 H, N*H*−Ar) ppm. ^{13}C NMR (DMSO-d_6): 28.0 (CH_3), 78.7 ($C(CH_3)_3$), 118.2, 128.6, 128.7, 134.8, 135.1, 137.3, 137.5 (24 C-aryl), 152.5 (NH−CO−NH), 152.7 (NH−CO-*t*-Bu) ppm. $C_{37}H_{42}N_4O_5$: calcd C 71.36, H 6.80, N 9.00 (622.76); found C 71.54, H 6.83, N 9.01.

HPLC analyses were performed on a Beckman System Gold equipped with a Phenomenex Bondclone C-18 300 × 3.9 mm column. Effluents were monitored at 254 and 215 nm. A 1 mL/min flow rate of the following mobile phase was used: solvent A, H_2O containing 0.1% (v/v) CF_3COOH; solvent B, CH_3CN/H_2O mixture (80/20, v/v) containing 0.1% (v/v) CF_3COOH. 0−2 min 100% A; 2−21 min linear change to 100% B starting with 30% B; 21−25 min 100% A.

Solvolysis Studies. Compounds **3**, **8**, and **9** (5×10^{-3}mmol each) were dissolved separately in 10 mL (resulting concentration 0.5 mmol/L) of one or more of the following solutions: PBS (0.2 mol/L, pH 6.5 or 7.4), $NaHCO_3$ solution (0.1 mol/L, pH 8.2), or $NaHCO_3$/glutathione solution (0.1 mol/L $NaHCO_3$, 10 mmol/L glutathione, pH 8.2), and held at a constant temperature of 25 °C. At selected time points, samples were taken and immediately frozen in liquid nitrogen until analyzed. A 20 μL aliquot of each sample was analyzed by HPLC. The concentration of each compound was determined by comparison of the integral of its chromatographic peak with the integral of this peak at point zero (c = 0.5 mmol/L). Rate constants and half-times were calculated according to standard procedures (*14*).

Reaction of MDI with GSH under Quasi-physiological Conditions. GSH (10 mmol) and $NaHCO_3$ (20 mmol) were dissolved in either 200 mL of water (C_{GSH} = 50 mmol/L, pH 7.0) or 200 mL of 0.1 M $NaHCO_3$ solution (C_{GSH} = 50 mmol/L, pH 8.2). After the addition of 25 mmol of MDI in 100 mL of toluene (5-fold excess), the solutions were vigorously stirred with a stirring bar. Samples of the aqueous phase were taken after 2, 5, and 24 h, diluted 1:100 with water, and analyzed by HPLC. The formation of the GSH−MDI bis adduct could be shown qualitatively by comparison of the resulting HPLC chromatograms with that of an authentic sample of compound **3**.

Results

Chemical Synthesis. Numerous instructions for the reaction of thiols with isocyanates are given in the literature, e.g., reactions in organic solvents with (*5*) or without (*15*) catalysts (tertiary amines), reactions in water at various pH values and temperatures (*16*, *17*), or reaction in water/solvent mixtures (*13*). In the specific case of GSH (**1**) and MDI (**2**), none of these conditions was found to be satisfying, either due to the sensitivity of MDI and the formed thiocarbamate toward water, or due to the nonsolubility of GSH in most organic solvents. For example, no reaction could be observed in solvents such as acetone, acetonitrile, or DMF due to the nonsolubility of GSH.

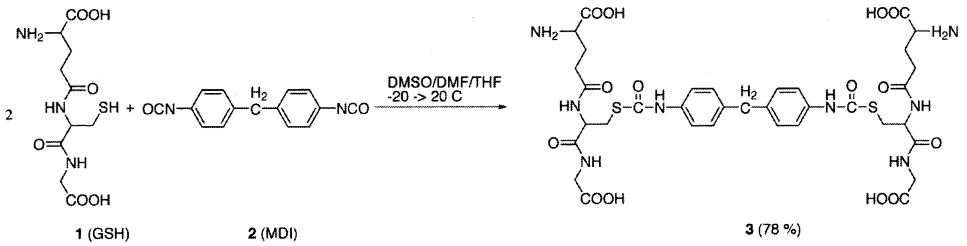
Reaction in hydrogen carbonate solution according to a protocol given by Day et al. for TDI (*6*) resulted in the degradation of the desired thiocarbamate, although it was initially formed under these conditions. Very recently the synthesis of the bis adduct of MDI **3** in an acetonitrile/water mixture has been published (*18*). The characterization of the compound was done by solid-state carbon NMR. The purity of the product was not given. In our hands, however, this methodology yields, in addition to the desired product, several other compounds as shown by HPLC and proton NMR spectroscopy. Since these byproducts have almost identical ^1H NMR spectra to the bis adduct, except for the aromatic region, it is most likely that di- and oligomeres resulting from the partial hydrolysis of MDI as well as monoadduct **9** are formed.

To circumvent these problems, an anhydrous approach was undertaken. DMSO is one of the very few organic solvents that is able to solubilize GSH. DMSO, however, catalyzes the reaction of nucleophiles with isocyanates (*19*); therefore, the reaction of GSH with MDI in this solvent at room-temperature involves both nucleophilic groups of GSH (the thiol and the amino groups); thus, thiocarbamate and urea bonds are formed. In principle, this problem could be prevented by a protective group strategy (*13*). This, however, leads to a complicated synthesis for a relativly simple molecule. Fortunately, kinetic control (low temperature, excess GSH) allows specific reaction with the thiol group with little interference from the amino group. A solvent mixture of DMSO/DMF/THF, keeping GSH in solution and preventing DMSO from freezing at the reaction temperature of −25 °C, was found to be suitable for this purpose (Scheme 1). The product obtained under these conditions was estimated by ^1H NMR spectroscopy to be 90−95% pure, and could be further purified by column chromatography. Compound **3** is practically insoluble in water alone or organic solvents (except DMSO); however, it is soluble in buffer solutions at pH values >6 (PBS, HCO_3^-) and water/acetonitrile/TFA mixtures.

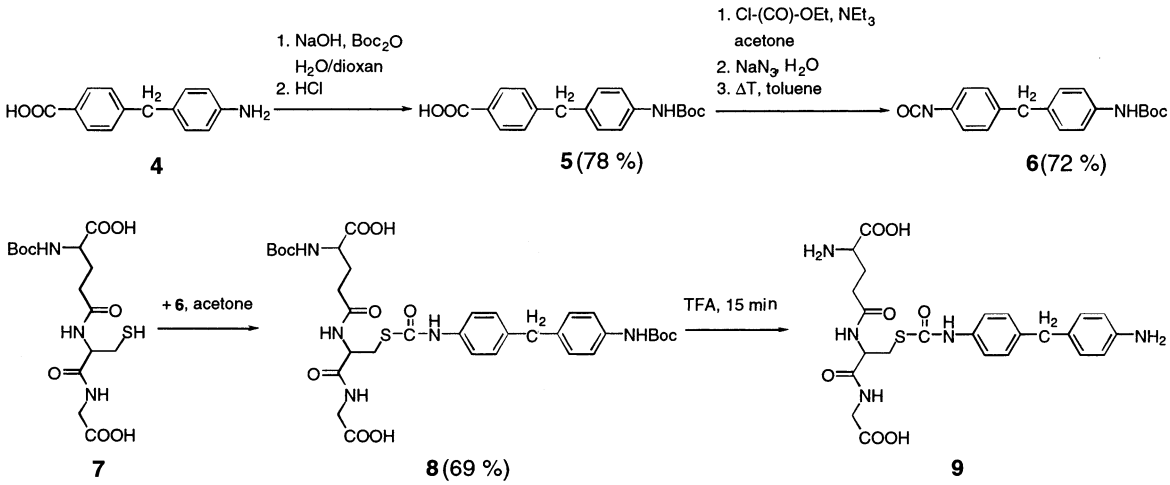
Monoadduct. The preparation of this compound was achieved by the reaction of N-Boc-protected GSH **7** (*20*) with the mono-Boc-protected MDI derivative **6**. The Boc-protected MDI derivative **6** was prepared starting with the literature known amino acid **4** (*21*). Reaction with excess Boc_2O under standard conditions resulted in good yields of compound **5**, which was converted in a straightforward manner to the isocyanate **6** under Curtius conditions (*22*). The Boc-protected monoadduct **8** was then obtained by direct reaction of the isocyanate **6** with **7** in acetone. Dissolution of this compound in TFA resulted in the removal of the protective groups and yielded the desired monoadduct **9** after evaporation of excess TFA (Scheme 2).

Solvolysis Studies. All investigated thiocarbamates were found to degradate upon dissolution in PBS (pH 6.5

Scheme 1. Synthesis of Bis Adduct 3 from Glutathione (1) and 4,4′-Methylene-bis-(phenylisocyanate) (2)



Scheme 2. Synthesis of Monoadducts 8 and 9



and 7.4) or hydrogen carbonate solution (pH 8.2). Each reaction follows pseudo-first-order kinetics. For the disappearance of the bis adduct **3** at 25 °C at a physiological pH of 7.4 (PBS), a $k_{app,unimolecular}$ of 2×10^{-4} s^{-1} and a half-life of $t_{(1/2)} = 59$ min were determined. This shows compound **3** to be more labile than the literature known bis adducts of glutathione with 2,4-TDI [$k_{app,unimolecular} = 4.7 \times 10^{-5}$ s^{-1}, $t_{(1/2)} = 245$ min] and 2,6-TDI [$k_{app,unimolecular} = 4.7 \times 10^{-6}$ s^{-1}, $t_{(1/2)} = 2460$ min] under the same conditions (*6*). A marked increase in reaction rate was observed in $NaHCO_3^-$ solution (pH 8.2), leading to a $k_{app,unimolecular} = 1.96 \times 10^{-3}$ s^{-1} and a half-life of only 5.9 min. By comparison, at pH 6.5 (PBS) the disappearance of compound **3** was significantly slower with a half-life of 440 min ($k_{app,unimolecular} = 2.6 \times 10^{-5}$ s^{-1}). The kinetics of these reactions are depicted in Figure 1. Despite its lability at elevated pH values, compound **3** could be stabilized strongly by the addition of glutathione to the solution. A concentration of 10 mmol/L GSH, which can be reached in the human epithelial lining fluid, for example (*23*), was found to increase the half-life in hydrogen carbonate solution (pH 8.2, 25 °C) by a factor of 34 compared to the degradation in hydrogen carbonate solution only. Thus, under these conditions $k_{app,unimolecular}$ was determined to be 5.8×10^{-5} s^{-1} [$t_{(1/2)} = 200$ min]. The kinetics of this reaction are shown in Figure 1. In each of the above-described cases, several unstable intermediates were observed during the course of the reaction. Though not investigated in detail, the monoadduct **11** as the initial degradation product of **3** and the amine monoadduct **9** as the hydrolysis product of **11** could be identified among them. The monoadduct **11** appears as a steady-state intermediate that disappears when the bis adduct disappears. It has to be noted that the concentration of **9** was found to be dependent on the pH value. Based on the assumption of equal absorbance of **3**

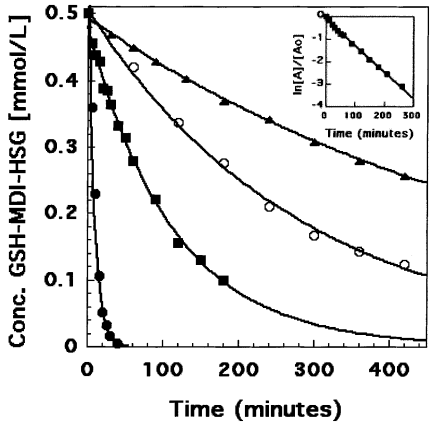


Figure 1. Time dependencies of the degradation of bis adduct **3** in PBS at pH values of 6.5 (filled triangles) and 7.4 (filled squares), hydrogen carbonate at pH 8.2 (filled circles), and hydrogen carbonate (0.1 mol/L, pH 8.2) containing GSH (10 mmol/L)(open circles). $T = 25$ °C. The inset provides the log relationship for the degradation of the bis adduct at pH 7.4.

and **9**, the maximal concentration of **9** was determined to be 3×10^{-5} mol/L at pH 6.5 (i.e., 6% of **3**) and 1×10^{-5} mol/L at pH 7.4 (i.e., 2% of **3**). No free methylene-bis-(phenylamine) (MDA) was detectable. Figure 2 shows an example chromatogram of the degradation of **3** in PBS, taken after 180 min.

The monoadducts **8** and **9** were found to be significantly more stable than the bis adduct **3**. For the unprotected monoadduct **9** in PBS (pH 7.4) at 25 °C, a $k_{app,unimolecular}$ of 2.46×10^{-5} s^{-1} and a half-life $t_{(1/2)} = 470$ min were determined. This is very similar to the values found for the Boc-protected monoadduct **8** which are $k_{app,unimolecular} = 3.45 \times 10^{-5}$ s^{-1} and $t_{(1/2)} = 335$ min (Figure 3). In both cases, as the exclusive product a colorless precipitate was formed during the reaction. The degrada-

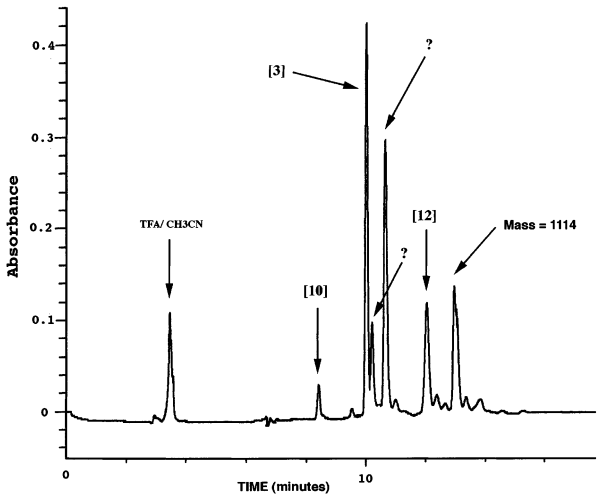


Figure 2. Example HPLC chromatogram of the degradation of bis adduct **3** in PBS, pH 7.4. The sample was taken after 180 min. Peaks are labeled according to Schemes 1 and 2.

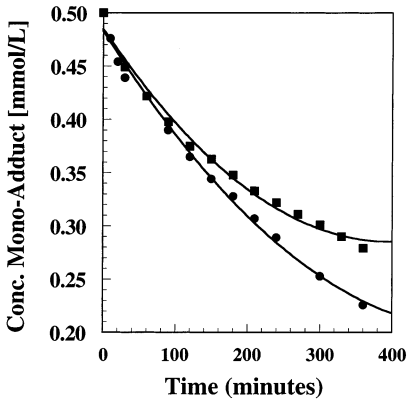
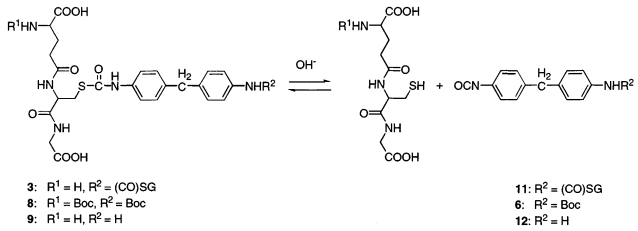


Figure 3. Time dependencies of the degradation of the monoadducts **8** (circles) and **9** (squares) in PBS (pH 7.4) at 25 °C.

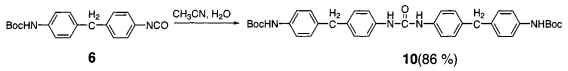
Scheme 3. Reversible, Base-Catalyzed Formation/ Degradation of Thiocarbamates in Aqueous Solution



3: R^1 = H, R^2 = (CO)SG
8: R^1 = Boc, R^2 = Boc
9: R^1 = H, R^2 = H

11: R^2 = (CO)SG
6: R^2 = Boc
12: R^2 = H

Scheme 4. Formation of Urea 10 upon Solution of Isocyanate 6 in Aqueous Acetonitrile



6 **10**(86 %)

tion product of **8** was identified as the urea **10** (Scheme 4) by comparison with an authentic sample of this compound. No free amine (MDA as hydrolysis product of **9**, and mono-Boc-protected MDA as hydrolysis product of **8**, respectivly) was detectable in either case.

Reaction of MDI with GSH in a Biphasic Solvent System at Neutral pH. MDI was found to react with GSH in a two-phase system (water/toluene) under neutral (pH 7) and slightly basic (pH 8.2) conditions. HPLC

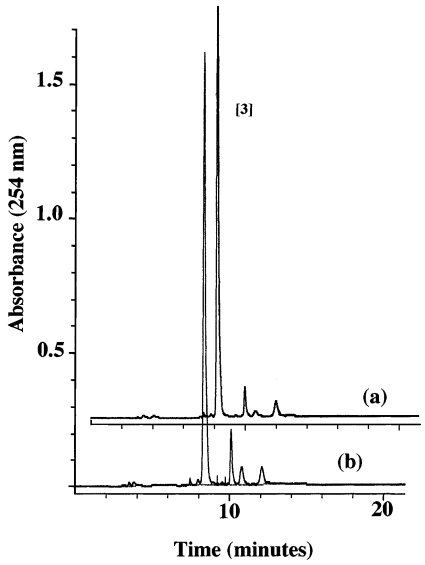


Figure 4. Example HPLC chromatogram of the formation of **3** taken after a reaction time of 2 h. (a) pH 7.0, (b) pH 8.2. Note that the excess of GSH is not visible at 254 nm.

analysis of the aqueous phase shows the bis adduct **3** to be the almost exclusive product at the begining of the reaction (excess of GSH). Figure 4 shows example chromatograms taken after 2 h. With the ongoing consumption of GSH, more and more degradation products of **3** were detectable. Samples taken after 24 h showed complete disappearance of **3** in both cases. As expected, the degradation reaction is faster at pH 8.2. The formation of di- and oligomeric ureas of MDI attached to GSH, which should be soluble in the aqueous phase, could not be established, although insoluble material, most likely polyureas as hydrolysis products of excess MDI, was formed in the toluene layer.

Discussion

The ability of isocyanates to cause modification at sites in the body which have not been directly exposed has led to the search for masked equivalents of these highly reactive and hydrolysis-sensitive compounds. Glutathione adducts with thiocarbamate bonds could represent these masked equivalents since these compounds, which are able to release the isocyanate at physiological pH (*24*), may serve as a carrier (*25*, *26*). Adducts of isocyanates with GSH have since been made under physiological conditions (*6*), though the possibility of the formation of thiocarbamates in hydrogen carbonate solution has been questioned (*27*). Even in vivo the formation of thiocarbamates from administered isocyanates or as metabolites from drugs (*28*) could be successfully demonstrated in several cases; e.g., Baillie and co-workers identified MIC−glutathione and MIC−(*N*-acetylcysteine) adducts in bile and urine of rats after exposure to MIC (*29*). Several studies concerning the stability of these thiocarbamates in aqueous solution have been performed, indicating a reversible, base-catalyzed degradation for which a E1cb mechanism is most likely (*25*, *30*).

In the present study, the reaction products of MDI, the most widely used isocyanate in the manufacturing of polyurethanes, with GSH, the monoadduct **9** and the bis adduct **3**, have been synthesized and characterized (Schemes 1 and 2). Based on their chromatographic properties, the rapid formation of bis adduct **3** between

GSH and MDI under neutral and slightly basic conditions in a two-phase system could be established. Furthermore, it could be shown that (amine) monoadduct **9**, as hydrolysis product of the initially formed monoadduct **11**, may not be formed in significant amounts due to the rapid reaction of **11** with excess GSH (Scheme 3). From a chemical point of view, these findings indicate the likeliness of the formation of bis adduct **3** under physiological conditions from inhaled MDI, since it was found that GSH in epithelial lining fluid can reach high levels. (*23*). In fact, the adduct of toluene diisocyanate with glutathione has been implicated by the loss of glutathione during in vivo inhalation experiments (*12*).

The degradation of all of the synthesized compounds **3**, **8**, and **9** was shown to be of pseudo-first-order. With a half-life of 59 min ($k_{app,unimolecular} = 2 \times 10^{-4}$ s^{-1}) determined for the bis adduct **3** at pH 7.4 and $T = 25$ °C, and a $t_{(1/2)} = 470$ min ($k_{app,unimolecular}$ of 2.46×10^{-5} s^{-1}) for the monoadduct **9**, their stability is well within the range expected for such compounds [compare to the literature known 2,4-TDI-GSH bis adduct with $t_{(1/2)} = 245$ min and $k_{app,unimolecular} = 4.7 \times 10^{-5}$ s^{-1}]. A first-order decay and the strong dependence of the stability of bis adduct **3** on the pH of the medium, established by solvolysis at pH values of 6.5, 7.4, and 8.2, support a base-catalyzed E1cb mechanism for the degradation of the thiocarbamate moiety as proposed by Baillie (*25*, *29*) and others (*30*). Furthermore, the reversibility of this reaction, which could be shown by a remarkable stabilization of **3** upon the addition of excess glutathione, indicates the presence of free isocyanate (Scheme 3) as demanded by this mechanism. Additional support for this mechanism comes from the formation of urea **10** during the degradation of the Boc-protected monoadduct **8**, which is certainly formed in analogy to the preparative method from released isocyanate **6** and its hydrolysis product (Scheme 4).

Though monoadduct **11** and its hydrolysis product **9** were not detectable during the reaction of MDI with GSH in a two-phase system (excess of GSH), the appearance of **9** could be established as an intermediate during the degradation of bis adduct **3**. The concentration was found to be significantly higher at lower pH, which is consistent with a decreasing fraction of reactive, nonprotonated amine being present for reaction with excess isocyanate as the pH decreases. The appearance of the monoadduct **9** as an intermediate in the degradation of **3**, though in low concentration, coupled with the differential rate constants for the cleavage of the thiocarbamate bond between the bis adducts and monoadducts of GSH, suggests a pathway for formation of the monoacetylated MDA, a metabolite found in the urine of rats after inhalation exposure to MDI aerosols (*31*) without the need for free MDA being present. Acetylation of **9** with subsequent release of the second amino group would lead to the same result. Furthermore, acetylation of the monoadduct **9** in vivo would be consistent with either formation of AcMDA adducts with hemoglobin through sulfinamide adducts (*11*) upon reversal of the second thiocarbamate and subsequent hydrolysis to the amine, or direct isocyanate adduct formation by transcarbamylation to tyrosine or cysteine by the monoadduct **9** following in vivo acetylation of the free aromatic amine on **9**. In either case, the products would yield AcMDA upon mild base hydrolysis (*11*). The current results support the observation that the thiocarbamate links in the mono and bis adducts of glutathione with MDI are not equivalent.

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