Synthesis of Adducts with Amino Acids as Potential Dosimeters for the Biomonitoring of Humans Exposed to Toluenediisocyanate

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Toluenediisocyanates (TDI) are important intermediates in the chemical industry. Among the main damages after low levels of TDI exposure are lung sensitization and asthma. Protein adducts of TDI might be involved in the etiology of sensitization reactions. Blood protein adducts are used as dosimeters for modifications of macromolecules in the target organs where the disease develops. The functional groups of cysteine, tyrosine, serine, lysine, tryptophan, histidine, and N-terminal amino acids are potential reaction sites for isocyanates. Especially the N-terminal amino acids, valine, and aspartic acid of hemoglobin and albumin, respectively, are reactive toward electrophilic xenobiotics. To develop methods for the quantitation of protein adducts of 2,4- and 2,6-TDI, we reacted 3-nitro-4-methylphenyl isocyanate (**1a**) with single amino acids and reduced the nitro group using catalytic hydrogenation or ammonium formate with palladium on carbon yielding *N*-[(3-amino-4-methylphenyl)carbamoyl]valine (**2a**), *N*-[(3-amino-4-methylphenyl)carbamoyl]aspartic acid (**8a**), N^{α}-acetyl-N^{ϵ}-[(3-amino-4-methylphenyl)-carbamoyl]lysine (**12a**), and N^{α}-acetyl-*O*-[(3-amino-4-methylphenyl)carbamoyl]serine (**15a**). The same reactions were performed with 5-nitro-2-methylphenyl isocyanate (**1b**) and 3-nitro-2-methylphenyl isocyanate (**1c**). The valine adducts were boiled in acid to obtain the corresponding hydantoins: 3-(3-amino-4-methylphenyl)-5-isopropylimidazoline-2,4-dione (**5a**), 3-(5-amino-2-methylphenyl)-5-isopropylimidazoline-2,4-dione (**5b**), and 3-(3-amino-2-methylphenyl)-5-isopropylimidazoline-2,4-dione (**5c**). A method for the detection of N-terminal adducts with valine in biological samples was developed. The tripeptide adduct *N*-[(3-amino-4-methylphenyl)-carbamoyl]valyl-glycyl-glycine (**19a**) was hydrolyzed with acid in the presence of globin and the internal standard *N*-[(3-amino-4-methylphenyl-d_6)carbamoyl]valyl-glycyl-glycine (**19d**). The released hydantoins were determined by LC/MS/MS and after derivatization with pentafluoropropionic anhydride by GC/MS. The determination limit was 0.16 pmol/sample. The same N-terminal adduct with valine was found in globin of a TDI-worker and in two women with polyurethane covered breast implants.

Introduction

Toluenediisocyanate (TDI)[1] is the most important isocyanate product after methylene diphenyl diisocyanate (MDI) in industry. TDI is an intermediate in the manufacturing of polyurethanes, dyes, pigments, and adhesives. Higher concentrations of isocyanates cause respiratory irritation. Among, the main damages after low levels of isocyanates exposures are lung sensitization and asthma (*1*−*6*). The sensitization properties of TDI are well documented (*2*−*6*). One of the corresponding aromatic amine of TDI, 2,4-toluendiamine (2,4-TDA), is carcinogenic in animal experiments (*7*, *8*). Commercial-grade TDI has been classified by the International Agency for Research on Cancer (IARC) as a carcinogen in animals, with "sufficient evidence" and as a potential occupational carcinogen for humans (*8*). Isocyanates and arylamines can bind with proteins and/or DNA (Figure 1) and lead to cytotoxic and genotoxic effects (*4*−*7*). For arylamines, it has been shown that blood protein adducts are a dosimeter for the adducts in the target organ (*9*−*11*). Protein adducts of isocyanates might be involved in the etiology of sensitization reactions (*12*). An established method to biomonitor exposed people is the determination of blood protein adducts. Such adducts are a marker of exposure and possibly a dosimeter for modifications of macromolecules in the target organs where the disease develops. To improve the risk assessment for workers exposed to TDI, it is important to develop dosimeters to establish if the toxic reactive intermediate is TDI or a metabolite of TDA.

Arylamines are metabolized to highly reactive *N*-hydroxyarylamines (*9*, *10*) by mixed mono-oxygenases. *N*-Hydroxyarylamines can be further metabolized to *N*-(sulfonyloxy)arylamines, *N*-acetoxy-arylamines, or *N*-hydroxyarylamine *N*-glucuronides. These highly reactive intermediates which bind covalently to biomolecules are responsible for the genotoxic and cytotoxic effects of this class of compounds. In exposed animals, arylamines such

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[1] Abbreviations: TDI, toluenediisocyanates; MDI, methylene diphenyl diisocyanate; 24TDA, 2,4-toluenediamine; Pd/C, palladium 10% on carbon; DEPT, distortionless enhancement by polarisation transfer; CH-COSY, CH correlation method; ESI-MS, electrospray ionization-mass spectrometry; TBME, *tert*-butyl methyl ether; PFPA, pentafluoro propionic anhydride; PU, polyurethane.

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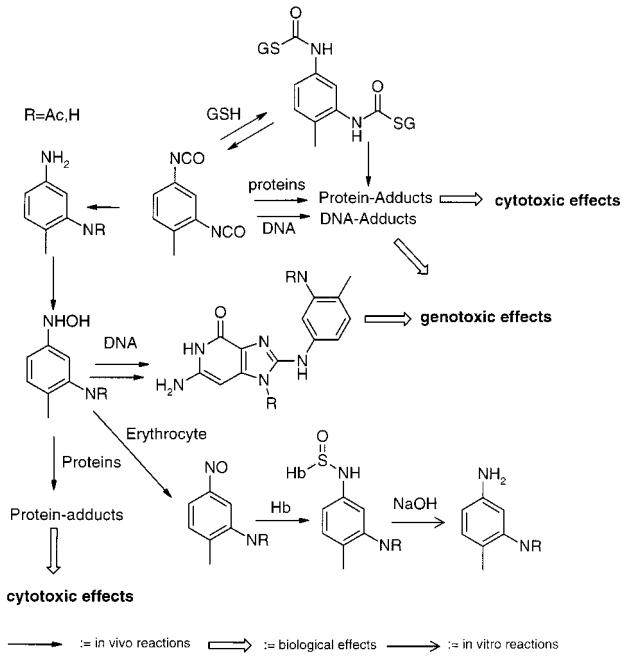

Figure 1. Putative DNA adducts and protein-adducts of 2,4-toluendiamine and 2,4-toluenediisocyanate.

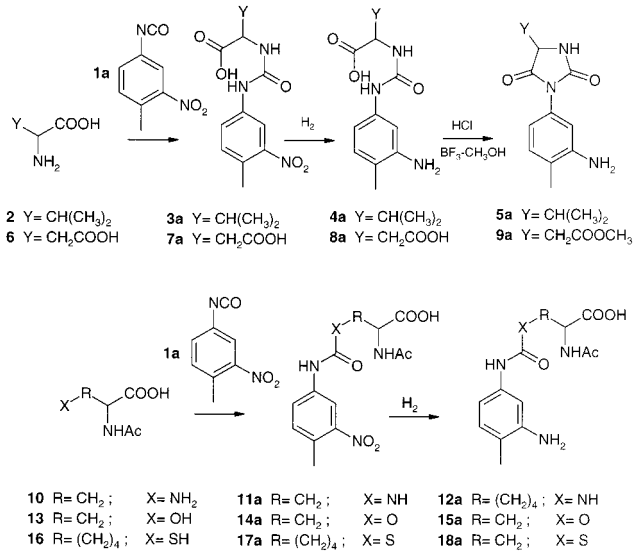

Figure 2. Reactions of amino acids with 3-nitro-4-methylphenylisocyanate.

as 4-aminobiphenyl (*11*), a human bladder carcinogen, is known to form adducts with DNA as well as with tissue proteins and the blood proteins albumin and hemoglobin in a dose-dependent manner. In contrast, isocyanates do not need any further activation to react with biomolecules (Figure 1).

Arylamine specific adducts are of the sulfinic acid amide type (*13*). Mild base hydrolysis of such adducts releases the parent aromatic amine (*14*). At a dose of 50 mg of 2,4-TDA/kg body weight, ca. 0.82 nmol was covalently bound to hemoglobin (*15*). The determination of such hydrolyzable hemoglobin adducts is well-established (*14*). 2,4-TDA binds covalently with DNA (*16*). The chemical structure of TDI adducts with DNA (*17*) and plasma proteins found in vivo is unknown (*17*), since for the quantification thereof harsh hydrolyses conditions were used in order to determine the released parent aromatic amine (*18*−*20*). The distribution after oral dosage or inhalation has been studied in rats with radiolabeled TDI (*21*, *22*). Recently, Day et al. (*23*) found carbamylation products with hemoglobin isolated from guinea pigs exposed by inhalation to 2,4-TDI. For all adducts, one of the two original isocyanato groups had been hydrolyzed to the amine. In addition, Day et al. found an amine-nitroso adduct on the α chain of hemoglobin. These results indicate that at least one of the isocyanato moieties (or a masked derivative) of 2,4-TDI survived passage through the lung, into the serum, and through the erythrocyte membrane to form adducts with hemoglobin that were stable to dialysis, gel filtration, and reversed-phase HPLC separation under acidic conditions. Carbamylation of the N-terminal valine of hemoglobin with methyl isocyanate in rats and rabbits has been demonstrated in vitro and in vivo by gas chromatography (*24*, *25*). Recently, Mraz and Bouskova described the reaction of TDI with single amino acids (*26*). None of the reaction products were isolated and characterized by NMR or MS. Sabbioni et al. (*27*) found an N-terminal valine adduct with hemoglobin after chronic exposure of rats to MDI.

Virtually all functional groups on proteins can react with isocyanates (*28*), but under physiological conditions, the potential sites of reaction are restricted: (i) the α-amino-group of the N-terminal amino acids, (ii) the sulfhydryl group of cysteine (*29*, *30*), (iii) the hydroxyl groups of tyrosine (*31*) and especially serine (*32*), (iv) the ϵ-amino-group of lysine, and (v) the imidazole ring of histidine. Although the pK_a of lysine is around 10.5, especially reactive lysines have been located in proteins (*33*, *34*). In vivo adducts of lysine have been found with acetaldehyde (*35*), glycated proteins (*36*), and aflatoxin B_1 (*37*). Carbamylation of cysteine has been noticed as an artifact in peptide mapping of hemoglobins in the presence of urea (*38*). Carbamylated hemoglobin is formed by the reaction of hemoglobin with cyanate, a product of in vivo urea dissociation. It is found in high levels in patients with renal failure and may be useful in their clinical evaluation (*39*).

The main goal of the present study is to synthesize typical TDI protein adducts in order to assess the exposure to isocyanates. According to the present literature, it is not likely that both isocyanate groups of one molecule would react at the same time with proteins. Therefore, in the present work we investigated the reaction products resulting from the reaction of one isocyanate group (Figure 2). To develop methods for the quantification of protein adducts of 2,4- and 2,6-TDI, amino acids were reacted with monoisocyanate derivatives of TDI. 3-Nitro-4-methylphenyl isocyanate (**1a**) was reacted with single amino acids: L-valine (**2**), L-aspartic acid (**6**), N^{α}-acetyl-L-lysine (**10**), *N*-acetyl-DL-serine (**13**), and *N*-acetyl-L-cysteine (**16**). The resulting *N*-[(3-nitro-4-methylphenyl)carbamoyl]valine (**3a**), *N*-[(3-nitro-4-methylphenyl)carbamoyl]aspartic acid (**7a**), N^{α}-acetyl-N^{ϵ}-[(3-nitro-4-methylphenyl)carbamoyl]lysine (**11a**), *N*-acetyl-*O*-[(3-nitro-4-methylphenyl)carbamoyl]serine (**14a**), and *N*-acetyl-*S*-[3-nitro-4-methylphenyl-carbamoyl]cysteine (**17a**) were reduced to the corresponding amino derivatives. As shown for other environmental pollutants, isocyanates might react with the N-terminal amino acids of hemoglobin and albumin, this would be L-valine and L-aspartic acid, respectively. Therefore, an adduct with a tripeptide containing L-valine as the N-terminal amino acid was synthesized to mimic the release of the formed

hydantoin from a protein. The same reactions were performed with 2-methyl-5-nitrophenyl isocyanate (**1b**) and 2-methyl-3-nitrophenyl isocyanate (**1c**).

Materials and Methods

Chemicals. L-Aspartic acid, L-glutamic acid, BF_3-MeOH, ammonium formate, Palladium 10% on carbon (Pd/C), dry dioxane, 4-fluoroaniline, $NaHCO_3$ (analytical grade), 37% HCl TraceSelect, formic acid, triphosgene and DMSO-d_6 were purchased from Fluka (Neu-Ulm, Germany); *N*-acetyl-L-cysteine from Merck (Darmstadt, Germany); N^{α}-acetyl-L-lysine and *N*-acetyl-DL-serine from Sigma (Deisenhofen, Germany); L-valine from Serva (Heidelberg, Germany); Celite Hyflo Super Cel by Johns Manville from Roth (Karlsruhe, Germany); *tert*-butyl methyl ether (TBME) picograde from Promochem (Wesel, Germany); 4-methyl-3-nitrophenyl isocyanate (**1a**), 2-methyl-5-nitrophenyl isocyanate (**1b**), 2-methyl-3-nitrophenyl isocyanate (**1c**), and 2-fluoro-nitrophenyl isocyanate from Lancaster (Mühlheim am Main, Germany).

Instrumentation. NMR Spectra: Bruker-AC-250 or Bruker -DPX-400 (CH-COSY-experiments) instrument; DMSO-d_6 as solvent and as internal standard (^{1}H: 2.50 ppm; ^{13}C: 39.43 ppm); δ in ppm, *J* in Hz; C-substitution determined using the distortionless enhancement by polarization transfer (DEPT) method or the CH correlation method (CH-COSY). The raw NMR data were processed with the program MestRe-C (Magnetic Resonance Companion, J. C. Cobas, J. Cruces, and F. J. Sordina, Departamente de Quimica Organica, Universidad de Santiago de Compostela, 15706 Santjago de Compostela, Spain). mp, melting points, apparatus by Dr. Tottoli (510) from Büchi, Glasapparatefabrik Flawil, Switzerland, uncorrected. Positive electrospray ionization-mass spectrometry (ESI-MS): quadrupole ion trap tandem mass spectrometer (LCQ Duo, Thermoquest). The samples were dissolved in methanol and introduced with a syringe pump into the MS (flow = 25 μL/min). The parameters were set as follows: capillary temperature of 220 °C, sheath gas flow of 20 (arbitrary units) and auxiliary gas flow of 0, full-scan MS mode. MS/MS spectra were obtained by ion trap collision-induced dissociation. HPLC analyses were performed on a Hewlett-Packard 1100 system with a quaternary HPLC pump and a photodiode array detector. GC/MS: Hewlett-Packard GC (HP 5890II) equipped with an autosampler (HP7236) and interfaced to MS (HP 5989A); *m*/*z* (rel.%).

Ureas from Isocyanates and Amino Acids. General Procedure 1. To a solution of amino acid (2.0 mmol) in 0.25 M $NaHCO_3$ (40 mL) was added the isocyanate (4.0 mmol), and the solution was stirred at 80 °C for 3 h. After cooling the solution with ice, the precipitate was filtered off and washed with 0.25 M $NaHCO_3$ (5 mL). The filtrate was acidified to pH 1−2 with 2 M HCl, extracted with ethyl acetate (3 × 50 mL). The organic phase was extracted with 0.5 M $NaHCO_3$ (3 × 50 mL). The water phase was made acidic to pH 1−2 with 2 M HCl and reextracted with ethyl acetate (3 × 50 mL). The organic extract was dried ($MgSO_4$), filtered, and evaporated.

***N*-[(3-Nitro-4-methylphenyl)carbamoyl]valine (3a). 3a** was obtained according to general procedure 1, with L-valine (372 mg, 3.18 mmol). Crystallization from EtOH/H_2O yielded **3a** (465 mg, 50%). White solid, mp 136−138 °C. ^{1}H NMR (DMSO-d_6): δ 9.01 (*s*, N*H*-Ar); 8.25 (*d*, *J* = 2.2, H−C(2)); 7.42 (*dd*, *J* = 8.4, 2.2, H−C(6)); 7.35 (*d*, *J* = 8.4, H−C(5)); 6.53 (*d*, *J* = 8.6, N*H*CH); 4.12 (*dd*, *J* = 8.6, 4.7, NHC*H*); 2.42 (*s*, Me); 2.10 (m_c, Me_2CH); 0.92 (*d*, *J* = 6.8, *Me*CH); 0.87 (*d*, *J* = 6.8, *Me*CH). ^{13}C NMR (DMSO-d_6): δ 173.5 (COOH); 154.7 (NHCONH); 148.7 (C, C(3)); 139.1 (C, C(1)); 132.9 (CH, C(5)); 124.7 (C, C(4)); 122.1 (CH, C(6)); 112.3 (CH, C(2)); 57.2 (NHCH); 30.1 (Me_2CH); 19.1 (Me); 18.9 (Ar-Me); 17.4 (Me). ESI-MS: 296 $[M + H]^+$, 278 $[(M - H_2O) + H]^+$, 118 $[Val + H]^+$.

***N*-[(5-Nitro-2-methylphenyl)carbamoyl]valine (3b). 3b** was obtained according to general procedure 1, with L-valine (367 mg, 3.13 mmol). White solid **3b** (570 mg, 62%), mp 124 °C. ^{1}H NMR (DMSO-d_6): δ 12.7 (*s*, COOH); 8.98 (*d*, *J* = 2.5, H−C(6)); 8.27 (*s*, Ar−N*H*); 7.71 (*dd*, *J* = 8.3, 2.5, H−C(4)); 7.40 (*d*, *J* = 8.3, H−C(3)); 7.21 (*d*, *J* = 8.6, N*H*CH); 4.17 (*dd*, *J* = 8.6, 4.6, NHC*H*); 2.32 (*s*, Me); 2.13 (m_c, Me_2CH); 0.93 (*d*, *J* = 7.4, *Me*CH); 0.91 (*d*, *J* = 7.4, *Me*CH). ^{13}C NMR (DMSO-d_6): δ 173.7 (COOH); 154.9 (NHCONH); 146.1 (C, C(5)); 139.3 (C, C(1)); 133.3 (C, C(2)); 130.9 (CH, C(3)); 115.8 (CH, C(4)); 112.6 (CH, C(6)); 57.5 (NH*C*H); 30.4 (Me_2CH); 19.2 (Me); 18.2 (Ar-*Me*); 17.6 (Me). ESI-MS: 296 $[M + H]^+$, 278 $[(M - H_2O) + H]^+$, 118 $[Val + H]^+$.

***N*-[(3-Nitro-2-methylphenyl)carbamoyl]valine (3c). 3c** was obtained according to general procedure 1, with L-valine (365 mg, 3.12 mmol). White solid **3c** (660 mg, 72%), mp 167 °C. ^{1}H NMR (DMSO-d_6): δ 8.28 (*s*, Ar−N*H*); 8.12 (*dd*, *J* = 8.2, 1.2, H−C(6)); 7.48 (*d*, *J* = 8.2, 1.2, H−C(4)); 7.35 (*t*, *J* = 8.2, H−C(5)); 7.03 (*d*, *J* = 8.7, N*H*CH); 4.15 (*dd*, *J* = 8.7, 4.8, NHC*H*); 2.25 (*s*, Me-Ar); 2.10 (m_c, Me_2CH); 0.94 (*d*, *J* = 7.3, *Me*CH); 0.92 (*d*, *J* = 7.3, *Me*CH). ^{13}C NMR (DMSO-d_6): δ 173.4 (*C*OOH); 154.9 (NH*C*ONH); 150.9 (C, C(3)); 139.6 (C, C(1)); 126.5 (CH, C(5)); 124.5 (CH, C(6)); 120.7 (C, C(2)); 117.1 (CH, C(4)); 57.3 (NHCH); 30.3 (Me_2CH); 19.1 (Me); 17.5 (Me); 13.2 (Me-Ar). ESI-MS: 296 $[M + H]^+$, 278 $[(M - H_2O) + H]^+$, 118 $[Val + H]^+$.

***N*-[(3-Nitro-4-methylphenyl)carbamoyl]aspartic Acid (7a). 7a** was obtained according to general procedure 1, with L-aspartic acid (413 mg, 3.10 mmol). Yellowish solid (316 mg, 33%), mp 152−153 °C. ^{1}H NMR (DMSO-d_6): δ 12.65 (*s*, broad, 2H, COO*H*); 9.26 (*s*, Ar−N*H*); 8.24 (*d*, *J* = 2.2, H−C(2)); 7.44 (*dd*, *J* = 8.3, 2.2, H−C(6)); 7.39 (*d*, *J* = 8.3, H−C(5)); 6.67 (*d*, *J* = 8.4, N*H*CH); 4.51 (m_c, NHC*H*CH_2); 2.81 (*dd*, *J* = 16.9, 5.6, 1H, NHCH*C*H_2); 2.70 (*dd*, *J* = 16.9, 4.8, 1H, NHCH*C*H_2); 2.42 (*s*, Me). ^{13}C NMR (DMSO-d_6): δ 172.9; 172.3 (COOH); 154.5 (NHCONH); 148.7 (C, C(3)); 139.2 (C, C(1)); 133.0 (CH, C(5)); 124.9 (C, C(4)); 122.3 (CH, C(6)); 112.5 (CH, C(2)); 48.8 (NH-*C*HCH$_2$); 36.6 (NHCH*C*H$_2$); 19.0 (Me). ESI-MS: 312 $[M + H]^+$, 134 $[Asp + H]^+$.

***N*$^{\alpha}$-Acetyl-*N*$^{\epsilon}$-[(3-nitro-4-methylphenyl)carbamoyl]-lysine (11a). 11a** was obtained according to general procedure 1, with N^{α}-acetyl-L-lysine (566 mg, 3.01 mmol). Crystallization from ethyl acetate/*n*-heptane yielded **11a** (383 mg, 35%). White solid, mp 149 °C. ^{1}H NMR (DMSO-d_6): δ 12.52 (*s*, COO*H*); 8.80 (*s*, Ar−N*H*); 8.23 (*d*, *J* = 2.2, H−C(2)); 8.12 (*d*, *J* = 7.9, AcN*H*CH); 7.43 (*dd*, *J* = 8.5, 2.2, H−C(6)); 7.29 (*d*, *J* = 8.5, H−C(5)); 6.26 (*t*, *J* = 5.4, N*H*CH$_2$); 4.14 (m_c, AcNHC*H*); 3.06 (m_c, NHC*H*$_2$); 2.39 (*s*, Me); 1.84 (*s*, Ac); 1.55−1.80 (*m*, C*H*$_2$); 1.25−1.45 (*m*, 2 CH$_2$). ^{13}C NMR (DMSO-d_6): δ 173.7 (COOH); 169.3 (MeCO); 154.9 (NHCONH); 148.8 (C, C(3)); 139.5 (C, C(1)); 132.7 (CH, C(5)); 124.4 (C, C(4)); 122.2 (CH, C(6)); 112.5 (CH, C(2)); 51.8 (AcNH*C*H); 39.1 (CH$_2$(ϵ)); 30.7 (CH$_2$(β)); 29.2 (CH$_2$-(δ)); 22.8 (CH$_2$(γ)); 20.6 (Me); 18.8 (Ar-*Me*). ESI-MS: 367 $[M + H]^+$, 349 $[(M - H_2O) + H]^+$, 215 $[(M -$ toluidine-$NO_2) + H]^+$, 173.

***N*$^{\alpha}$-Acetyl-*N*$^{\epsilon}$-[(5-nitro-2-methylphenyl)carbamoyl]-lysine (11b). 11b** was obtained according to general procedure 1, with N^{α}-acetyl-L-lysine (565 mg, 3.00 mmol). Beige solid **11b** (561 mg, 51%), mp 154−156 °C. ^{1}H NMR (DMSO-d_6): δ 8.93 (*d*, *J* = 2.5, H−C(6)); 8.10 (*d*, *J* = 7.8, AcN*H*CH); 7.97 (*s*, Ar−N*H*); 7.69 (*dd*, *J* = 8.3, 2.5, H−C(4)); 7.41 (*d*, *J* = 8.3, H−C(3)); 6.88 (*t*, *J* = 5.4, N*H*CH$_2$); 4.15 (m_c, AcNHC*H*); 3.10 (m_c, NHC*H*$_2$); 2.28 (*s*, Me); 1.83 (*s*, Ac); 1.80−1.55 (*m*, CH$_2$); 1.41−1.25 (*m*, 2 CH$_2$). ^{13}C NMR (DMSO-d_6): δ 173.8 (COOH); 169.2 (Me*C*O); 154.9 (NHCONH); 146.0 (C, C(5)); 139.4 (C, C(1)); 133.3 (C, C(2)); 130.8 (CH, C(3)); 115.6 (CH, C(4)); 112.7 (CH, C(6)); 51.8 (AcNH*C*H); 39.1 (CH$_2$(ϵ)); 30.8 (CH$_2$(β)); 29.2 (CH$_2$(δ)); 22.9 (CH$_2$-(γ)); 22.3 (Me); 18.0 (Me). ESI-MS: 367 $[M + H]^+$, 349 $[(M - H_2O) + H]^+$, 215 $[(M -$ toluidine-$NO_2) + H]^+$.

***N*$^{\alpha}$-Acetyl-*N*$^{\epsilon}$-[(3-nitro-2-methylphenyl)carbamoyl]-lysine (11c). 11c** was obtained according to general procedure 1, with N^{α}-acetyl-L-lysine (568 mg, 3.02 mmol). Crystallization from H_2O yielded **11c** (729 mg, 66%). White solid, mp 149 °C. ^{1}H NMR (DMSO-d_6): δ 12.54 (*s*, COOH); 8.13 (*d*, *J* = 7.7, AcN*H*CH); 8.08 (*dd*, *J* = 8.0, 1.0, H−C(6)); 8.04 (*s*, Ar−N*H*); 7.48 (*dd*, *J* = 8.0, 1.0, H−C(4)); 7.34 (*t*, *J* = 8.0, H−C(5)); 6.67 (*t*, *J* = 5.5, N*H*CH$_2$); 4.18 (m_c, AcNHC*H*); 3.10 (m_c, NHC*H*$_2$); 2.24 (*s*, Me); 1.86 (*s*, Ac); 1.75−1.50 (*m*, C*H*$_2$); 1.47−1.22 (*m*, 2

CH_2). ^{13}C NMR (DMSO-d_6): δ 173.8 (COOH); 169.3 (Me*C*O); 155.1 (NHCONH); 151.0 (C, C(3)); 140.0 (C, C(1)); 126.5 (CH, C(5)); 125.2 (CH, C(6)); 121.2 (C, C(2)); 117.1 (CH, C(4)); 51.8 (AcNH*C*H); 38.8 ($CH_2(\epsilon)$); 30.8 ($CH_2(\beta)$); 29.3 ($CH_2(\delta)$); 22.9 ($CH_2(\gamma)$); 22.3 (Me); 13.3 (Ar-*Me*). ESI-MS: 367 $[M + H]^+$, 349 $[(M - H_2O) + H]^+$, 215 $[(M - \text{toluidine-}NO_2) + H]^+$.

Carbamates from Isocyanates and *N*-Acetyl-DL-serine. General Procedure 2. To a solution of *N*-acetyl-DL-serine (0.5 mmol) in dry pyridine (3 mL) was added the isocyanate (0.55 mmol) , and the solution was stirred at 80 °C for 3 h. The solvent was evaporated. The residue was taken up in 0.5 M $NaHCO_3$ (15 mL). The insoluble residue was separated by filtration. The water phase was washed with ethyl acetate (2 × 15 mL) acidified with HCl to pH 1.5 and extracted with ethyl acetate (3 × 30 mL). The organic phase was dried over $MgSO_4$ and the solvent was evaporated at reduced pressure.

***N*-Acetyl-*O*-[(3-nitro-4-methylphenyl)carbamoyl]-serine (14a). 14a** was obtained according to general procedure 2, with *N*-acetyl-DL-serine (500 mg, 3.40 mmol). Crystallization from ethyl acetate/*n*-heptane yielded **14a** (774 mg, 70%). White solid, mp 174−175 °C. ^1H NMR (DMSO-d_6): δ 10.11 (*s*, Ar−N*H*); 8.22 (*d*, *J* = 7.7, AcN*H*); 8.19 (*d*, *J* = 1.8, H−C(2)); 7.50 (*dd*, *J* = 8.2, 1.8, H−C(6)); 7.04 (*d*, *J* = 8.2, H−C(5)); 4.54 (m_c, C*H*CH_2O); 4.44 (*dd*, *J* = 11.0, 4.5, 1H, CHC*H*$_2$O); 4.22 (*dd*, *J* = 11.0, 6.6, 1H, CHC*H*$_2$O); 2.45 (*s*, Me); 1.86 (*s*, Ac). ^{13}C NMR (DMSO-d_6): δ 170.9 (COOH); 169.4 (Me*C*O); 153.0 (NHCOO); 148.6 (C, C(3)); 138.0 (C, C(1)); 133.1 (CH, C(5)); 126.3 (C, C(4)); 122.8 (CH, C(2)); 113.2 (CH, C(6)); 63.6 (CH*C*H$_2$O); 51.4 (*C*HCH$_2$O); 22.3 (Me); 19.0 (Me). ESI-MS: 326 $[M + H]^+$, 130 $[(M - \text{toluidine-}NO_2COO) + H]^+$.

***N*-Acetyl-*O*-[(5-nitro-2-methylphenyl)carbamoyl]-serine (14b). 14b** was obtained according to general procedure 2, with *N*-acetyl-DL-serine (500 mg, 3.40 mmol). White solid **14b** (830 mg, 75%), mp 177 °C. ^1H NMR (DMSO-d_6): δ 9.34 (*s*, Ar−N*H*); 8.34 (*d*, *J* = 2.5, H−C(6)); 8.33 (*d*, *J* = 7.0, AcN*H*); 7.91 (*dd*, *J* = 8.4, 2.5, H−C(4)); 7.47 (*d*, *J* = 8.4, H−C(3)); 4.59 (m_c, C*H*CH_2O); 4.46 (*dd*, *J* = 11.1, 4.6, 1H, CHC*H*$_2$O); 4.24 (*dd*, *J* = 11.1, 6.8, 1H, CHC*H*$_2$O); 2.34 (*s*, Me); 1.89 (*s*, Ac). ^{13}C NMR (DMSO-d_6): 170.9 (COOH); 169.4 (Me*C*O); 153.8 (NHCOO); 145.8 (C, C(5)); 139.0 (C, C(1)); 137.4 (C, C(2)); 131.3 (CH, C(3)); 119.0 (CH, C(4)); 118.0 (CH, C(6)); 63.7 (CH*C*H$_2$O); 51.4 (*C*HCH$_2$O); 22.3 (Me); 18.0 (Me). ESI-MS: 326 $[M + H]^+$, 130 $[(M - \text{toluidine-}NO_2COO) + H]^+$.

***N*-Acetyl-*O*-[(3-nitro-2-methylphenyl)carbamoyl]-serine (14c). 14c** was obtained according to general procedure 2, with *N*-acetyl-DL-serine (500 mg, 3.40 mmol). White solid **14c** (894 mg, 81%), mp 140 °C. ^1H NMR (DMSO-d_6): δ 9.41 (*s*, Ar−N*H*); 8.33 (*d*, *J* = 7.9, AcN*H*); 7.69 (*d*, *J* = 8.1, 1.0, H−C(6) or H−C(4)); 7.63 (*d*, *J* = 8.1, 1.0, H−C(4) or H−C(6)); 7.41 (*t*, *J* = 8.1, H−C(5)); 4.57 (m_c, C*H*CH_2O); 4.43 (*dd*, *J* = 11.1, 4.6, 1H, CHC*H*$_2$O); 4.21 (*dd*, *J* = 11.1, 6.8, 1H, CHC*H*$_2$O); 2.25 (*s*, Me); 1.88 (*s*, Ac). ^{13}C NMR (DMSO-d_6): δ 171.0 (COOH); 169.5 (Me*C*O); 154.0 (NH*C*OO); 150.9 (C, C(3)); 138.2 (C, C(1)); 129.7 (CH, C(6)); 126.7 (CH, C(5)); 126.4 (C, C(2)); 120.5 (CH, C(4)); 63.8 (CH*C*H$_2$O); 51.5 (*C*HCH$_2$O); 22.4 (Me); 13.7 (Ar-*Me*). ESI-MS: 326 $[M + H]^+$, 130 $[(M - \text{toluidine-}NO_2COO) + H]^+$.

Carbamic Acid S-Esters. General Procedure 3. To a solution of *N*-acetyl-L-cysteine (163 mg, 1.0 mmol) in 0.25 M $NaHCO_3$ (10 mL) was added the isocyanate (2.0 mmol). The mixture was treated by ultrasound during 3 min in 10 min intervals at 25 °C for 2 h. The reaction mixture was cooled on ice. The precipitate was separated by filtration. The pH of the filtrate was adjusted to 2.5 and extracted with ethyl acetate (3 × 30 mL). The combined organic phases were extracted with 0.6 M $NaHCO_3$ (3 × 50 mL). The pH of the basic water solution was then adjusted to 2.5 and extracted with ethyl acetate (3 × 40 mL). The extract was dried ($MgSO_4$) and evaporated, and the residue was recrystallized with EtOH/H_2O.

***N*-Acetyl-*S*-[3-nitro-4-methylphenyl-carbamoyl]cysteine (17a). 17a** was obtained according to general procedure 3, with *N*-acetyl-L-cysteine (1.0 mmol). Crystallization from EtOH/H_2O yielded **17a** (205 mg, 60%). White solid, mp 187−188 °C. ^1H NMR (DMSO-d_6): δ 12.5 (br. *s*, COO*H*); 10.76 (*s*, Ar−N*H*); 8.33 (*d*, *J* = 8.2, AcN*H*); 8.25 (*d*, *J* = 2.3, H−C(2)); 7.64 (*dd*, *J* = 8.5, 2.3, H−C(6)); 7.45 (*d*, *J* = 8.5, H−C(5)); 6.29 (m_c, AcN*H*); 4.16 (m_c, NHC*H*CH$_2$); 3.42 (*dd*, *J* = 13.8, 5.0, 1H, CHC*H*$_2$S); 3.07 (*dd*, *J* = 13.8, 8.7, 1H, CHC*H*$_2$S); 2.45 (*s*, Me); 1.85 (*s*, Me). ^{13}C NMR (DMSO-d_6): δ 171.7 (COOH); 169.2 (Me*C*O); 164.6 (NHCOS); 148.5 (C, C(3)); 137.4 (C, C(1)); 133.3 (CH, C(5)); 127.3 (C, C(4)); 123.3 (CH, C(6)); 114.0 (CH, C(2)); 51.9 (*C*HCH$_2$S); 30.9 (CH*C*H$_2$S); 22.2 (Me); 19.1 (Me). ESI-MS: 342 $[M + H]^+$, 164 $[\text{N-AcCys} + H]^+$.

Reduction of Nitro-Compounds. General Procedure 4. The nitro compound (0.3 mmol) and Pd/C (30 mg) in dry MeOH (6 mL) was stirred under a hydrogen atmosphere at room temperature overnight. After 20 h the reaction mixture was filtered over Celite to remove the Pd/C and washed twice with MeOH (3 mL each). The solvent was evaporated under reduced pressure yielding the desired product.

Reduction of Nitro-Compounds. General Procedure 5. Pd/C (50 mg) and ammonium formate (540 mg, 8.56 mmol) were added under nitrogen at room temperature to a solution of the nitro compound (0.3 mmol) in dry MeOH (10 mL). After 3 h the reaction mixture was filtered over Celite. The solvent was evaporated. To eliminate ammonium formate, the residue was dried overnight at 0.01 Torr and 30 °C.

***N*-[(3-Amino-4-methylphenyl)carbamoyl]valine (4a). 4a** was obtained according to general procedure 4 with **3a** (280 mg, 1.0 mmol). Crystallization from EtOH yielded **4a** (138 mg, 51%). White solid, mp 114−115 °C. ^1H NMR (DMSO-d_6): δ 8.35 (*s*, Ar−N*H*); 6.71 (*d*, *J* = 2.0, H−C(2)); 6.71 (*d*, *J* = 8.0, H−C(5)); 6.48 (*dd*, *J* = 8.0, 2.0, H−C(6)); 6.28 (*d*, *J* = 8.4, NH*C*H); 4.04 (*dd*, *J* = 8.4, 4.5, NHC*H*); 2.06 (m_c, Me$_2$C*H*); 1.95 (*s*, *Me*-Ar); 0.89 (*d*, *J* = 6.8, *Me*CH); 0.84 (*d*, *J* = 6.8, *Me*CH). ^{13}C NMR (DMSO-d_6): δ 174.0 (COOH); 155.0 (NHCONH); 146.4 (C, C(3)); 138.8 (C, C(1)); 129.7 (CH, C(5)); 114.2 (C, C(4)); 106.0 (CH, C(6)); 103.6 (CH, C(2)); 57.4 (NHCH); 30.4 (Me*C*H); 19.2 (*Me*CH); 17.6 (*Me*CH); 16.8 (*Me*-Ar). ESI-MS: 266 $[M + H]^+$, 248 $[(M - H_2O) + H]^+$, 118 $[\text{Val} + H]^+$.

***N*-[(5-Amino-2-methylphenyl)carbamoyl]valine (4b). 4b** was obtained according to general procedure 5 with **3b** (280 mg, 1.0 mmol). Crystallization from ethyl acetate/*n*-heptane yielded **4b** (117 mg, 44%). Beige solid, mp 205 °C. ^1H NMR (DMSO-d_6): δ 7.76 (*s*, Ar−N*H*); 7.14 (*d*, *J* = 2.2, H−C(6)); 6.72 (*d*, *J* = 8.0, H−C(3)); 6.67 (*d*, *J* = 8.5, N*H*CH); 6.11 (*dd*, *J* = 8.0, 2.2, H−C(4)); 3.94 (*dd*, *J* = 8.5, 4.3, NHC*H*); 2.08 (m_c, Me$_2$C*H*); 2.03 (*s*, Me); 0.87 (*d*, *J* = 7.0, *Me*CH); 0.84 (*d*, *J* = 7.0, *Me*CH). ^{13}C NMR (DMSO-d_6): δ 174.1 (COOH); 155.2 (NHCONH); 146.7 (C, C(5)); 138.3 (C, C(1)); 130.2 (CH, C(3)); 113.7 (C, C(2)); 108.2 (CH, C(4)); 106.8 (CH, C(6)); 57.6 (NHCH); 30.5 (Me$_2$C*H*); 19.3 (Me); 17.7 (Me); 17.1 (Ar-*Me*). ESI-MS: 266 $[M + H]^+$, 248 $[(M - H_2O) + H]^+$, 118 $[\text{Val} + H]^+$.

***N*-[(3-Amino-2-methylphenyl)carbamoyl]valine (4c). 4c** was obtained according to general procedure 4 with **4c** (280 mg, 1.0 mmol). White solid **4c** (238 mg, 90%), mp 194 °C. ^1H NMR (DMSO-d_6): δ 12.6 (*s*, COO*H*); 7.75 (*s*, Ar−N*H*); 6.92 (*dd*, *J* = 7.9, 1.0, H−C(6)); 6.76 (*t*, *J* = 7.9, H−C(5)); 6.35 (*dd*, *J* = 7.9, 1.0, H−C(4)); 6.61 (*d*, *J* = 8.8, N*H*CH); 4.15 (*dd*, *J* = 8.8, 4.9, NHC*H*); 2.12 (*s*, *Me*-Ar); 2.12 (m_c, Me$_2$C*H*); 0.95 (*d*, *J* = 6.7, *Me*CH); 0.90 (*d*, *J* = 6.7, *Me*CH). ^{13}C NMR (DMSO-d_6): δ 173.6 (COOH); 154.8 (NHCONH); 146.8 (C, C(3)); 137.8 (C, C(1)); 125.4 (CH, C(5)); 112.0 (C, C(2)); 110.7 (CH, C(6)); 109.5 (CH, C(4)); 57.3 (NHCH); 30.3 (Me$_2$C*H*); 19.1 (Me); 17.6 (Me); 11.1 (Me). ESI-MS: 266 $[M + H]^+$, 248 $[(M - H_2O) + H]^+$, 118 $[\text{Val} + H]^+$.

***N*-[(5-Amino-2-fluorophenyl)carbamoyl]valine (4e). 4e** was obtained according to general procedure 1 and 5 starting with 1 mmol of valine. White solid 118 mg (44%). ^1H NMR (DMSO-d_6): δ 8.83 (s, NH); 7.15 (*dd*, *J* = 2.8, 7.3, H−C(6)); 6.84 (*d*, *J* = 8.2, N*H*-CH); 6.54 (*dd*, *J* = 8.6, 11.3, H−C(3)); 5.82 (*ddd*, *J* = 2.8, 3.9, 8.6, H−C(4)); 4.10 (*dd*, *J* = 8.1, 4.2, C*H*-COOH); 2.03 (m_c, MeC*H*); 1.92 (*s*, *Me*-Ar); 0.83 (*dd*, *J* = 6.9, *Me*-CH); 0.79 (*d*, *J* = 6.9, *Me*-CH). ^{13}C NMR (DMSO-d_6): δ 172.8 (COOH); 154.8 (Me*C*O); 145.0 (C, C(5)); 144.3 (*d*, *J* = 228.5 (C, C(2)); 128.3

(*d*, J = 11.1, C, C(1)); 114.3 (*d*, J = 19.5, CH, C(3)); 106.0 (*d*, J = 6.5, CH, C(4)); 105.8 (C, C(6)); 58.2 (NHCH); 30.6 (Me_2CH); 19.5 (*Me*CH); 17.7 (*Me*CH). ESI-MS: 270 $[M + H]^+$, 252 $[(M - H_2O) + H]^+$, 118 $[Val + H]^+$.

***N*-[(3-Amino-4-methylphenyl)carbamoyl]aspartic Acid (8a). 8a** was obtained according to general procedure 4 with **7a** (300 mg, 0.96 mmol). Beige solid **8a** (239 mg, 88%), mp 110 °C. ^{1}H NMR (DMSO-d_6): δ 8.59 (*s*, Ar−N*H*); 6.72 (*d*, J = 2.1, H−C(2)); 6.72 (*d*, J = 8.0, H−C(5)); 6.48 (*dd*, J = 8.0, 2.1, H−C(6)); 6.38 (*d*, J = 5.2, N*H*CH); 4.03 (m_c, NHC*H*); 2.53−2.49 (*m*, NHCHCH_2); 1.95 (*s*, Me).^{13}C NMR (DMSO-d_6): δ 174.2 (COOH); 172.9 (COOH); 154.4 (NHCONH); 146.4 (C, C(3)); 138.9 (C, C(1)); 129.7 (CH, C(5)); 114.0 (C, C(4)); 106.1 (CH, C(6)); 103.7 (CH, C(2)); 49.4 (NH*C*H); 41.2 (NHCH*C*H$_2$); 16.7 (Me). ESI-MS: 282 $[M + H]^+$, 134 $[Asp + H]^+$.

***N*$^\alpha$-Acetyl-*N*$^\epsilon$-[(3-amino-4-methylphenyl)carbamoyl]-lysine (12a). 12a** was obtained according to general procedure 4 with **11a** (120 mg, 0.33 mmol). Crystallization from MeOH/ethyl acetate yielded, **12a** (60 mg, 52%). Beige solid, mp 89 °C. ^{1}H NMR (DMSO-d_6): δ 8.14 (*s*, Ar−N*H*); 7.99 (*d*, J = 7.7, AcN*H*); 6.73 (*d*, J = 1.9, H−C(2)); 6.73 (*d*, J = 7.9, H−C(5)); 6.48 (*dd*, J = 7.9 1.9, H−C(6)); 6.14 (*t*, J = 5.4, N*H*CH$_2$); 4.11 (m_c, NHC*H*); 3.02 (m_c, NHCH_2); 1.95 (*s*, Me); 1.84 (*s*, Me); 1.77−1.45 (*m*, CH_2); 1.45−1.20 (*m*, 2 CH_2). ^{13}C NMR (DMSO-d_6): δ 174.1 (COOH); 169.1 (Me*C*O); 155.3 (NHCONH); 146.4 (C, C(3)); 139.0 (C, C(1)); 129.7 (CH, C(5)); 114.0 (C, C(4)); 106.2 (CH, C(6)); 103.8 (CH, C(2)); 52.3 (NHCH); 39.2 (CH$_2$(ϵ)); 31.1 (CH$_2$-(β)); 29.6 (CH$_2$(δ)); 22.9 (Me); 22.4 (CH$_2$(γ)); 16.7 (Me). ESI-MS: 337 $[M + H]^+$, 319 $[(M - H_2O) + H]^+$, 215 $[(M -$ toluenediamine$) + H]^+$, 189 $[AcLys + H]^+$, 123 $[toluenediamine + H]^+$.

***N*$^\alpha$-Acetyl-*N*$^\epsilon$-[(5-amino-2-methylphenyl)carbamoyl]-lysine (12b). 12b** was obtained according to general procedure 4 with **11b** (90 mg, 0.246 mmol). Beige solid **12b** (61 mg, 74%), mp 130−132 °C. ^{1}H NMR (DMSO-d_6): δ 8.07 (*d*, J = 7.7, AcN*H*CH); 7.33 (*s*, Ar−N*H*); 7.12 (*d*, J = 2.2, H−C(6)); 6.73 (*d*, J = 8.0, H−C(3)); 6.47 (*t*, J = 5.4, CH$_2$N*H*); 6.11 (*dd*, J = 2.2, 8.0, H−C(4)); 4.15 (m_c, NHC*H*); 3.05 (m_c, NHCHCH_2); 2.00 (*s*, Me); 1.85 (*s*, Me); 1.77−1.45 (*m*, CH_2); 1.40−1.20 (*m*, 2 CH$_2$). ^{13}C NMR (DMSO-d_6): δ 173.9 (COOH); 169.3 (Me*C*O); 155.4 (NHCONH); 146.7 (C, C(5)); 138.4 (C, C(1)); 130.1 (CH, C(3)); 113.9 (C, C(2)); 108.2 (CH, C(4)); 107.0 (CH, C(6)); 52.0 (NHCH); 39.1 (CH$_2$(ϵ)); 30.9 (CH$_2$(β)); 29.5 (CH$_2$(δ)); 23.0 (Me); 22.3 (CH$_2$-(γ)); 17.0 (Me). ESI-MS: 337 $[M + H]^+$, 319 $[(M - H_2O) + H]^+$, 215 $[(M -$ toluenediamine$) + H]^+$, 189 $[AcLys + H]^+$, 123 $[toluenediamine + H]^+$.

***N*$^\alpha$-Acetyl-*N*$^\epsilon$-[(3-amino-2-methylphenyl)carbamoyl]-lysine (12c). 12c** was obtained according to general procedure 5 with **11c** (70 mg, 0.193 mmol). Beige solid **12c** (61 mg, 92%), mp >174 °C decomp. ^{1}H NMR (DMSO-d_6): δ 8.07 (*d*, J = 7.6, AcN*H*CH); 7.50 (*s*, Ar−N*H*); 6.86 (*d*, J = 7.8, H−C(6)); 6.75 (*t*, J = 7.8, H−C(5)); 6.34 (*d*, J = 7.8, H−C(4)); 6.28 (*t*, J = 5.4, N*H*CH$_2$); 3.05 (m_c, NHC*H*); 1.90 (*s*, Me); 1.84 (*s*, Me); 1.77−1.45 (*m*, CH$_2$); 1.45−1.20 (*m*, 2 CH$_2$). ^{13}C NMR (DMSO-d_6): δ 174.7 (COOH); 168.5 (Me*C*O); 155.8 (NHCONH); 146.7 (C, C(3)); 138.2 (C, C(1)); 125.2 (CH, C(5)); 112.5 (C, C(2)); 111.2 (CH, C(6)); 109.3 (CH, C(4)); 53.4 (NH*C*H); 39.1 (CH$_2$(ϵ)); 32.0 (CH$_2$(β)); 29.8 (CH$_2$(δ)); 22.8 (Me); 22.7 (CH$_2$(γ)); 11.3 (Me). ESI-MS: 337 $[M + H]^+$, 319 $[(M - H_2O) + H]^+$, 215 $[(M -$ toluenediamine$) + H]^+$, 189 $[AcLys + H]^+$, 123 $[toluenediamine + H]^+$.

***N*-Acetyl-*O*-[(3-amino-4-methylphenyl)carbamoyl]-serine (15a). 15a** was obtained according to general procedure 5 with **14a** (100 mg, 0.31 mmol). Beige solid **15a** (70 mg, 77%), mp 148 °C. ^{1}H NMR (DMSO-d_6): δ 9.22 (*s*, Ar−N*H*); 7.58 (*d*, J = 7.0, AcN*H*); 6.81 (*d*, J = 1.8, H−C(2)); 6.74 (*d*, J = 8.2, H−C(5)); 6.52 (*dd*, J = 8.2, 1.8, H−C(6)); 4.36 (*dd*, J = 9.8, 2.8, 1H, CHCH_2O); 4.13 (m_c, C*H*CH$_2$O); 4.07 (*dd*, J = 9.8, 6.3, 1H, CHCH_2O); 1.96 (*s*, Me); 1.86 (*s*, Me). ^{13}C NMR (DMSO-d_6): δ 171.4 (COOH); 168.6 (Me*C*O); 153.5 (NHCOO); 146.5 (C, C(3)); 137.6 (C, C(1)); 129.6 (CH, C(5)); 115.1 (C, C(4)); 107.8 (CH, C(6)); 105.4 (CH, C(2)); 65.2 (CHCH$_2$O); 53.8 (*C*HCH$_2$O); 22.9 (Me); 16.8 (Me). ESI-MS: 296 $[M + H]^+$, 130 $[(M -$ toluidine-NH−COO$) + H]^+$.

***N*-Acetyl-*O*-[(5-amino-2-methylphenyl)carbamoyl]-serine (15b). 15b** was obtained according to general procedure 4 with **14b** (150 mg, 0.46 mmol). Beige solid **15b** (113 mg, 85%), mp 136 °C. ^{1}H NMR (DMSO-d_6): δ 8.45 (*s*, Ar−N*H*); 7.68 (*d*, J = 7.6, AcN*H*); 6.77 (*d*, J = 8.0, H−C(3)); 6.62 (*d*, J = 2.2, H−C(6)); 6.27 (*dd*, J = 8.0, 2.2, H−C(4)); 4.36 (*dd*, J = 10.3, 3.7, 1H, CHCH_2O); 4.24 (m_c, C*H*CH$_2$O); 4.10 (*dd*, J = 10.3, 6.3, 1H, CHCH_2O); 2.00 (*s*, Me); 1.85 (*s*, Me). ^{13}C NMR (DMSO-d_6): δ 171.6 (*C*OOH); 168.8 (Me*C*O); 154.3 (NHCOO); 146.7 (C, C(5)); 136.6 (C, C(1)); 130.2 (CH, C(3)); 118.2 (C, C(2)); 110.8 (CH, C(4)); 110.5 (CH, C(6)); 65.1 (CHCH$_2$O); 53.6 (*C*HCH$_2$O); 22.8 (Me); 16.8 (Me). ESI-MS: 296 $[M + H]^+$, 130 $[(M -$ toluidine-NH-COO$) + H]^+$.

***N*-Acetyl-*O*-[(3-amino-2-methylphenyl)carbamoyl]-serine (15c). 15c** was obtained according to general procedure 4 with **14c** (150 mg, 0.46 mmol). Beige solid **15c** (109 mg, 83%), mp 212 °C. ^{1}H NMR (DMSO-d_6): δ 8.76 (*s*, Ar−N*H*); 8.29 (*d*, J = 7.9, AcN*H*); 6.82 (*t*, J = 7.9, H−C(5)); 6.47 (*d*, J = 7.9, H−C(4), H−C(6)); 4.53 (m_c, C*H*CH$_2$O); 4.34 (*dd*, J = 11.0, 4.6, CHCH_2O); 4.14 (*dd*, J = 11.0, 6.9, 1H, CHCH_2O); 1.89 (*s*, broad, 6H, 2 *Me*). ^{13}C NMR (DMSO-d_6): δ 171.1 (COOH); 169.4 (Me*C*O); 154.1 (NHCOO); 147.1 (C, C(3)); 136.2 (C, C(1)); 125.4 (CH, C(5)); 116.6 (C, C(2)); 113.9 (CH, C(6)); 111.4 (CH, C(4)); 63.2 (CHCH$_2$O); 51.6 (*C*HCH$_2$O); 22.4 (Me); 11.8 (Me). ESI-MS: 296 $[M + H]^+$, 130 $[(M -$ toluidine-NH-COO$) + H]^+$.

***N*$^\alpha$-Acetyl-*S*-[3-amino-4-methylphenyl-carbamoyl]cysteine (18a). 18a** was obtained according to general procedure 4 with **17a** (90 mg, 0.26 mmol). Yellowish solid (74 mg, 90%), mp >230 °C decomp. ^{1}H NMR (DMSO-d_6): δ 9.93 (*s*, Ar−N*H*); 8.23 (*d*, J = 8.0, AcN*H*); 6.85 (*d*, J = 1.8, H−C(2)); 6.80 (*d*, J = 8.1, H−C(5)); 6.54 (*dd*, J = 8.1, 1.8, H−C(6)); 4.35 (m_c, NH-C*H*CH$_2$); 3.36 (*dd*, J = 13.7, 5.0, 1H, CHCH_2S); 3.00 (*dd*, J = 13.7, 8.7, 1H, CHCH_2S); 1.98 (*s*, Me); 1.85 (*s*, Me). ^{13}C NMR (DMSO-d_6): δ 171.9 (COOH); 169.3 (Me*C*O); 163.2 (NHCOS); 146.7 (C, C(3)); 137.3 (C, C(1)); 129.8 (CH, C(5)); 116.6 (C, C(4)); 107.3 (CH, C(6)); 105.0 (CH, C(2)); 52.3 (*C*HCH$_2$S); 30.6 (CHCH_2S); 22.4 (Me); 16.9 (Me). ESI-MS: 312 $[M + H]^+$, 164 $[N\text{-}AcCys + H]^+$.

***N*-[(3-Amino-4-methylphenyl)carbamoyl]valyl-glycyl-glycine (19a). 19a** was obtained according to general procedure 1 and 4 starting with 1 mmol of valyl-glycyl-glycine. Crystallization from ethyl acetate yielded **19a** (95 mg, 25%). Beige solid, mp >300 °C. ^{1}H NMR (DMSO-d_6): 8.34 (*s*, NH); 8.32 ("*t*", N*H*CH$_2$); 8.06 ("*t*", N*H*CH$_2$); 6.72 (*d*, J = 8.2, H−C(5)); 6.68 (*d*, J = 2.0, H−C(2)); 6.48 (*dd*, J = 8.2, 2.0, H−C(6)); 6.31 (*d*, J = 8.4, N*H*CH), 4.07 (*dd*, J = 8.4, 5.6, NHC*H*), 3.73 (m_c, 4H, CH$_2$-(Gly); 1.97 (m_c, Me$_2$C*H*), 1.95 (*s*, *Me*-Ar); 0.89 (*d*, J = 6.8, *Me*CH); 0.84 (*d*, J = 6.8, *Me*CH). ^{13}C NMR (DMSO-d_6): δ 172.2, 171.2, 169.0 (2NHCO, COOH); 155.2 (NHCONH); 146.6 (C, C(3)); 138.8 (C, C(1)); 129.8 (CH, C(5)); 114.3 (C, C(4)); 106.0 (CH, C(6)); 103.6 (CH, C(2)); 57.8 (NHCH); 41.7 (CH$_2$NH); 41.0 (CH$_2$NH); 30.8 (Me_2CH); 19.3 (Me*C*H); 17.7 (Me*C*H); 16.8 (Ar-*Me*). ESI-MS: 380 $[M + H]^+$, 248 $[(M -$ glycyl-glycine$) + H]^+$.

***N*-[(3-Amino-4-methylphenyl-*d*$_6$)carbamoyl]valyl-glycyl-glycine (19d).** 2,4-Dinitrotoluene-d_6 was synthesized according to Sabbioni and Beyerbach (*40*). 4-Amino-2-nitrotoluene-d_6 was synthesized according to the procedure described for the synthesis of 4-amino-2-nitrotoluene (*41*). 4-Amino-2-nitro-toluene-d_6 (1 mg, 6.32 μmol) in dry dioxane (0.5 mL) was reacted with triphosgene (2 mg) for 2 h at 80 °C, then valyl-glycyl-glycine (10 mg) in 0.25 M $NaHCO_3$ (1 mL) was added. After 2 h at 80 °C, 4 mL of water was added and the reaction mixture was extracted with dichloromethane (1 × 3 mL) and ethyl acetate (2 × 3 mL). The pH was then adjusted to pH 2. The water phase was extracted with ethyl acetate (2 × 4 mL). The organic extracts were dried over a pipet filled with dry Na_2SO_4. The filtrate was evaporated at the speed vac. The residue was taken up in dry MeOH (1 mL). Pd/C (2 mg) was added and the reaction mixture was placed under a hydrogen atmosphere following general procedure 4. After 8 h at room temperature the reaction mixture was filtered over a pipet filled with Celite. The concentration of the filtrate was determined by HPLC. HPLC

analyses were performed on a Lichrospher RP18 (125 mm × 4 mm, 5 μm) column with a 20 min, 20 to 80% MeOH gradient in 0.1% formic acid [flow rate 1 mL/min, t_R (*N*-[(3-amino-4-methylphenyl-d_6)carbamoyl]valyl-glycyl-glycine) = 6.3 min, t_R (*N*-[(3-nitro-4-methylphenyl-d_6)carbamoyl]valyl-glycyl-glycine) = 13.4 min]. The yield was determined by HPLC with calibration line obtained with standard solutions of the undeuterated corresponding compound **19a**. The purity and identity of the compound were checked by HPLC with a photodiode array detector [UV (0.1% formic acid/MeOH) λ_{max} 244 nm] in comparison to the corresponding undeuterated compound **19a**. Yield 472 μg (19.3%) **19d**. ESI-MS: 386 $[M + H]^+$ (100), 385 (79), 384 (28), 254 (3), 253 (4) [M-glycyl-glycine]$^+$, 252 (3).

Hydantoins. General Procedure 6. The urea derivatives (**4a**−**c**,**e**) (0.2 mmol) were dissolved in 6 M HCl (10 mL) and stirred for 2 h at 80−90 °C. The solvent was evaporated, the residue was dissolved in EtOH and filtrated. Then H_2O was added dropwise to the filtrate until precipitation occurred. After cooling, the precipitate was filtered off and dried.

3-(3-Amino-4-methylphenyl)-5-isopropylimidazoline-2,4-dione (5a). 5a was obtained according to general procedure 6 with **4a** (100 mg, 0.38 mmol). Crystallization from EtOH yielded **5a** (65 mg, 70%). White solid, mp 191 °C. ^{1}H NMR (DMSO-d_6): δ 8.39 (*s*, 1H, N*H*); 6.97 (*d*, *J* = 7.9, H−C(5)); 6.50 (*d*, *J* = 2.1, H−C(2)); 6.34 (*dd*, *J* = 7.9, 2.1, H−C(6)); 5.03 (*s*, broad, NH_2); 4.09 (dd, *J* = 3.3, 1.2, C*H*CHMe$_2$); 2.17−2.07 (*m*, Me$_2$C*H*); 2.07 (*s*, Me); 1.01 (*d*, *J* = 7.1, *Me*CH); 0.87 (*d*, *J* = 6.7, *Me*CH). ^{13}C NMR (DMSO-d_6): δ 172.8 (*C*O); 156.3 (NCONH); 146.9 (C, C(3)); 130.3 (C, C(1)); 129.7 (CH, C(5)); 120.8 (C, C(4)); 114.1 (CH, C(6)); 112.0 (CH, C(2)); 61.1 (*C*HCHMe$_2$); 29.9 (Me$_2$*C*H); 18.4(Me); 17.0 (*Me*-Ar); 15.6 (Me). EI-MS: 248 (13), 247 (84, M^+), 149 (25), 148 (100), 147 (21), 106 (11). PFPA-derivative: EI-MS: 394 (14), 393 (84, M^+), 351 (74), 295 (19), 294 (21), 274 (21), 175 (65), 147 (22), 132 (14), 72 (100), 55 (18). NCI-MS: 374 (21), 373 (100). ESI-MS: 248 $[M + H]^+$. MS/MS 248 (40% collision energy) → 149 (100%).

3-(5-Amino-2-methylphenyl)-5-isopropylimidazoline-2,4-dione (5b). 5b was obtained according to general procedure 6 with **4b** (100 mg, 0.38 mmol). Beige solid **5b** (70 mg, 75%), mp 176−177 °C. ^{1}H NMR (DMSO-d_6): δ 8.39 and 8.36 (*s*, 1H, N*H*, diast.); 6.948[b] and 6.939[a] (*d*, *J* = 8.2, 1H, H−C(3)); 6.566 (*dd*, *J* = 8.2, 2.2, 1H, H−C(4)); 6.344[a] and 6.256[b] (*d*, *J* = 2.2, H−C(6)); 4.172[b] and 4.137[a] (*d*, *J* = 3.3, NHC*H*CH); 2.25−2.08 (*m*, 1H, CHC*H*Me$_2$); 1.91[b] and 1.90[a] (*s*, *Me*); 1.019 (*d*, *J* = 7.0, *Me*CH); 0.906 (*d*, *J* = 6.8, *Me*CH). Ratio isomers = a/b = 1.2/1. ^{13}C NMR (DMSO-d_6): δ 172.89 and 172.79 (CO); 156.3 (NCONH); 147.34 and 147.28 (C, C(5)); 131.42 and 131.07 (C, C(1)); 130.6 and 130.5 (CH, C(3)); 122.21 and 121.71 (C, C(2)); 114.60 and 114.43 (CH, C(4)); 114.06 and 113.60 (CH, C(6)); 61.72 and 61.18 (NH*C*HCH); 29.97 and 29.57 (CH*C*H*Me*$_2$); 18.62 and 18.33 (Me); 16.49 and 16.20 (Ar-*Me*); 16.10 and 15.70 (Me). EI-MS: 248 (15), 247 (95, M^+), 149 (29), 148 (100), 147 (27), 120 (14), 106 (34), 72 (16), 55(14). PFPA-derivative: 394 (18), 393 (99, M^+), 352 (15), 351 (100), 253 (13), 295 (38), 294 (46), 175 (15), 72 (63), 57 (36), 55 (20). NCI-MS: 374 (21), 373 (100).

3-(3-Amino-2-methylphenyl)-5-isopropylimidazoline-2,4-dione (5c). 5c was obtained according to general procedure 6 with **4c** (100 mg, 0.38 mmol). White solid **5c** (61 mg, 65%), mp 160 °C. ^{1}H NMR (DMSO-d_6): δ 8.374[b] and 8.342[a] (*s*, 1H, N*H*); 6.95 (*t*, *J* = 7.8, H−C(5)); 6.69 (*d*, *J* = 7.8, H−C(4)); 6.37[a] and 6.23[b] (*d*, *J* = 7.8 H-C(6)); 5.03 (*s*, broad, NH_2); 4.19[b] and 4.14[a] (d, *J* = 2.7, NHC*H*CH); 2.17−2.07 (*m*, Me$_2$C*H*); 1.80 and 1.78 (*s*, Me); 1.03 and 1.02 (*d*, *J* = 6.8, *Me*CH); 0.92 and 0.89 (*d*, *J* = 6.6, *Me*CH). Ratio isomer a/isomer b = 1.2/1 ^{13}C NMR (DMSO-d_6): δ 172.96 and 172.88 (*C*O); 156.38 and 156.32 (NCONH); 147.64 and 147.52 (C, C(3)); 131.54 and 131.44 (C, C(1)); 126.10 and 126.03 (CH, C(5)); 119.68 and 119.36 (C, C(2)); 116.49 and 115.99 (CH, C(6)); 114.3 (CH, C(4)); 61.82 and 61.34 (*C*HCHMe$_2$); 29.96 and 29.58 (Me$_2$*C*H); 18.68 and 18.40(Me); 16.24 and 15.77 (Me); 11.9 (Ar-*Me*). EI-MS: 248 (15), 247 (100, M^+), 149 (24), 148 (61), 147 (60), 120 (11), 106 (23), 72 (11). EI-MS: PFPA derivative: 394 (15), 393 (79, M^+), 351 (39), 295 (33), 294 (20), 274 (28), 175 (27), 147 (18), 72 (100), 55 (34). NCI-MS: 374 (21), 373 (100, M-HF).

3-(3-Amino-4-methylphenyl-d_6)-5-isopropylimidazoline-2,4-dione (5d). 5d was obtained according to general procedure 6 with **19b** (900 ng). The identity of the sample was confirmed by positive ESI-MS and by GC-MS after derivatization with PFPA: ESI-MS: 254 $[M + H]^+$ (66), 253 (100), 252 (86), 251 (47). ESI-MS: MS/MS (40% collision energy): 253 → 251 (6), 210 (6), 209 [M-isopropyl]$^+$ (11), 208 (6), 155 (11), 154 [$CD_3(NH_2)$-C_6D_3−NCO]$^+$ (60), 153 (100), 152 (56). GC-MS of the PFPA derivative: NCI-MS: 379 (58, M-HF), 378 (100), 377 (87), 376 (45).

3-(5-Amino-2-fluorophenyl)-5-isopropylimidazoline-2,4-dione (5e). 5e was obtained according to general procedure 6 with (100 mg, 0.38 mmol). Slightly rosa solid (61 mg, 65%), mp 174 °C. ^{1}H NMR (DMSO-d_6): δ 8.70 (*s*, 1H, N*H*); 7.8−7.4 (*m*, 3H, aromatic-H); 4.24 (*d*, *J* = 2.7, NHC*H*CH); 2.25−2.00 (*m*, 1H, CHC*H*Me$_2$); 2.13 (*s*, *Me*); 1.02 (*d*, *J* = 6.9, *Me*CH); 0.90 (*d*, *J* = 6.8, *Me*CH). ^{13}C NMR (DMSO-d_6): δ 172.2 (CO); 155.7 (*d*, *J* = 249.2, C, C(2)); 154.9 (NCONH); 130.4 (C, C(5)); 124.7 (CH, C(4), *d*, *J* = 8.8); 124.0(CH, C(6)); 120.2 (C, C(1), *d*, *J* = 14.6); 117.4 (CH, C(3), *d*, *J* = 21.5); 61.9 (NH*C*HCH); 29.9 (Me$_2$*C*H); 18.3 (Me); 15.8 (Me). EI-MS: 252 (6), 251 (51), 153 (20), 152 (100), 72 (21). PFPA-derivative: 398 (8), 397 (47), 352 (15), 355 (100), 298 (30), 257 (16), 179 (18), 151 (18), 72 (56). NCI: 378 (18), 377 (100).

1-(3-Amino-4-methylphenyl)-2,5-dioxoimidazolidine-4-acetic acid methylester (9a). 9a was obtained according to general procedure 6 with **8a** (150 mg, 0.53 mmol). The residue was then derivatized with a 10% BF_3-MeOH (2 mL) solution at room temperature. After 1 h, water (1 mL) was added and the reaction mixture was evaporated to dryness. The residue was taken up in water (5 mL) and extracted with ethyl acetate (3 × 5 mL). Evaporation of the solvents yielded a beige solid (78 mg, 50%), mp 199 °C. ^{1}H NMR (DMSO-d_6): δ 8.31 (*s*, N*H*); 6.97 (*d*, *J* = 8.0, H−C(5)); 6.54 (*d*, *J* = 2.0, H−C(2)); 6.39 (*dd*, *J* = 8.0, 2.0, H−C(6)); 5.05 (*s*, broad, NH_2); 4.43 (*dt*, *J* = 1.2, 4.9, NHC*H*CH$_2$); 3.64 (*s*, OMe); 2.92 (*dd*, *J* = 17.0, 5.4, 1H, NHCHC*H*$_2$); 2.81 (*dd*, *J* = 17.0, 4.6, 1H, NHCHC*H*$_2$); 2.07 (*s*, Me). ^{13}C NMR (DMSO-d_6): δ 172.6 (*C*OOMe); 169.9 (N*C*OCH); 156.2 (NCONH); 146.8 (C, C(3)); 130.6 (CH, C(5)); 129.7 (C, C(1)); 120.8 (C, C(4)); 114.3 (CH, C(6)); 112.1 (CH, C(2)); 52.6 (OMe); 51.7 (*C*H); 35.1 (*C*H$_2$); 17.1 (*Me*). ESI-MS: 278 $[M + H]^+$, 246 $[(M - MeOH) + H]^+$, 204 $[(M - CH_2COOMe) + H]^+$. EI-MS: 278 (17), 277 (100, M^+), 218 (19), 149 (31), 148 (66), 147 (24), 122 (21), 106 (21), 74 (13).

GC/MS Analysis and Calibration Line of the Hydantoin (5a). 19a (0, 0.66, 1.32, 6.59, and 13.2 pmol), **19d** (6.59 pmol) in MeOH (10 μL), and 4-fluoroaniline (1 μg) in MeOH (10 μL) were added to 2 M HCl (4 mL), and the mixture was heated for 2 h at 100 °C in a centrifuge tube with a Teflon-lined screw cap. The experiments were performed in triplicate. The hydrolysate was adjusted to pH 9 with $NaHCO_3$, and then extracted with *tert*-butyl methyl ether (TBME) (6 mL). The organic layer was passed through a pipet filled with anhydrous Na_2SO_4 (1 g). The Na_2SO_4 was rinsed with TBME (1.5 mL). The dried organic phase was collected in a tapered tube. Decane (10 μL) was added as a keeper to the organic phase prior to evaporation with the speed evacuator. The residue was taken up in ethyl acetate (2 × 100 μL) and derivatized with pentafluoropropionic anhydride (PFPA) (5 μL). After 20 min at room temperature, the derivatization was stopped by adding a methanol solution (10 μL)). After evaporation to dryness, the residue was taken up in ethyl acetate (10 μL). The extracts were analyzed on a fused silica capillary column [ZB-5 (Phenomenex, Aschaffenburg, Germany), 0.25 mm i.d., 15 m long, 0.5 μm film thickness] attached to a methyl-deactivated tubing precolumn (Supelco, 0.25 mm, 1 m long) with a Hewlett-Packard chromatograph (model 5890II) coupled to a mass spectrometer as a detector (HP 5989A). An aliquot (1 μL) was injected splitless (inactivated liner with glass wool) at 80 °C. The transfer line temperature and the injector temperature were set at 280 °C. The oven temperature was kept for 1 min at 70 °C and then increased at a rate

of 50 °C/min to 300 °C. The compounds were identified and quantified using negative chemical ionization mass spectrometry, with methane as the reactant gas (1.5 Torr, ion source temperature of 250 °C, electron energy of 200 eV) and SIM of *m*/*z* 373 and 377 (dwell time of 150 ms). The derivatized internal standard **5d** and **5a** eluted at 6.27 min. With a shorter column and lower film thickness [Rtx-5MS (Restek, Bellefonte, PA), 0.25 mm i.d., 12 m long, 0.25 μm film thickness], the retention time of **5d** was 5 min. The calibration line was generated from the amount (expressed in picomoles) of **19a** (*x*) and the peak ratio (*y*) of **5a** and **5d**: $y = 0.22x$, $r^2 = 0.99$.

LC/MS/MS Analysis and Calibration Line of the Hydantoin (5a). The same work-up procedure described for the determination by GC/MS was applied for the LC/MS/MS method. After extraction with TBME 100 μL of 0.1% formic acid was used as a keeper instead of decane. The extracts were evaporated at the speed vac to a volume of 60−80 μL. The residue volume was increased to 85 μL by adding further 0.1% formic acid. Then 20 μL of MeOH were added and the solution was ultrasonicated for 5 min. An aliquot (10−25 μL) was injected on a C18 reversed-phase column [150 × 1 mm, 3 μm, Luna C18-(2) Column by Phenomenex, Aschaffenburg, Germany] and analyzed with a linear gradient from 20 to 72% MeOH in 15 min and with isocratic conditions at 72% MeOH for 4 min and a flow of 50 μL/min. The flow of 50 μL/min (=$^1/_5$ of total flow) was produced using a high-pressure micro-splitter (UpChurch Scientific, Oak Harbor, WA). To obtain this flow, a second column Hypersil BDS 18, 125 × 2 mm, 3 μm) with a similar back pressure had to be attached to the second channel (waste) of the splitter. The detector was a quadrupole ion trap tandem mass spectrometer (LCQ Duo, Thermoquest). The MS parameters were optimized in the electrospray ionization mode (ESI). Positive ions were detected. Parameter optimization was carried out with a 4 pg/μL solution and a flow rate of 50 μL/min. The signal abundance of *m*/*z* 248 $[M + H]^+$ was maximized with the autotune program. The other parameters were set as follows: capillary temperature of 220 °C, sheath gas flow of 80 (arbitrary units) and auxiliary gas flow of 0. For maximum yield of the daughter ions the collision energy was set at 40% and the following masses were chosen: *m*/*z* 253 (isolation width ± 1.25 amu) → 153 and 248 (isolation width ± 0.75 amu) → 149. The calibration line was linear over the range (0, 0.66, 1.32, 6.59, and 13.2 pmol) (**19a**). The calibration line was generated from the amount (expressed in picomoles) of **19a** (*x*) and the peak ratio (*y*) of **5a** and **5d**: $y = 0.29x$, $r^2 = 0.99$.

Analysis of Human Samples by GC/MS and LC/MS/MS. Globin was isolated as previously described in ref *27*. The procedures described above were followed 50−100 mg of globin were used.

Results and Discussion

Synthesis of Isocyanate Adducts. The urea derivatives of **1a**−**c** with the free amino group of L-valine, L-aspartic acid, and N^{α}-acetyl-L-lysine were synthesized according to Figure 2. The compounds **3a**−**c**, and **7a**−**c** were obtained by adding **1a**−**c** to the corresponding amino acid in 0.5 M $NaHCO_3$ at 80 °C. The products were obtained in yields ranging from 33 to 75% and were fully characterized. The yields were lower than similar reactions in organic solvents because of the concurring hydrolysis of the isocyanate in aqueous solution to the unstable carbamic acid, which decarboxylates and then reacts with another molecule of **1a**−**c** to the symmetric substituted urea. At a more basic pH than the carbonate buffer system or in organic solvents such as pyridine, possibly the yields of the wished products would be higher. For the amino acids with functional groups (**10**, **13**, **16**), the α-amino group was protected with an acetyl group. We did not investigate the optical purity of the products. Under the given reaction conditions, the presence of the negative charge at the carboxylate moiety, and the presence of the N^{α}-acyl group, the racemization of C(α) is facilitated (*42*). However, for the present study, the enantiomeric purity of the products was not important.

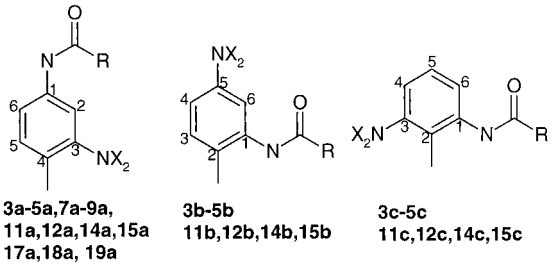

Figure 3. Hydrolysis of N-terminal-valine adduct **19a** in the presence of the internal standard **19d**.

The reaction of **1a**−**c** with functional groups other than the α-amino group was done under various conditions. The synthesis of carbamoyl-adducts from **1a** with thiol groups was performed with *N*-acetyl-cysteine and **1a** at 30 °C in 0.25 M $NaHCO_3$. The carbamic acid *S*-ester **17a** was achieved with an acceptable yield of 60%. *N*-Acetyl-D,L-serine did not react with **1a**−**c** in the hydrogen−carbonate buffer system. The lack of reactivity of the functional groups of these amino acids under the given conditions raises questions about the physiological relevance of these types of adducts, although the reaction with serine is supposed to deactivate cholinesterase in in vitro reactions and in exposed workers (*28*, *32*). Therefore, the reaction of *N*-acetyl-D,L-serine with **1a**−**c** was performed in pyridine. The carbamate **14a**−**c** was obtained in good yield (53−77%).

The nitro group was reduced using two methods: (i) catalytic hydrogenation with hydrogen on Pd/C, and (ii) ammonium formate and Pd/C in methanol. With both methods the reduced compounds are obtained (**4a**−**c**, **8a**−**c**, **12a**−**c**, **15a**−**c**), except for **18a**. The carbamic S-ester **17a** has to be reduced with catalytic hydrogenation to obtain the product **18a**.

Hydantoin formation was achieved by heating the adducts **4a**−**c** in 6 M HCl at 90 °C. The hydantoins (**5a**−**c**) were obtained in ca. 70% yield. The same hydantoin **5a** was generated from the tripeptide adduct *N*-[(3-amino-4-methylphenyl)carbamoyl]valyl-glycyl-glycine (**19a**). The hydantoin **9a** of the aspartic acid adduct **8a** was synthesized in the same way. For GC/MS analysis, **9a** was derivatized with BF_3-MeOH. The purity of the products was determined by GC/MS and EI mode on a fused silica column Rtx5-MS (Restek) 12 m × 0.25 mm internal diameter with a film thickness of 0.25 μm.

Spectroscopic Characterization of the Products. All products were characterized with MS, ^{1}H NMR, and ^{13}C NMR (Figure 3). EI-MS was performed with the hydantoins **5a**−**c** and **9a**. The molecular ion *m*/*z* 247 is present in all three hydantoins **5a**−**c**. The base peak corresponds to the mass of the isocyanates **1a**−**c**. All the other compounds were analyzed using ESI-MS with positive ionization. The $[M + H]^+$ is the base peak for all compounds. Another major fragment is after loss of water or after the loss of the amino acid.

In the ^{1}H NMR spectra, the signals of the aromatic protons of the arylamine moiety are assigned according to the chemical shifts estimated with the increment rules (*43*, *44*). In the following the ^{1}H NMR and ^{13}C NMR shifts are discussed. The three protons of the aromatic ring can be assigned unambiguously with the coupling constants,

except for the products resulting from **1c**. For the compounds synthesized from **1a** the 1,3 coupling constant between H-C(2) and H-C(6) amounts to ca. 2 Hz. The vicinal coupling constants are all ca. 8.0 Hz. The chemical shift of H-C(2) is lower than H-C(6) and H-C(5). The protons of the amino acids were assigned according to the literature (*45*). In all cases, the protons represented an *ABXY*-spectrum except for the valine-adducts with an *AXY*-spectrum. The N−H protons of the arylamine moiety were found between 8.8 and 9.3 ppm for the urea compounds **2a**, **7a**, and **11a**, and above 10 ppm for the carbamate **14a** and for the carbamate S-ester **17a**. The amino protons of the amino acids were found at 6.2−6.7 when attached to the carbamoyl group and at 8.3−8.6 ppm when attached to the *N*-acetyl-group. In the compounds with the reduced nitro compound (**4a**, **8a**, **12a**, **15a**, **18a**): H-C(6), H-C(2), and H-C(5) are shifted with 1.0, 1.5, and 0.5 ppm, respectively, to higher field in comparison to the parent nitro derivatives. The chemical shifts of these compounds are not predicted correctly with the substituent increments or with the ^{1}H NMR predictor by ACD version 4.5 (Advanced Chemistry Development Inc., Toronto, Canada). However, the signals can be assigned with the coupling constants. In the ^{13}C NMR of the nitro-derivatives (**3a**, **7a**, **11a**, **14a**, **17a**), the order of the chemical shifts is the same for all compounds: C(3) > C(1) > C(5) > C(4) > C(6) > C(2). In combination with a DEPT experiment C(4) and C(6), which chemical shifts are only 2 ppm apart, can be distinguished. In the nitro-reduced compounds (**4a**, **8a**, **12a**, **15a**, **18a**), the C(2) and C(6) are only 2.4 ppm apart. For an unambiguous assignment in these cases, a CH-COSY experiment was performed with **4a**. According to the increment rules (*46*) and to the predicted ^{13}C NMR by ACD, the following signal order was elucidated: C(3) > C(1) > C(5) > C(4) > C(6) > C(2). The same interpretations were applied for the interpretation of NMR of adducts of **1b** and **1c**.

In the ^{1}H and ^{13}C NMR of the nitro derivatives (**3b**, **11b**, **14b)** the signal order correspond to the predicted order: H-C(6) > H-C(4) > H-C(3), and C(5) > C(1) > C(2) > C(3) > C(4) > C(6). In the nitro-reduced compounds (**4b**, **12b**, **15b)**, the signal order of the aromatic protons was assigned with the help of the coupling constants H-C(6) > H-C(3) > H-C(4), which does not correspond to the predicted order. For the ^{13}C NMR, the signals could be assigned from the predicted order: C(5) > C(1) > C(3) > C(2) > C(4) > C(6). However, the observed signals of C(4) and C(6) are approximately one ppm. The assignment of these signals was confirmed with a CH-COSY experiment performed with **4b**. For the adducts of **1c** (**3c**, **11c**, **14c**), the signal order of the ^{1}H and ^{13}C NMR corresponded to the prediction: H-C(6) > H-C(4) > H-C(5) and C(3) > C(1) > C(5) > C(6) > C(5) > C(4). The assignments of the carbons C(5) and C(6) which are only ca. 2 ppm apart were confirmed with a CH-COSY experiment. For the reduced derivatives (**4c**, **12c**, **15c)** the predicted and observed order was H-C(6) > H-C(5) > H-C(4) except for **15c** and C(3) > C(1) > C(5) > C(2) > C(6) > C(4). However, the carbons in C(6) and C(2) were observed at 12.0 and 8.5 ppm lower field than predicted.

The spectroscopic properties of the hydantoins need to be discussed separately. In the hydantoin **5a**, H-C(2) and H-C(6) are shifted with ca. 0.2 ppm to higher field and H-C(5) 0.28 ppm to lower field in comparison to the precursor compounds **4a** and **8a**. The vicinal coupling constant of the amino acid NH with the α-CH is around 8 Hz for the precursor compounds **4a** and **8a**. In the case of the hydantoins, this coupling constant is ca. 1 Hz for **5a** and **9a**. This can be explained with the change of the dihedral angle CHNH to near 90°. Semiempirical (AM1, HyperChem 3.5) showed a dihedral angle H−C−N−H of 104°. Compared to the protons of the aliphatic NH group in **4a** and **8a** at ca. 6.3 ppm, the corresponding NH signal shifts 2 ppm downfield to ca. 8.3 ppm for the hydantoins **5a** and **9a**. In the ^{13}C NMR, the shift difference between the hydantoins and the corresponding precursor compounds are more pronounced: C(2) and C(6) shift by ca. 8.0 ppm to lower filed, C(3) and C(5) do not change and C(4) is shifted by 7.0 ppm to lower field. In the hydantoins **5b**−**c** resulting from the isocyanates **1b**−**c**, most signals for the hydrogen or the carbons are doubled, with a shift difference of 0−0.15 ppm and 0−0.5 ppm, respectively. The largest differences are noted for the H-C(6) with 0.14 ppm and C(6) with 0.5 ppm in ortho position to the hydantoin ring. The NMR of the hydantoin **5e** synthesized from 2-fluoro-5-methyl-phenylisocyanate and L-valine did show the expected amount of signals. These results suggest the presence of rotamers for the hydantoins **5b**−**c**. Force field calculations (MM+, HyperChem 3.5) were performed with the compounds **5a** and **5b**. The most stable conformer was found by starting the force field calculations with different rotamers. The most stable rotamer of **5a** has torsion angles of 45° and 50° between the two rings. For **5b** these torsion angles are 93° and 94°. The molecule was then rotated in 10° steps between the two rings. The resulting torsion angles between the two ring systems were kept restraint. The rest of the molecule was optimized without any further restraints. The most unstable rotamer was with both ring systems in the same plane. The maximum energy difference between two rotamers was 19 kcal/mol for **5b** and 6 kcal/mol for **5a**. Similar rotamers have been found in hydantoins obtained from 2-methylphenyl isocyanate (*46*−*50*). Differences were only noted in the ^{13}C NMR-spectra. The ^{1}H NMR spectra were the same for both rotamers.

Analysis of Isocyanate Adducts and Biological Applications. According to the present literature the measurement of N-terminal adducts with blood proteins is the most promising to biomonitor exposure to isocyanates. Therefore, experiments were performed for the detection of such adducts. The hydantoins **5a**−**c** were first analyzed by GC-MS and EI detection. The detection limit was investigated without derivatization by measuring the major single ions, e.g., *m*/*z* 247, and 148. Unfortunately, after a few injections the peak shape of the chromatogram deteriorated drastically. Therefore, quantification without derivatization is not possible. For low level detection (<40 pmol) the hydantoins **5a**−**c** were derivatized with pentafluoropropionic anhydride. This enables the analysis of the compounds by GC-MS with negative chemical ionization (NCI). Only one major fragment is seen in the NCI mode, $[M - HF]^-$. To apply the method for the analysis of human samples we synthesized the N-terminal adduct of **1a** with the tripeptide valyl-glycyl-glycine, *N*-[(3-amino-4-methylphenyl)carbamoyl]valyl-glycyl-glycine (**19a**). As an internal standard we used *N*-[(3-amino-4-methylphenyl-d_6)carbamoyl]valyl-glycyl-glycine (**19d**). The internal standard **19d** and 0−13.2 pmol of **19a** were added to 2 M HCl and globin of an unexposed worker (Figure 4). After 2 h at 100 °C, the solution was neutralized extracted with TBME, derivatized, with pentafluoropropionic anhydride

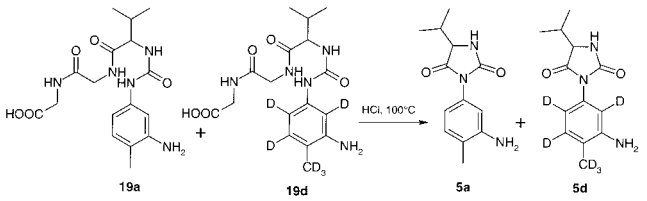

Figure 4. Synthesized products with carbon numbers for the NMR characterization.

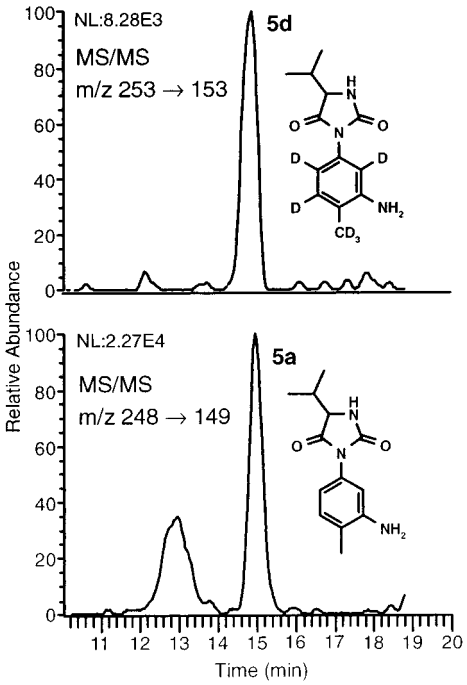

Figure 5. LC/MS/MS chromatogram of a globin extract from a worker exposed to TDI. Adduct **5a** was quantified against **5d**.

(PFPA), evaporated to dryness, and analyzed by GC-MS in the NCI mode. For the quantification of the PFPA-derivatized internal standard **5d** (d-4-TDA-Val-Hyd-PFPA) and of the PFPA-derivatized **5a** (4-TDA-Val-Hyd-PFPA) the single ions 377 and 373 were monitored. A calibration curve was obtained which is linear over the range of 0−13.2 pmol. After ca. 10 injections the glass wool in the liner had to be replaced, since the sensitivity dropped. As an alternative internal standard, we suggest the valine adduct **4e** of 2-fluoro-5-methyl-phenylisocyanate. The obtained calibration curve is linear; however, a background level of 1 pmol **5a** is seen taking **4e** as internal standard.

A LC/MS/MS method was developed. The same work-up described for the GC/MS approach was followed. The derivatization step with PFPA is not necessary in this case. The hydrolysate was analyzed on a RP18 column with 1 mm diameter. The MS/MS ions of *m/z* 248 → 149 and of *m/z* 253 → 153 were monitored for **5a** and for the product **5d** of the internal standard **19d**, respectively (Figures 5−7). The calibration line for the method was linear from 0 to 13.2 pmol. The determination limit was 0.16 pmol/sample. Under the present conditions, not more than one-quarter of the hydrolysate can be injected on the column because of ion suppression effects. By injecting larger amounts the size of the peaks decreases. The method was applied to the analysis of a few biological samples. Blood of control workers and of a worker exposed to TDI was obtained and blood from women with polyurethane (PU)-covered breast implants were avail-

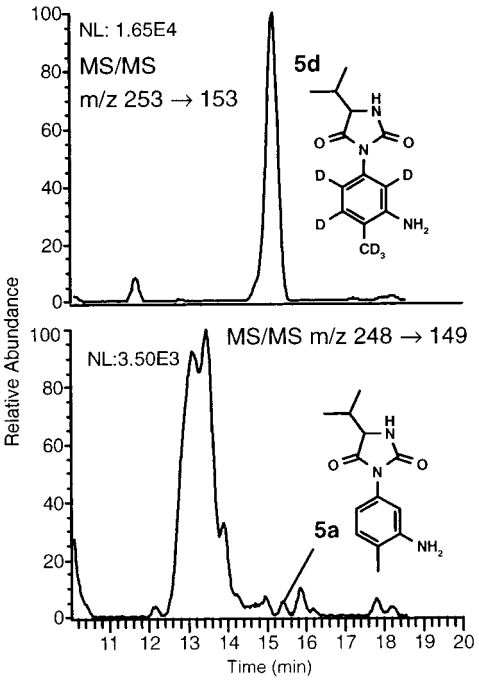

Figure 6. LC/MS/MS chromatogram of a globin extract from patient B: prior to PU-covered-breast implant surgery.

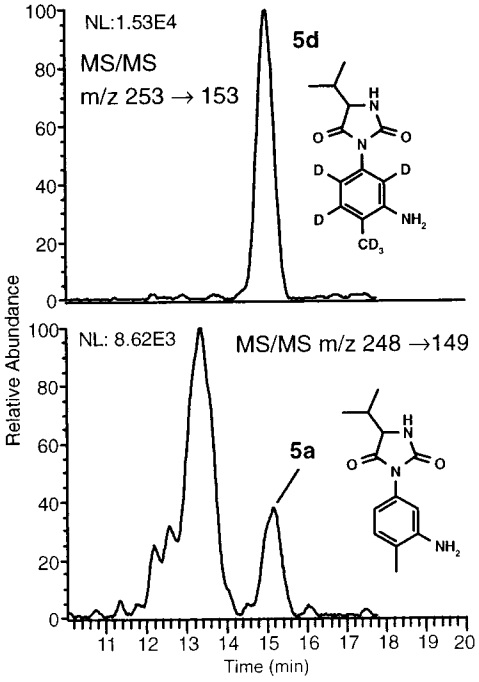

Figure 7. LC/MS/MS chromatogram of a globin extract from patient B: 113 days after PU-covered-breast implant surgery.

able. After isolation of globin the samples were hydrolyzed in the presence of the internal standard **19b**. The highest level was found in the worker with 7.1 pmol of **5a**/100 mg of globin (Figure 5). The possible isomers **5b**,**c** of TDI would elute at 12. 5 and 13 min with no base peak separation. Unfortunately, these peaks are covered by coeluting impurities. The presence of the adduct **5a** was confirmed by GC/MS, t_R = 6.25 min. The PFPA derivatives of the possible products **5b** and **5c** could not be detected by GC-MS at, t_R = 6.15 min. In control workers no adduct could be found. For the breast implant patient A [corresponds to patient A in Sepai et al. (*20*)] no adduct (Figure 6) was found in the first days after surgery (day

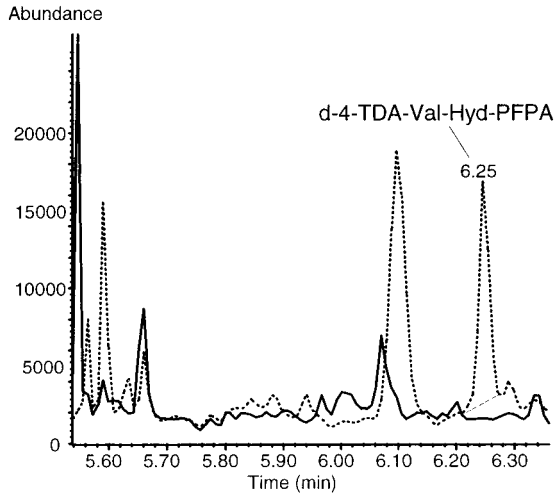

Figure 8. GC/MS chromatogram of a globin extract from patient B: prior to PU-covered-breast implant surgery.

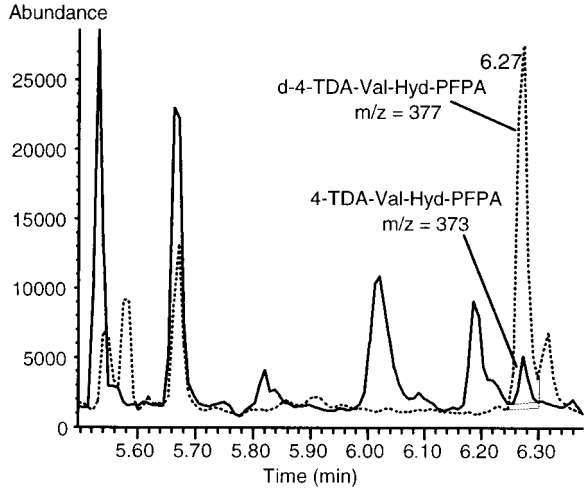

Figure 9. GC/MS chromatogram of a globin extract from patient B: 82 days after PU-covered-breast implant surgery.

0 and day 3). In the following samples taken at 82 and 113 days after surgery, 0.75 and 0.55 pmol (Figure 7) of adduct were found. The presence of the adduct was confirmed by GC-MS (Figures 8 and 9). For patient B [corresponds to patient B in Sepai et al. (*20*)], in the first week after surgery (day 0 and day 7), no adduct with N-terminal valine could be detected. In the next sample taken at day 39, postoperation, 0.46 pmol of **5a** could be found per 100 mg of globin. At day 95 and day 151, the levels increased to 0.54 and 0.70 pmol **5a**/100 mg of Hb. The implant was removed after 151 days. The levels at day 173 dropped to 0.37 pmol. Therefore, to form such adducts, TDI, toluene isocyanate amine, or TDI covalently bound to GSH (*50*) are biologically available. This has been shown in in vitro experiments by Benoit (*51*). The polyurethane foam covers as retrieved from the implants were contaminated on average with 2,4- and 2,6-TDA, 2,4- and 2,6-TDI, and toluene isocyanate amine. In addition several studies showed that the PU-cover of the implants is degraded yielding several unknown products (*52*−*55*). Chan et al. (*56*) determined TDA in urine of women with PU-covered breast implant. Sepai et al. (*20*) have confirmed the presence of TDA in hydrolyzed urine and the presence of plasma protein adducts. TDA from unknown adducts could be released by hydrolysis with HCl. The structure of the parent adduct was not investigated. After 30 days of surgery, 54 pmol of 2,4-TDA/mg of albumin were released. Therefore, binding to albumin is ca. 100 times larger than with globin.

Conclusions

The synthesized adducts can be used for the analysis of biological samples obtained from animals or human exposed to TDI. The analysis of with the N-terminal valine of hemoglobin was applied successfully for the analysis of human samples. The N-terminal amino acid analysis of albumin will be more difficult. After acid hydrolysis, the released hydantoin has to be extracted at acidic pH. For GC/MS analysis, the carboxylic acid has to be esterified with diazomethane or with BF_3/MeOH. To obtain a better response in the NCI-mode, we suggest to derivatize the carboxylic acid with pentafluoroethanol/BF_3. The quantification of adducts with other amino acids will be more difficult. The proteins have to be digested to the single amino acids as demonstrated for albumin adducts of aflatoxin G_1 (*57*). The major problem consists of the enrichment of the modified amino acid. In addition the synthesized adducts might be used to quantify and to test the specificity of the TDI-antibodies present in exposed workers.

Note Added after ASAP Posting

This article was inadvertently released ASAP on 11/20/01 before final corrections were made. For **5b**, ratio of isomers was changed to 1.2/1. The corrected version was posted 12/10/01.

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