Synthesis and Spectroscopic Characterization of 4-Chlorophenyl Isocyanate (= 1-Chloro-4-isocyanatobenzene) Adducts with Amino Acids as Potential Dosimeters for the Biomonitoring of Isocyanate Exposure

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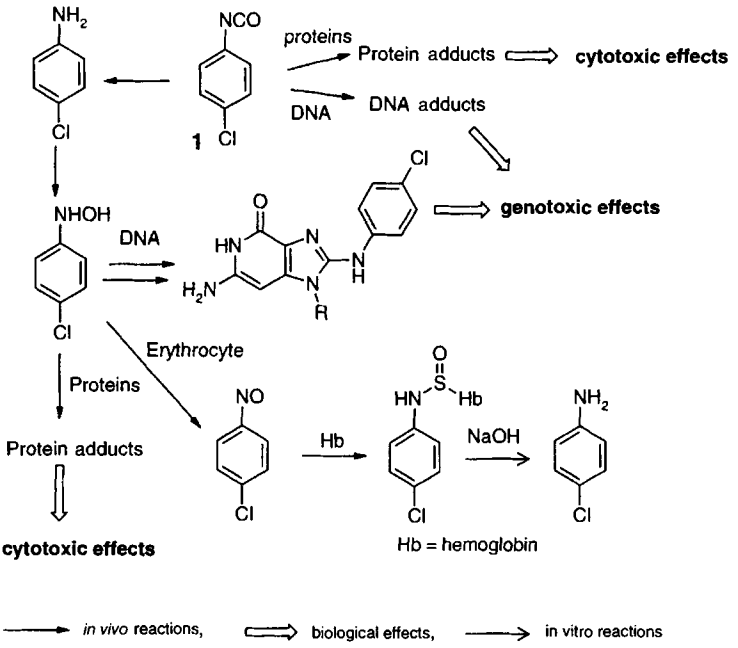
Blood-protein adducts are used as dosimeter for modifications of macromolecules in the target organs where the disease develops. The functional groups of cysteine, tyrosine, serine, lysine, tryptophan, histidine and N-terminal amino acids are potential reaction sites for isocyanates. Especially the N-terminal amino acids, valine and aspartic acid of hemoglobin and albumin, respectively, are reactive towards electrophilic xenobiotics. To develop methods for the quantification of such blood-protein adducts, we treated 4-chlorophenyl isocyanate (**1**) with the tripeptide L-valyl-glycyl-glycine (**2a**) and with single amino acids yielding *N*-[(4-chlorophenyl)carbamoyl]valyl-glycyl-glycine (**3a**), *N*-[(4-chlorophenyl)carbamoyl]valine (**3b**), *N*-[(4-chlorophenyl)carbamoyl]aspartic acid (**3c**), *N*-[(4-chlorophenyl)carbamoyl]glutamic acid (**3d**), *N*-acetyl-*S*-[(4-chlorophenyl)carbamoyl]cysteine (**3e**), and *N*-acetyl-*O*-[(4-chlorophenyl)carbamoyl]serine (**3f**), N^{α}-acetyl-N^{ε}-[(4-chlorophenyl)carbamoyl]lysine (**3g**). For several chemicals, it was shown that blood-protein adducts are good dosimeters of exposure and dosimeters for the target dose. The hydrolysis of the N-terminal adducts of isocyanates release hydantoins which can be separated from the rest of the protein and analyzed using GC/MS or HPLC. This was achieved with **3a**. The released hydantoin could be analyzed using GC/MS. We propose to analyze the N-terminal adducts of isocyanates with blood protein to distinguish between arenamine and arylisocyanate exposure.

1. Introduction. – Monoisocyanates are important intermediates in the manufacturing of polyurethanes, dyes, pigments, pharmaceuticals, and pesticides [1]. Isocyanates cause severe irritation to the mucous membranes of the eyes and respiratory tract on inhalation exposure. The main target organ is the lung where damage to the bronchi, bronchioles, and alveoli occur, depending on the concentration and duration of exposure [2]. The main damage after low levels of isocyanate exposure is, among others, lung sensitization in the form of asthma. The sensitization properties of 4-chlorophenyl isocyanate (= 1-chloro-4-isocyanatobenzene; **1**) are currently under investigation. The corresponding aromatic amine of **1**, 4-chloroaniline (= 4-chlorobenzenamine), is carcinogenic in animal experiments [3]. Aryl isocyanates and arenamines can bind with proteins and/or DNA (*Scheme 1*) and lead to cytotoxic and genotoxic effects. For arenamines, it has been shown that blood-protein adducts are a dosimeter for the adducts in the target organ [4]. Protein adducts of isocyanates might be involved in the etiology of sensitization reactions [5]. An established method to biomonitor exposed people is the determination of adducts with biomolecules. Blood-protein adducts are used as dosimeter for modifications of macromolecules in the target organs where the disease develops. To improve the risk assessment for isocyanate-exposed workers, it is important to develop dosimeters and to establish whether the toxic reactive intermediate is the isocyanate **1** or a metabolite of

Scheme 1. *DNA Adducts and Protein Adducts of Arenamines, Nitroarenes, and Isocyanoarenes*



4-chloroaniline, which may be formed from **1**. Aromatic amines are metabolized to highly reactive *N*-hydroxyarenamines [6] by mixed mono-oxygenases. *N*-Hydroxyarenamines can be further metabolized to *N*-(sulfonyloxy)arenamines, *N*-acetoxyarenamines, or *N*-hydroxyarenamine *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, aromatic amines like biphenyl-4-amine [7], 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 to aromatic amines, isocyanates do not need any further activation to react with biomolecules (*Scheme 1*).

4-Chloroaniline binds with 0.063% of the dose to hemoglobin [8]. The determination of such adducts is well-established [9]. Arenamine-specific adducts are of the sulfinamide type [10]. The chemical structure of isocyanate adducts found *in vivo* is unknown. The main goal of the present study is to synthesize isocyanate typical-protein adducts in order to assess the exposure to isocyanates.

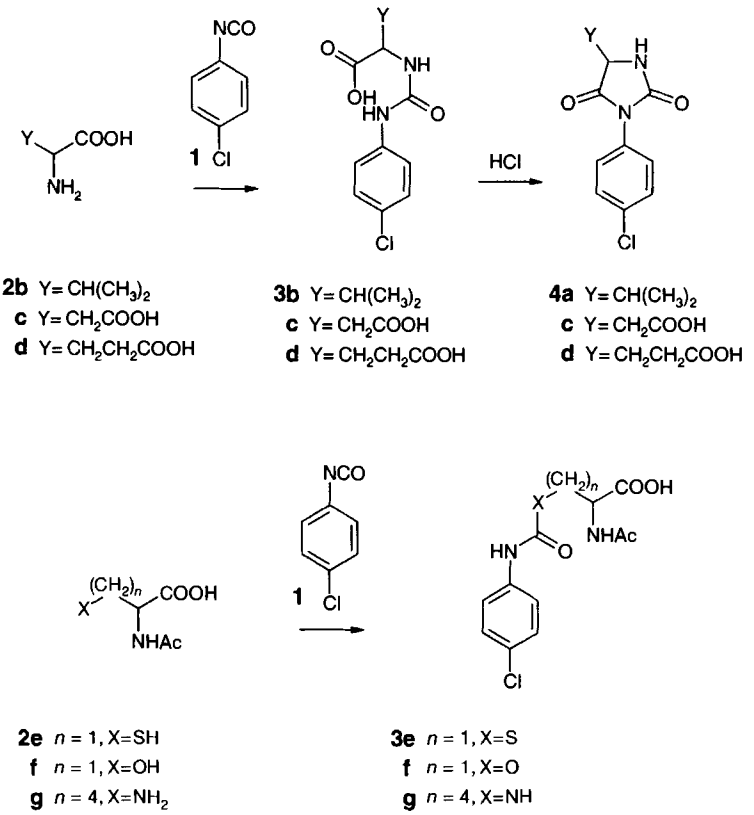
Several methods have been described for the derivatization of proteins with isocyanates and or isothiocyanates. *Stark* and *Smith* [11] used the carbamoylation with potassium cyanate for the N-terminal analysis of proteins. The reaction of phenyl isothiocyanate with amino acids has been used for the *Edman* degradation and sequence analysis of proteins [12]. *Törnqvist et al.* [13] utilized pentafluorophenyl isothiocyanate to analyze the N-terminal-alkylated valines of hemoglobin. Virtually, all functional groups on proteins can react with isocyanates [14]. Under physiological conditions, the potential sites of reaction are restricted to *i*) the N^{α}-amino groups of the N-terminal amino acids,

ii) the mercapto group of cysteine [15], *iii*) the hydroxy groups of tyrosine [16] and especially serine [17], *iv*) the ε-amino group of lysine, and *v*) the imidazole ring of histidine. In proteins, especially reactive lysines have been located [18]. Adducts of lysine have been found *in vivo* with acetaldehyde [19], glycated proteins [20], and aflatoxin B_1 [21].

Karol et al. modified human serum albumin *in vitro* with 4-methylphenyl isocyanate to produce an antigen to assay immunoglobulin IgE of workers exposed to diisocyanatotoluene [22]. These adducts have never been characterized by spectroscopic methods. To biomonitor isocyanate-exposed workers, several research groups have hydrolyzed urine, plasma, albumin, and hemoglobin under acidic and basic conditions and quantified the released parent arenamine with GC/MS [23]. However, the chemical structure of the adducts cleaved from biomolecules is unknown. Putative adducts of isocyanates with biomolecules have to be synthesized to establish whether the aryl isocyanate or the corresponding arenamine is the reacting intermediate. This will improve significantly the risk assessment for isocyanate-exposed workers. Therefore, putative adducts of the isocyanate **1** with proteins were synthesized from amino acids and tripeptides. The following N^{α}-acetyl-protected amino acids were chosen for the reaction with **1**: *N*-acetyl-L-cysteine (**2e**), *N*-acetyl-DL-serine (**2f**), and N^{α}-acetyl-L-lysine (**2g**). As shown for other environmental pollutants, isocyanates might react with the N-terminal amino acids of blood proteins; for hemoglobin and albumin, this would be L-valine (**2b**) and L-aspartic acid (**2c**), respectively. Therefore, the isocyanate **1** was also reacted with **2b** and **2c**. An adduct of **1** with a tripeptide **2a** containing L-valine as the N-terminal amino acid was synthesized to mimic the release of the formed hydantoin from a protein and to have an internal standard for the analyses of hemoglobin obtained from workers exposed to other isocyanates than **1**. The adduct of the substrate **1** with L-glutamic acid (**2d**) was synthesized to obtain a stable marker for the analysis of glutathione (= L-glutamyl-L-cysteinylglycine) adducts of **1** in biological fluids.

2. Results and Discussion. – 2.1. *Synthesis of Isocyanate Adducts.* The urea derivatives with the free amino group of valine, aspartic acid, glutamic acid, and N^{α}-acetyllysine were synthesized according to *Scheme 2*. The compounds **3b**–**d** and **3g** were obtained by adding **1** to the corresponding amino acid **2** in 0.25M $NaHCO_3$ at 80° following a procedure described for the reactions of *N,N*-diphenylcarbamoyl chloride with amino acids [24]. The products were obtained in satisfactory yields (62–91%) and were fully characterized (see *Sect. 2.3*). Two equivalents of **1** were added to the aqueous solution to compensate the loss of **1** by the formation of the symmetrically substituted urea. At a more basic pH than the carbonate buffer system or in organic solvents like pyridine, possibly the yields of the desired products would be higher. However, we chose the aqueous medium to assess the possibility that these adducts may be formed *in vivo* on exposure to isocyanates. Raising the temperature to 80° increased the yield by a factor of 2 compared to the reaction at 37°. For the amino acids **2e**–**g** with functional groups, 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 at C(α) is facilitated. However, for the present study, the enantiomeric purity of the products was not important [25].

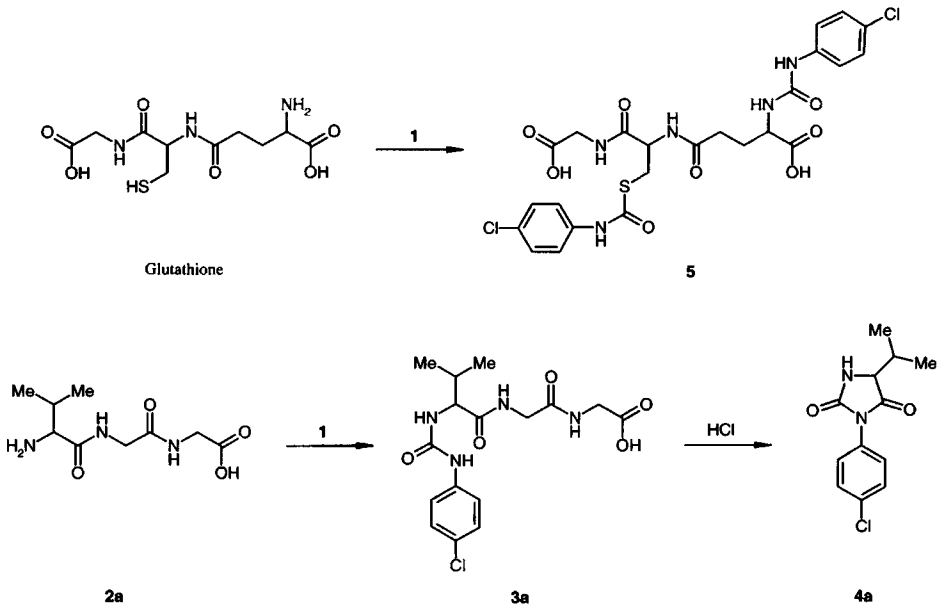
Scheme 2. *Reactions of Amino Acids with 4-Chlorophenyl Isocyanate* (**1**)



The reaction of **1** with other functional groups than the α-amino group were realized by varying the conditions. The carbamic acid *S*-ester **3e** was synthesized from **1** and N^{α}-acetyl-L-cysteine (**2e**) at 30° in sodium hydrogencarbonate buffer. 4-Chloroaniline was the main product if the reaction temperature was kept at 80°. Glutathione (Glu(-Cys-Gly)) was reacted with 2 mol-equiv. of **1** to yield 45% of the bis-adduct **5** (*Scheme 3*), by addition of **1** to the amino group of the Glu moiety and the thiol group of the Cys unit. *N*-Acetyl-L-serine, *N*-acetyl-L-tyrosine, N^{α}-acetyl-L-histidine, and N^{α}-acetyl-L-tryptophan did not react with **1** in the hydrogencarbonate 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 L-serine is supposed to deactivate cholinesterase in *in vitro* reactions and in exposed workers [17][26]. Therefore, the reaction of *N*-acetyl-DL-serine (**2f**), N^{α}-acetyl-L-histidine, and N^{α}-acetyl-L-tryptophan with **1** was performed in pyridine. While the synthesis of the adducts failed for the N^{α}-acetyl-L-histidine and N^{α}-acetyl-L-tryptophan, the carbamate **3f** from **2f** was obtained in 53% yield.

2.2. *Analysis of Isocyanate Adducts.* Hydantoin formation was achieved by heating the adducts **3b–d** in a mixture of dioxane and conc. HCl acid at 90° for 3 h. The hydantoins **4a, c, d** were obtained in 60–85% yield. Compound **4a** was analyzed by

Scheme 3. *Reaction of Glutathione (Glu(-Cys-Gly)) and Val-Gly-Gly with 4-Chlorophenyl Isocyanate* (**1**)



GC/MS using an unpolar capillary column. The major single ions *m/z* 153, 210, and 252 (M^+) were monitored after electron-impact ionization (EI). Injections of 20 pg of product **4a** yielded a peak-to-noise ratio better than 10 monitoring the ion at 252. The same hydantoin **4a** should be released from isocyanate adducts of **1** with the N-terminal L-valine of the α-globin chain of hemoglobin. To develop a method for the quantification of *in vivo* material, we synthesized the tripeptide adduct **3a** from L-valyl-glycyl-glycine (**2a**) and **1**. Similarly, *N*-[(4-methylphenyl)carbamoyl]valyl-glycyl-glycine was synthesized from **2a** and 4-methylphenyl isocyanate (the details of this synthesis will be published elsewhere). For biological samples from workers exposed to **1**, the *N*-[(4-methylphenyl)carbamoyl]valyl-glycyl-glycine can be used as internal standard. In a preliminary experiment, 100 ng of the internal standard and **1**, 10, and 100 ng of the adduct **3a** were added in MeOH to a HCl solution. After 1 h at 100°, the hydrolysate was extracted with CH_2Cl_2, the extract evaporated, and the residue analyzed by GC/MS. Both hydantoins could be monitored, at 5.2 (5-isopropyl-3-(4-methylphenyl)imidazolidine-2,4-dione) and 5.4 min (**4a**, using GC/MS with single-ion monitoring in the EI mode). The method is not sensitive enough to detect the lowest concentration (1 ng).

2.3. *Spectroscopic Characterization of the Products* **3a–g**, **4a, c, d**, *and* **5**. All products are characterized by MS and ^{1}H- and ^{13}C-NMR (see *Exper. Part*). In the EI-MS, the molecular ion is observed for the isocyanate adducts **3b** and the hydantoins **4a, d**. For all compounds, the base peak is at *m/z* 153 (except for **3b, g** (*m/z* 127)) which results from the cleavage of the urea bond with the amino acid (→ $\mathbf{1}^+$). For the other compounds, *i.e.*, **3a, c, e–g**, **4c**, and **5**, FAB-MS are measured. The $[M + 1]^+$ ion is detected for all compounds, except for the glutamic acid adduct **3d**. In the FAB-MS, the $[M + 1]^+$ fragment is the base peak for all compounds, except for the serine adduct **3f** and the glutathione adduct **5**. For the aspartic-acid adduct **3c**, the urea bond is mainly

cleaved between the N(α) of aspartic acid and the carbamoyl group to yield a fragment for aspartic acid (*m/z* 134). In the case of the carbamic acid *S*-ester **3e**, the S-carbamoyl bond is cleaved yielding a fragment for *N*-acetylcysteine (*m/z* 164). For the carbamate adduct **3f** of serine, the main fragments result from the cleavage of the O-alkyl bond giving rise to a serine fragment at *m/z* 130. For the adduct **3a**, the *m/z* of **3b** minus H_2O is the most intensive fragment.

In the ^{1}H-NMR spectra ((D$_6$)DMSO, 25°), the signals of the aromatic protons of the arenamine moiety are assigned according to the chemical shifts estimated with the increment rules [27]. The chemical shift of H–C(2)/H–C(6) is at lower field than that of H–C(3)/H–C(5). The coupling of these aromatic protons should correspond to an *AA'XX'* spectrum. Mostly only 6 lines are visible instead of 20. Only J_{AX} ($J_{A'X'}$) can be assigned, but not $J_{AA'}$ ($J_{XX'}$) and $J_{AX'}$ ($J_{A'X}$). The protons of the amino acids are assigned according to [28]. In all cases, the protons represent an *ABXY* spectrum except for the valine adducts with an *AXY* spectrum. The NH protons of the arenamine moiety are observed at 8.5–9.0 ppm for the urea compounds **3a–d, g**, while for the carbamate **3f** and for the carbamic acid *S*-ester **3e**, the NH protons are observed at 9.87 and 10.51 ppm, respectively. The amino protons of the amino acids are found at 6.4–6.8 ppm when attached for the carbamoyl group and at 7.9–8.3 ppm when attached for the *N*-acetyl group. The vicinal coupling constant of the amino acid NH with H–C(α) is *ca.* 8 Hz for all compounds. In the case of the hydantoins **4a, c**, this coupling constant is 0 Hz. This can be explained with the change of the dihedral angle C–H–N–H to near 90°. Compared to the protons of the aliphatic NH group in **3b, d** at 6.4–6.7 ppm, the corresponding NH signal of the hydantoins **4a, c** is shifted by 2 ppm downfield to 8.4–8.6 ppm.

The signals of the ^{13}C-NMR spectra are estimated and assigned using the increment rules [29]. The degree of C-substitution is determined with a DEPT experiment. The signals for the aromatic C-atoms (C(1) to C(6), C(1) being bound to the amino group) are almost identical for all urea derivatives, except for the hydantoins **4a, c, d** where C(4) is shifted downfield and C(1) upfield, as compared to the corresponding urea compounds **3b–d**. The signals for C(1) and C(4) are so closed together that an unambiguous assignment is not possible without further experiments. The order of chemical shifts is the same for all other compounds: C(1) > C(3), C(5) > C(4) > C(2), C(6). C(1) appears at the lowest field and C(2) and C(6) at the highest field. The signal of the carbamoyl C-atom is around 154–156 ppm for the urea compounds **3b–d, g** and **5** and the hydantoins **4a, c, d**. The carbamoyl signal is shifted by 2 ppm upfield in the case of **3f** and by 8 ppm downfield in the case of **3e**. The C-signals of the amino-acid residues are assigned according to [30]. The resonances of the carboxylic acid and amide moieties are observed at 170–175 ppm.

3. Conclusions. – The synthesized adducts can be used to optimize the analysis of biological samples obtained from animals or humans exposed to **1**. The analysis of the adducts with the N-terminal amino acids of hemoglobin can be performed easily using GC/MS analysis of the organic extract of the acid hydrolysate. 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, and for GC/MS analysis, the carboxylic acid must be esterified with diazomethane or with BF_3/MeOH. To obtain a better response in the CI-MS (neg.-ion mode), we suggest to derivatize the carboxylic acid with penta-

fluoroethanol/BF_3. The quantification of adducts with other amino acids will be more difficult, since the proteins would have to be digested to the single amino acids. The problem consists in the enrichment of the modified amino acid among the large excess of unmodified amino acids.

Experimental Part

1. *General.* L-Aspartic acid, 4-chlorophenyl isocyanate, L-glutathione (reduced), L-glutamic acid, and (D_6)DMSO were purchased from *Fluka* (Neu-Ulm, Germany), *N*-acetyl-L-cysteine from *Merck* (Darmstadt, Germany), N^{α}-acetyl-L-lysine, and L-valyl-glycyl-glycine from *Sigma* (Deisenhofen, Germany), and L-valine from *Serva* (Heidelberg, Germany). HPLC: quaternary HPLC pump with a UV detector both from *Hewlett-Packard series 1050*. M.p.: melting-point apparatus by Dr. *Tottoli* (*510*) from *Büchi*, Glasapparatefabrik Flawil, Switzerland; uncorrected. IR Spectra: *Perkin-Elmer 1420*; in cm^{-1}. NMR Spectra: *Bruker-AC-250* instrument; (D_6)DMSO as solvent and as internal standard (^1H: 2.50 ppm; ^{13}C: 39.43 ppm), δ in ppm, J in Hz; C-substitution determined using the distorsionless enhancement by polarization transfer (DEPT) method; the spectra were evaluated with the program SwaNMR 3.12 by Dr. *Giuseppe Balacco* (*A. Menarini Industrie Farmaceutiche Riunite s.r.l.*, Via Sette Santi 3, I-50131 Firenze). Gaschromatography/mass spectrometry (GC/MS): *Hewlett-Packard GC* (*HP 5890II*) equipped with an autosampler (*HP7276*) and interfaced to a MS (*HP 5989A*); *m/z* (rel.%). Fast atom bombardment mass spectrometry (FAB-MS): *Fison-70-SEQ* mass spectrometer; Xe atoms at 8 keV acceleration; *p*-toluenesulfonic acid/glycerol matrix for pos. mode; *m/z* (rel.%). High-resolution MS (HR-MS): *Finnigan MAT 90*; perfluorokerosene as reference. Elemental analyses: Mikroanalytisches Labor, Institut für Anorganische Chemie, Universität Würzburg.

2. *Ureas from* **1** *and Amino Acids. General Procedure 1* (*GP1*). A soln. of amino acid (1 mmol) or peptide (1 mmol) in 0.25M $NaHCO_3$ (20 ml) was stirred and heated to 80°. Then **1** (307 mg, 2 mmol) was added. A precipitate (= *N,N'*-bis(4-chlorophenyl)urea according to ^1H-NMR) formed immediately. After 1 h reaction and after cooling with ice, the precipitate was filtered off and washed with H_2O (20 ml) and EtOH (1 ml). The filtrate was carefully acidified to pH 2 with 2M HCl and cooled. The precipitate was filtered and redissolved in AcOEt (50 ml). The org. phase was extracted twice with sat. $NaHCO_3$ soln. (20 ml), the aq. phase acidified and extracted with AcOEt, the extract dried ($MgSO_4$) and evaporated, and the residue recrystallized with EtOH/H_2O.

N-*[(4-Chlorophenyl)carbamoyl]valyl-glycyl-glycine* (**3a**). According to *GP1*, with L-valine-glycine-glycine (107 mg, 0.5 mmol). Crystallization from EtOH yielded **3a** (120 mg, 62%). White solid. M.p. 227° (dec.). ^1H-NMR ((D_6)DMSO): 8.83 (*s*, NH); 8.36 ('*t*', NHCH$_2$); 8.13 ('*t*', NHCH$_2$); 7.38 (*d*, J = 8.7, H–C(2), H–C(6)); 7.23 (*d*, J = 8.7, H–C(3), H–C(5)); 6.40 (*d*, J = 8.6, NHCH); 4.10 (*m*, NHCH); 3.74 (*s*, 2 H, CH$_2$(Gly)); 3.72 (*s*, 2 H, CH$_2$(Gly)); 1.95 (*m*, CH); 0.86 (*d*, J = 6.7, 1 Me); 0.82 (*d*, J = 6.6, 1 Me); signal for COOH not visible. ^{13}C-NMR ((D_6)DMSO): 171.8, 171.0, 169.0 (2 NHCO, COOH); 154.8 (NHCONH); 139.2 (C(1)); 128.5 (C(3), C(5)); 124.4 (C(4)); 118.8 (C(2), C(6)); 57.6 (CHCOOH); 41.5 (CH$_2$NH); 40.4 (CH$_2$NH); 30.9 (MeCH); 19.2 (MeCH); 17.6 (MeCH). FAB-MS: 387 (37), 385 (100, $[M + 1]^+$), 351 (24), 319 (22), 303 (32), 277 (44), 255 (54), 253 (75), 249 (44).

N-*[(4-Chlorophenyl)carbamoyl]valine* (**3b**). According to *GP1*, with L-valine (117 mg, 1 mmol). Crystallization from EtOH yielded **3b** (190 mg, 70%). White needles. M.p. 149°. IR (KBr): 3340 (NH), 1700 (COOH), 1630 (N–CO). ^1H-NMR ((D_6)DMSO): 8.77 (*s*, NH); 7.41 (*d*, J = 8.9, H–C(2), H–C(6)); 7.28 (*d*, J = 8.9, H–C(3), H–C(5)); 6.45 (*d*, J = 8.8, NHCH); 4.12 (*dd*, J = 4.9, 8.8, NHCH); 2.10 (*m*, CH); 0.92 (*d*, J = 6.8, Me); 0.87 (*d*, J = 6.8, Me); signal for COOH not visible. ^{13}C-NMR ((D_6)DMSO): 175.6 (COOH); 154.3 (NHCONH); 139.1 (C(1)); 128.5 (C(3), C5)); 124.6 (C(4)); 118.8 (C(2), C(6)); 57.1 (CHCOOH), 30.1 (Me$_2$CH); 19.1 (Me); 17.4 (Me). EI-MS: 272 (1.6), 270 (5, M^+), 254 (12), 252 (37, $[M - 18]^+$), 212 (15), 210 (43), 189 (11), 155 (27), 153 (86, [4-ClPhNCO]$^+$), 129 (29), 127 (100, [4-ClPhNH$_2$]$^+$), 125 (20), 101 (39), 90 (18), 65 (14), 55 (13), 44 (12), 43 (21), 41 (21). Anal. calc. for $C_{12}H_{15}ClN_2O_3$ (270.72): C 53.24, H 5.59, N 10.35; found: C 53.36, H 5.57, N 10.28. HR-MS: 270.077 (calc. 270.077).

N-*[(4-Chlorophenyl)carbamoyl]aspartic Acid* (**3c**). According to *GP1*, with L-aspartic acid (266 mg, 2 mmol). Crystallization from EtOH yielded **3c** (527 mg, 91%). White needles. M.p. 165°. ^1H-NMR ((D_6)DMSO): 12.65 (*s*, 2 COOH); 9.00 (*s*, NH); 7.44 (*d*, J = 9.0, H–C(2), H–C(6)); 7.27 (*d*, J = 9.0, H–C(3), H–C(5)); 6.58 (*t*, J = 8.5, CHNH); 4.50 (*ddd*, J = 4.9, 5.4, 8.5, CHNH); 2.74 (*dd*, J = 5.4, 16.8, 1 H, CH$_2$CH); 2.69 (*dd*, J = 4.9, 16.8, 1 H, CH$_2$CH). ^{13}C-NMR ((D_6)DMSO): 172.9 (COOH); 172.2 (COOH); 154.5 (NHCONH); 139.2 (C(1)); 128.5 (C(3), C(5)); 124.6 (C(4)); 118.9 (C(2), C(6)); 48.6 (CHCOOH); 36.6 (CH$_2$COOH). FAB-MS: 289 (38), 287 (100, $[M + 1]^+$), 173 (48), 157 (48), 145 (67), 139 (60), 134 (53), 129 (59), 103 (63).

N-*[(4-Chlorophenyl)carbamoyl]glutamic Acid* (**3d**). According to *GP1* with L-glutamic acid (147 mg, 1 mmol). Crystallization from EtOH yielded **3d** (240 mg, 80%). White needles. M.p. 168° (dec.). ^{1}H-NMR ((D$_6$)DMSO): 12.51 (*s*, 2 COOH); 8.78 (*s*, NH); 7.43 (*d*, *J* = 8.9, H–C(2, H–C(6)); 7.28 (*d*, *J* = 8.9, H–C(3), H–C(5)); 6.53 (*d*, *J* = 8.8, NHCH); 4.22 (*ddd*, *J* = 4.9, 8.8, NHC*H*); 2.30 (*m*, 2 H, CH$_2$); 2.04, 1.83 (2*m*, 2 H, CH$_2$). ^{13}C-NMR ((D$_6$)DMSO): 173.8 (CO$_2$H); 173.7 (CO$_2$H); 154.7 (NHCONH); 139.1 (C(1)); 128.5 (C(3), C(5)); 124.7 (C(4)); 119.1 (C(2), C(6)); 51.6 (CH); 29.9 (CH$_2$); 27.1 (CH$_2$). EI-MS: 284 (7), 282 (23, [*M* – 18]$^+$), 266 (9), 264 (28, [*M* – (2 × 18)]$^+$), 224 (11), 223 (10), 222 (30, [*M* – COCH$_2$]$^+$), 155 (33), 154 (12), 153 (100, [4-ClPhNCO]$^+$), 129 (25), 128 (7), 127 (68, [4-ClPhNH$_2$]$^+$), 125 (18), 92 (11), 90 (15), 84 (52), 65 (16), 55 (10), 44 (23), 41 (26). Anal. calc. for $C_{12}H_{13}ClN_2O_5$ (300.70): C 47.93, H 4.36, N 9.32; found: C 48.14, H 4.30, N 9.01.

N$^{\alpha}$-*Acetyl*-N$^{\varepsilon}$-*[(4-chlorophenyl)carbamoyl]lysine* (**3g**). According to *GP1*, with *N*$^{\alpha}$-acetyl-L-lysine (188 mg, 1 mmol). Crystallization from EtOH yielded **3g** (307 mg, 90%). White solid. M.p. 169°. ^{1}H-NMR ((D$_6$)DMSO): 8.55 (*s*, NH); 8.11 (*d*, *J* = 7.3, 1 NHCO); 7.40 (*d*, *J* = 8.8, H–C(2), H–C(6)); 7.24 (*d*, *J* = 8.8, H–C(3), H–C(5)); 6.17 (*t*, *J* = 5.7, CH$_2$N*H*); 4.14 (*m*, C*H*NH); 3.05 (*m*, C*H*$_2$NH); 1.84 (*s*, Me); 1.75–1.25 (*m*, 3 CH$_2$); signal for COOH not visible. ^{13}C-NMR ((D$_6$)DMSO): 173.7 (COOH); 169.3 (Me*C*O); 155.0 (NHCONH); 139.5 (C(1)); 128.3 (C(3), C(5)); 124.3 (C(4)); 118.9 (C(2), C(6)); 51.7 (*C*HCOOH); 40.6 (CH$_2$(*ε*)); 30.7 (CH$_2$(*β*)); 29.3 (CH$_2$(*δ*)); 22.8 (CH$_2$(*γ*)); 22.2 (*Me*CO). EI-MS: 155 (6), 153 (17), 129 (27), 127 (100), 125 (7), 100 (11), 92 (15), 65 (18), 44 (54).

3. *Carbamic Acid* S-*Esters*. N-*Acetyl*-S-*[(4-chlorophenyl)carbamoyl]cysteine* (**3e**). To a soln. of *N*-acetyl-L-cysteine (163 mg, 1 mmol) in 0.25M $NaHCO_3$ (10 ml), **1** (307 mg, 2 mmol) was added. The mixture was treated by ultrasound during 3 min in 10-min intervals at 25° for 1 h. Purification was achieved by short-column chromatography (*KG 60 Merck*, 63–200 μm, petroleum ether/Et$_2$O 1:4). Crystallization from EtOH yielded **3e** (235 mg, 74%). Yellowish-brown crystals. M.p. 156°. IR (KBr): 3300 (NH), 1665 (N–CO–S). ^{1}H-NMR ((D$_6$)DMSO): 10.51 (*s*, NHCOS); 7.95 (*d*, *J* = 7.9, AcN*H*); 7.53 (*d*, *J* = 8.9, H–C(2), H–C(6)); 7.35 (*d*, *J* = 8.9, H–C(3), H–C(5)); 4.30 (*m*, C*H*CH$_2$S); 3.44 (*dd*, *J* = 4.7, 13.3, 1 H, CH$_2$S); 3.08 (*dd*, *J* = 8.2, 13.3, 1 H, CH$_2$S); 1.84 (*s*, Ac); signal for COOH not visible. ^{13}C-NMR ((D$_6$)DMSO): 169.2, 169.0 (Me*C*O, COOH); 163.9 (NHCOS); 137.9 (C(1)); 128.7 (C(3), C(5)); 126.7 (C(4)); 120.4 (C(2), C(6)); 53.0 (*C*HCOOH); 31.9 (CH$_2$S); 22.6 (*Me*CO). FAB-MS: 319 (38), 317 (100, [*M* + 1]$^+$), 164 (58, [*M* – AcCys]$^+$).

N,S-*Bis[(4-chlorophenyl)carbamoyl]glutathione* (= N-*[(4-Chlorophenyl)carbamoyl]-γ-glutamyl*-S-*[(4-chlorophenyl)carbamoyl]cysteinyl-glycine*; **5**). According to *GP1* with GSH (307 mg, 1 mmol): **5** (280 mg, 45%). Yellowish powder. M.p. 164° (dec.). ^{1}H-NMR ((D$_6$)DMSO): 10.54 (*s*, NHCOS); 9.14 (*s*, NHCO); 8.36 (*d*, *J* = 8.5, N*H*CH(Cys)); 8.27 (*t*, *J* = 5.8, N*H*CH$_2$); 7.53 (*d*, *J* = 8.9, 2 H); 7.43 (*d*, *J* = 8.9, 2 H); 7.35 (*d*, *J* = 8.9, 2 H); 7.26 (*d*, *J* = 8.9, 2 H); 6.73 (*d*, *J* = 7.6, N*H*CHCH$_2$CH$_2$); 4.48 (*m*, C*H*CH$_2$CH$_2$); 4.16 (*m*, C*H*CH$_2$S); 3.73 (*m*, NOCC*H*$_2$*N*); 3.39 (*dd*, *J* = 4.8, 13.5, 1 H, CH$_2$S); 2.99 (*dd*, *J* = 9.5, 13.5, 1 H, CH$_2$S); 2.25 (*m*, 2 H, CH$_2$); 1.90, 1.70 (2*m*, 2 H, CH$_2$); signal for COOH not visible. ^{13}C-NMR ((D$_6$)DMSO): 174.0, 171.7, 171.0, 170.2 (2 NHCO, 2 COOH); 164.6 (NHCOS); 154.8 (NHCONH); 139.4 (C); 137.8 (C); 128.8 (CH); 128.5 (CH); 127.0 (C); 124.5 (C); 120.4 (CH); 119.0 (CH); 52.3 (CH); 52.1 (CH); 41.1 (CH$_2$); 31.5 (CH$_2$); 31.2 (CH$_2$); 28.0 (CH$_2$). FAB-MS: 616 (34), 614 (49, [*M* + 1]$^+$), 461 (35), 395 (72), 381 (34), 319 (75), 303 (100), 289 (89), 287 (89).

4. *Carbamates*. N-*Acetyl*-O-*[(4-chlorophenyl)carbamoyl]serine* (**3f**). *N*-Acetyl-DL-serine (**2f**; 84.5 mg, 0.5 mmol) was dissolved in dry pyridine (3 ml). After addition of **1** (0.55 mmol), the soln. was stirred and heated to 80° for 2 h. The solvent was evaporated, the residue taken up in 0.5M $NaHCO_3$ (15 ml), the insoluble residue separated by centrifugation, the aq. phase washed with AcOEt (2 × 15 ml), and the aq. phase acidified with HCl to pH 1.5 and extracted with AcOEt (3 × 30 ml). The org. phase was dried ($MgSO_4$) and evaporated and the residue recrystallized from EtOH/H_2O: **3f** (82 mg, 53%). Yellowish crystals. M.p. 180–183°. ^{1}H-NMR ((D$_6$)DMSO): 12.9 (br. *s*, COOH); 9.87 (*s*, ArN*H*); 8.33 (*d*, *J* = 7.8, AcN*H*); 7.50 (*d*, *J* = 8.9, H–C(2), H–C(6)); 7.33 (*d*, *J* = 8.9, H–C(3), H–C(5)); 4.54 (*m*, C*H*CH$_2$O); 4.42 (*dd*, *J* = 4.5, 11.0, 1 H, CHC*H*$_2$O); 4.21 (*dd*, *J* = 11.0, 6.7, 1 H, CHC*H*$_2$); 1.87 (*s*, Ac). ^{13}C-NMR ((D$_6$)DMSO): 170.9 (COOH); 169.4 (Me*C*O); 152.9 (NHCOO); 137.9 (C(1)); 128.5 (C(3), C(5)); 126.0 (C(4)); 119.7 (C(2), C(6)); 59.7 (COO*C*H$_2$CH); 51.4 (COOCH$_2$*C*H); 20.7 (*Me*CO). FAB-MS: 303 (25), 301 (74, [*M* + 1]$^+$), 267 (12), 130 (100).

5. *Hydantoins. General Procedure 2* (*GP2*). The urea derivatives **3b–d** (0.2 mmol) were dissolved in dioxane/conc. HCl soln. 10:3 (13 ml) and heated for 3 h at 80–90°. The mixture was evaporated, the residue 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-(4-Chlorophenyl)-5-isopropylimidazoline-2,4-dione (**4a**). According to *GP2*, with **3b** (54 mg, 0.2 mmol). Crystallization from EtOH yielded **4a** (41 mg, 81%). White needles. M.p. 156°. IR (KBr): 3210 (NH), 1760 (NCOC), 1710 (NCON). ^{1}H-NMR ((D$_6$)DMSO): 8.62 (*s*, NH); 7.55 (*d*, *J* = 8.8, H–C(2), H–C(6)); 7.38 (*d*, *J* = 8.8, H–C(3), H–C(5)); 4.14 (*d*, *J* = 3.5, CH); 2.10 (*m*, CH); 1.01 (*d*, *J* = 7.2, Me); 0.87 (*d*, *J* = 6.8, Me).

^{13}C-NMR ((D$_6$)DMSO): 172.7 (CO); 155.8 (NHCONH); 132.2 (C); 131.0 (C); 128.9 (CH); 128.3 (CH); 61.5 (CH); 30.0 (CH); 18.5 (Me); 15.9 (Me). EI-MS: 254 (29, $[M + 2]^+$), 252 (89, M^+), 212 (27), 210 (82, $[M - COCH]^+$), 189 (18), 155 (33), 154 (15), 153 (100, $ClPhNCO^+$), 125 (12), 112 (13), 43 (16). Anal. calc. for $C_{12}H_{13}ClN_2O_2$ (252.70): C 57.04, H 5.19, N 11.09; found: C 57.26, H 5.30, N 11.03. HR-MS: 252.067 (calc. 252.066).

1-(4-Chlorophenyl)-2,5-dioxoimidazolidine-4-acetic Acid (**4c**). According to *GP2*, with **3c** (57.3 mg, 0.2 mmol). Crystallization yielded **4c** (32 mg, 60%). M.p. 210°. ^{1}H-NMR ((D$_6$)DMSO): 8.47 (*s*, NH); 7.54 (*d*, *J* = 8.77, H–C(2), H–C(6)); 7.36 (*d*, *J* = 8.77, H–C(3), H–C(5)); 4.40 (*m*, C*H*CH$_2$); 2.82 (*dd*, *J* = 5.2, 17.2, 1 H, CHC*H*$_2$); 2.75 (*dd*, *J* = 4.4, 17.2, 1 H, CHC*H*$_2$); signal for COOH not visible. ^{13}C-NMR ((D$_6$)DMSO): 172.7, 170.9 (COOH, CONH); 155.6 (NCONH); 131.9 (C); 131.2 (C); 128.6 (CH); 128.1 (CH); 52.9 (*C*HCH$_2$); 35.3 (CH*C*H$_2$): FAB-MS: 271 (43), 269 (100, $[M + 1]^+$), 157 (89), 139 (75), 129 (56).

1-(4-Chlorophenyl)-2,5-dioxoimidazolidine-4-propanoic Acid (**4d**). According to *GP2*, with **3d** (61 mg, 0.2 mmol). Crystallization yielded **4d** (48 mg, 85%). White powder. M.p. 212°. ^{1}H-NMR ((D$_6$)DMSO): 8.60 (*s*, NH); 7.55 (*d*, *J* = 8.8, H–C(2), H–C(6)); 7.41 (*d*, *J* = 8.8, H–C(3), H–C(5)); 4.25 (*t*, *J* = 6.3, CH); 2.41 (*t*, *J* = 7.6, 2 H, CH$_2$); 2.03, 1.89 (2*m*, 2 H, CH$_2$). ^{13}C-NMR ((D$_6$)DMSO): 173.6, 172.8 (NHCO, COOH); 155.2 (NHCONH); 132.0 (C); 131.0 (C); 128.6 (CH); 128.3 (CH); 55.4 (CH); 29.0 (CH$_2$); 26.8 (CH$_2$). EI-MS: 284 (9), 282 (28, M^+), 266 (16), 264 (48, $[M - 18]^+$), 224 (18), 223 (19), 222 (56, $[M - COCH_2]^+$), 155 (36), 154 (18), 153 (100, $ClPhNCO^+$), 129 (6), 127 (22, $ClPhNH_2^+$), 125 (25), 111 (16), 90 (22), 73 (14), 55 (20), 54 (10), 42 (12), 41 (17), 39 (15). HR-MS: 282.041 (calc. 282.041). Anal. calc. for $C_{12}H_{11}ClN_2O_4$ (282.68): C 50.99, H 3.92, N 9.91; found: C 51.04, H 3.90, N 9.78.

Gas-Chromatographic Analyses of Hydantoin **4a**. To 6M HCl (1 ml), **3a** (100, 10, 1 ng) and *N*-[(4-methylphenyl)carbamoyl]valyl-glycyl-glycine (100 ng) in MeOH (100 µl) was added, and the mixture was refluxed for 1 h. The hydrolyzate was basified to pH 9 with NaOH and then extracted with CH_2Cl_2 (3 ml). The org. phase was evaporated under a stream of N_2. The residue was taken up in AcOEt (15 µl). Aliquots of 1 µl were analyzed by splitless injection onto a fused silica capillary column (*Rtx-5MS*; i.d. 0.25 mm, length 12 m, 0.5 µm film thickness) with a *Hewlett-Packard* chromatograph (model *5890II*) coupled to a mass spectrometer as detector (*HP 5989A*). The injector and the transfer-line temp. was set at 260°. The oven temp. was kept for 1 min at 50° and then increased at a rate of 50°/min to 260°.

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