Reactions of 4-methylphenyl isocyanate with amino acids

Gabriele Sabbioni, John H. Lamb, Peter B. Farmer and Ovnair Sepai

Arylisocyanates are important intermediates in the chemical industry. Amongst the main damage after low levels of isocyanate exposure are lung sensitization and asthma. Protein adducts of isocyanates might be involved in the aetiology of sensitization reactions. Blood protein adducts are used as dosimeters for modifications of macromolecules in the target organs where the disease develops. To develop methods for the quantitation of protein adducts we reacted 4-methylphenyl isocyanate (4MPI) with the tripeptide valyl-glycyl-glycine and with single amino acids yielding *N*-(4-methylphenyl-carbamoyl)-L-valyl-glycyl-glycine (4MPI-Val-Gly-Gly), *N*-(4-methylphenyl-carbamoyl)- L-valine (4MPI-Val), *N*-(4-methylphenyl-carbamoyl)- L-aspartic-acid (4MPI-Asp), N_α-acetyl-*S*-(4-methylphenyl-carbamoyl)- L-cysteine (4MPI-AcCys), N_α-acetyl-*N*-(4-methylphenyl-carbamoyl)- ε-lysine (4MPI-AcLys), N_α-acetyl-*O*-(4-methylphenyl-carbamoyl)-tyrosine (4MPI-AcTyr) and N_α-acetyl-*O*-(4-methylphenyl-carbamoyl)- D,L-serine (4MPI-AcSer). The hydrolysis of the adducts was tested under acidic and basic conditions, to obtain the maximum yield of 4-methylaniline (4MA). The isocyanates were hydrolysed for 1 h, 3h and 24h at 100°C with 6 M HCl in and/or 0.1 M NaOH at room temperature, following methods applied for the analyses of biological samples of arylisocyanate-exposed workers. In addition, we applied a new protocol: the adducts were hydrolyzed for 1–24 h in 0.3 M NaOH at 100°C. The hydrolysates were analysed using HPLC with UV-detection and quantified against the internal standard, 4-fluoroaniline or 4-chloroaniline. 4MA was obtained with the best yields using 0.3M NaOH; after 24 h all amino acid adducts were cleaved under these conditions. Acid hydrolysis of 4MPI-Val and 4MPI-Asp yielded the respective hydantoins 3-(4-methylphenyl)-5-isopropyl-1,3-imidazoline-2,4-dione and 2-(1-(4-methylphenyl)-2,5-dioxoperhydro-4-imidazolyl) acetic acid. For future studies, we propose to hydrolyse biological samples with 0.3 M NaOH at 100°C to release the maximum amount of 4MA from the adducts. However, in biological samples from workers, hydrolysable adducts can also result from arylamine exposure. Therefore, we propose to analyse the N-terminal adducts of isocyanates with blood protein to distinguish between arylamine and arylisocyanate exposure.

Keywords: *isocyanates, protein adducts, amino acid adducts.*

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Introduction

Monoisocyanates are important intermediates in the manufacturing of polyurethanes, dyes, pigments, pharmaceuticals and pesticides. Higher concentrations of isocyanates are presumed to be a potent respiratory irritant. Amongst the main damage after low levels of isocyanate exposure are lung sensitization and asthma. The sensitization properties of 4-methylphenylisocyanate (4MPI) are currently under investigation. The corresponding aromatic amine of 4MPI, 4-methylaniline (4MA), is carcinogenic in animal experiments. Arylisocyanates and arylamines can bind with proteins and/or DNA (Figure 1) and lead to cytotoxic and genotoxic effects. Protein adducts of isocyanates might be involved in the aetiology of sensitization reactions. An established method to biomonitor exposed people is the determination of adducts with biomolecules and blood protein adducts have been widely used as dosimeters for modifications of macromolecules in the target organs where the disease develops. To improve the risk assessment for workers exposed to 4MPI, it is important to develop dosimeters to establish if the toxic reactive intermediate is 4MPI or a metabolite of 4MA. Aromatic amines are metabolized to highly reactive *N*-hydroxy arylamines (Kadlubar and Beland 1985, Beland and Kadlubar 1990) by mixed function mono-oxygenases. *N*-Hydroxy arylamines can be further metabolized to *N*-sulphonyloxy arylamines, *N*-acetoxy arylamines or *N*-hydroxy arylamine *N*-glucuronides. These highly reactive intermediates are responsible for the genotoxic and cytotoxic effects of this class of compounds. In exposed animals aromatic amines such as 4-aminobiphenyl (4ABP) (Kadlubar *et al.* 1989), a human bladder carcinogen, are known to form adducts with DNA, with tissue proteins, and with the blood proteins albumin and haemoglobin in a dose-dependent manner. In contrast, isocyanates do not need any further activation to react with biomolecules (Figure 1).

4MA binds with 0.063% of the dose to haemoglobin (Birner and Neumann 1988, Sabbioni 1992). The determination of such adducts is well established (Sabbioni 1994a, b, Sabbioni and Beyerbach 1995, Sabbioni and Sepai 1995). Arylamine specific adducts are of the sulphinic acid amide type (Eyer 1988, Kazanis and McClelland 1992). The chemical structure of isocyanate adducts found *in vivo* is unknown. The main goal of the present study is to synthesize typical isocyanate protein adducts in order to assess the exposure to isocyanates.

Several applications have been described for the derivatization of proteins with isocyanates and/or isothiocyanates. Stark and Smith (1963) used the carbamoylation with potassium cyanate to the *N*-terminal analysis of protein. Phenylisothiocyanate reactions with amino acids have been used for the Edman degradation and sequence analysis of proteins (Edman 1949, Edman and Henschen 1975). Törnqvist *et al.* (1986) used pentafluorophenylisothiocyanate to analyse the *N*-terminal-alkylated valines of haemoglobin. Virtually all functional groups on proteins can react with isocyanates (Brown 1986, Brown *et al.* 1987) but under physiological conditions the potential sites of reaction are restricted: (i) the N_α-amino-groups of the *N*-terminal amino

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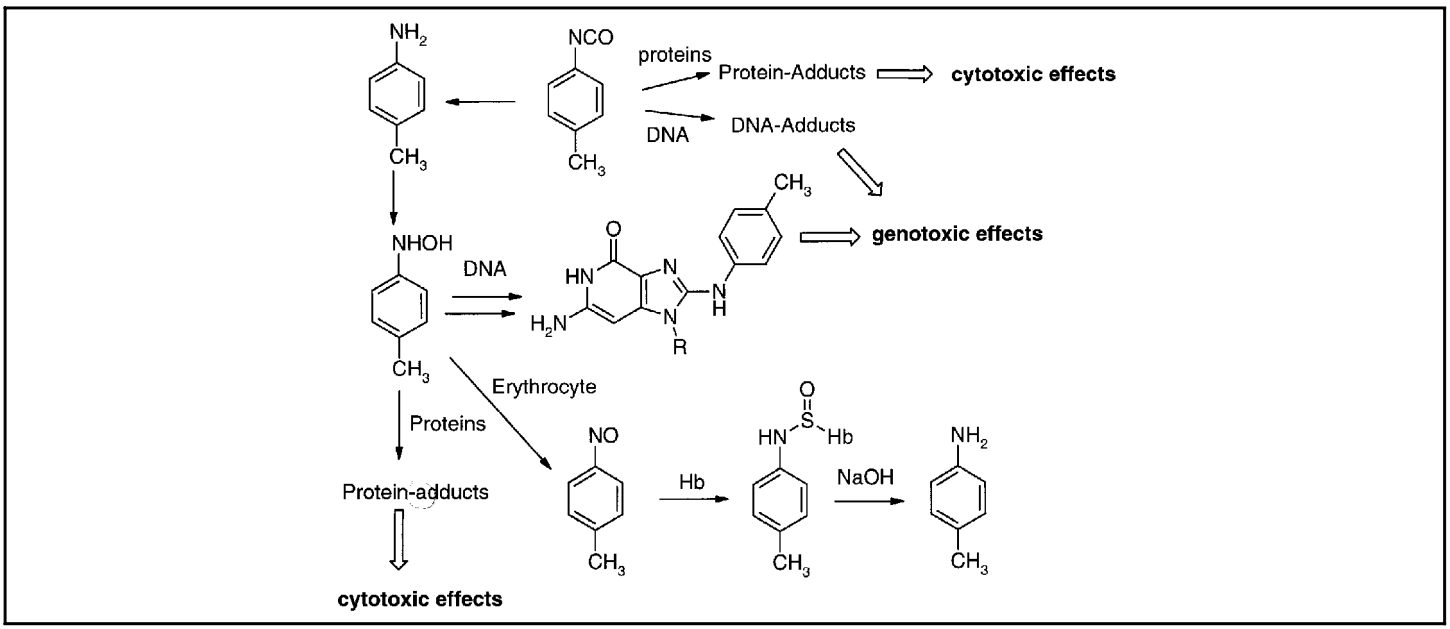

Figure 1. Reactions of 4MPI and 4MA with proteins and DNA.

acids, (ii) the sulphydryl group of cysteine (Hubbell and Casida 1977, Pearson *et al.* 1990, 1991, Slatter *et al.* 1991, 1993, Guan *et al.* 1994, Jochheim and Baillie 1994), (iii) the hydroxyl groups of tyrosine (Twu and Wold 1973) and especially serine (Brown and Wold 1973, Brown 1975), (iv) the ε-amino-group of lysine and (v) the imidazole ring of histidine. Although the pK_a of lysine is around 10.5, in proteins especially reactive lysines have been located (Brown and Shockley 1982, Skipper 1996). *In vivo* adducts of lysine have been found with acetaldehyde (Tuma and Sorrell 1985), glycated proteins (Vasan *et al.* 1996) and aflatoxin B1 (Sabbioni 1990).

In the past human serum albumin was modified *in vitro* with 4MPI to produce an antigen to assay immunoglobulin IgE of workers exposed to toluene diisocyanate (TDI) (Karol *et al.* 1978a, Game 1982). These adducts have never been chemically characterized. Furthermore, to biomonitor isocyanate-exposed workers, several research groups have hydrolysed urine, plasma, albumin and haemoglobin under acidic and basic conditions and quantified the released parent arylamine with GC–MS. The chemical structure of the adducts prior to cleavage is unknown. Putative adducts of isocyanates with biomolecules have to be synthesized to establish the reacting intermediate; arylisocyanate or arylamine. This will improve the risk assessment for isocyanate-exposed workers. Therefore, putative reaction products of 4MPI with proteins were synthesized. 4MPI was reacted with single amino acids and tripeptides. The following N_α-acetyl protected amino acids were chosen for the reactions with 4MPI: N_α-acetyl-cysteine, N_α-acetyl-lysine, N_α-acetyl-tyrosine, N_α-acetyl-serine, and N_α-acetyl-histidine. As shown for other environmental pollutants, isocyanates might react with the *N*-terminal amino acids of blood proteins; for haemoglobin and albumin this would be valine and aspartic acid, respectively. Therefore, 4MPI was reacted with valine and aspartic acid. An adduct of 4MPI with a tripeptide with valine as the *N*-terminal amino acid was synthesized to (i) mimic the release of the formed hydantoin from a protein, and to (ii) have an internal standard for the analyses of haemoglobin obtained from workers exposed to other isocyanates than 4MPI.

MATERIALS AND METHODS

L-Aspartic acid, 4-methylphenyl isocyanate and [D_6]DMSO were purchased from Fluka (Neu-Ulm, Germany), N_α-acetyl-L-cysteine from Merck (Darmstadt, Germany), N_α-acetyl-L-histidine, N_α-acetyl-L-lysine, N_α-acetyl-L-tryptophan, N_α-acetyl-L-tyrosine, and valyl-glycyl-glycine from Sigma (Deisenhofen, Germany), and L-valine from Serva (Heidelberg, Germany). The NMR spectra were recorded on a Bruker AC 250 instrument with [D_6]DMSO as solvent and as internal standard. The degree of substitution of the C atoms was determined using the distortionless enhancement by polarization transfer (DEPT) method. The raw NMR data were processed 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). Fast atom bombardment mass spectrometry (FAB-MS) was conducted on a VG 70-SEQ mass spectrometer with argon atoms at 8 keV acceleration. For positive FAB the samples were introduced in a *p*-toluene sulphonic acid/glycerol matrix. HPLC analyses were performed with a quaternary HPLC pump with a UV-detector both from Hewlett Packard 1050 series, and a fluorimeter from Kontron, SFM23. Gas chromatography–mass spectrometry (GC–MS) analyses were done on a Hewlett Packard GC (HP 5890II) equipped with a autosampler (HP7276) and interfaced to a MS (HP 5989A).

Synthesis of ureas from 4MPI and amino acids

Procedure 1: Amino acid (1 mmol) or peptide (1 mmol) was dissolved in 0.25 M $NaHCO_3$ (20 ml). The solution was stirred and heated to 80°C and 4MPI (266 mg, 2 mmol) was added. A precipitate formed immediately. Aft

and after cooling with ice the precipitate was filtered off and washed with water (20 ml) and ethanol (1 ml). The filtrate was carefully acidified to pH 2 with 2M HCl and cooled. The precipitate was filtered and redissolved in ethyl acetate (50 ml). The organic phase was extracted twice with saturated $NaHCO_3$ solution (20 ml), and the aqueous phase was separated and acidified for extraction with ethyl acetate. After drying over $MgSO_4$ the ethyl acetate was evaporated at reduced pressure. The residue was recrystallized with ethanol/water.

N-(4-Methylphenyl-carbamoyl)-L-valine (4MPI-Val)

L-Valine (117 mg, 1 mmol) was transformed according to procedure 1. Crystallization from ethanol yielded 4MPI-Val (165.2 mg, 66%) as white needles.

^{1}H-NMR: ([D_6]DMSO, 250 MHz): δ = 12.8 (broad s, 1H, COOH), 8.68 (s, 1H, NH—Ph), 7.26 (d, *J* = 8.4, 2H, H-2/6), 7.04 (d, *J* = 8.4, 2H, H-3/5), 6.35 (d, *J* = 8.8 Hz, 1H, N*H*—CH—COOH), 4.12 (dd, *J* = 8.8 Hz, *J* = 4.8 Hz, C*H*—COOH), 2.22 (s, 3H, CH_3—Ph), 2.08 (m, 1H, C*H*—CH_3), 0.92 (d, *J* = 6.8 Hz, 3H, CH—CH_3), 0.88 (d, *J* = 6.8 Hz, 3H, CH—CH_3).

^{13}C-NMR ([D_6]DMSO, 63 MHz): δ = 173.8 (COOH), 155.0 (NH—CO—NH), 137.6 (C-1), 129.8 (C-4), 129.0 (C-3/5), 117.4 (C-2/6), 57.1 (*C*H—COOH), 30.2 (*C*H—CH_3), 20.2 (CH_3—Ph), 19.1 (CH—*C*H_3), 17.5 (CH—*C*H_3).

FAB-MS: *m*/*z* (%): 251 (100, $[M+1]^+$), 250 (14, M^+), 205 (9), 118 (43), 107 (29), 106 (13), 72 (54).

N-(4-Methylphenyl-carbamoyl)-L-valyl-glycyl-glycine (4MPI-Val-Gly-Gly)

Val-Gly-Gly (115.5 mg, 0.5 mmol) was transformed according to procedure 1. Crystallization from ethanol yielded 4MPI-Val-Gly-Gly (109 mg, 60%) as a white solid.

^{1}H-NMR ([D_6]DMSO, 250 MHz): δ = 12.61 (s, 1H, COOH), 8.57 (s, 1H, NH—Ph), 8.36 (t, *J* = 5.8 Hz, 1H, CH_2—N*H*), 8.18 (t, *J* = 5.8 Hz, 1H, CH_2—N*H*), 7.25 (d, *J* = 8.4 Hz, 2H, H-2/6), 7.03 (d, *J* = 8.4, 2 H, H-3/5), 6.34 (d, *J* = 8.4 Hz, 1H, N*H*—CH—COOH), 4.12 (dd, *J* = 5.7 Hz, *J* = 8.4 Hz, 1H, NH—C*H*—COOH), 3.78 (m, 4H, 2 CH_2NH), 2.22 (s, 3H, Ph—CH_3), 2.01 (m, 1H, C*H*—CH_3), 0.91 (d, *J* = 6.8 Hz, 3H, CHCH_3), 0.88 (d, *J* = 6.8 Hz, 3H, CHCH_3).

^{13}C-NMR ([D_6]DMSO, 63 MHz): δ = 174.4, 173.4, 171.4 (2 NHCO, CO_2H), 157.5 (NH—CO—NH), 140.1 (C-1), 132.1 (C-4), 131.4 (C-3/5), 119.9 (C-2/6), 60.1 (NH—*C*H—COOH), 44.0 (CH_2—NH), 42.5 (CH_2—NH), 33.2 (CH—CH_3), 22.6 (CH_3—Ph), 21.6 (CH—*C*H_3), 20.0 (CH—*C*H_3).

FAB-MS: *m*/*z* (%): 365 (37, $[M+1]^+$) 264 (13), 251 (34, [4MPI-Val]$^+$), 233 (45), 205 (24), 118 (21), 107 (33), 86 (20), 72 (100).

N-(4-Methylphenyl-carbamoyl)-L-aspartic acid (4MPI-Asp)

L-Aspartic acid (146.4 mg, 1.10 mmol) was transformed according to procedure 1. Crystallization from ethanol yielded 4MPI-Asp (188 mg, 64%) as white needles.

^{1}H-NMR ([D_6]DMSO, 250 MHz): δ = 12.5 (s, 2H, 2 COOH), 8.50 (s, 1H, NH—Ph), 7.26 (d, *J* = 8.3 Hz, 2H, H-2/6), 7.03 (d, *J* = 8.3 Hz, 2H, H-3/5), 6.48 (d, *J* = 8.4 Hz, 1H, N*H*—CH—COOH), 4.49 (m, 1H, C*H*—COOH), 2.78 (dd, *J* = 16.8 Hz, *J* = 5.4 Hz, 1H, CH_2—COOH), 2.68 (dd, *J* = 16.8 Hz, *J* = 4.9 Hz, 1H, CH_2COOH), 2.29 (s, 3H, CH_3—Ph).

^{13}C-NMR ([D_6]DMSO, 63 MHz): δ = 174.0 (CH—*C*OOH), 173.1 (CH_2—*C*OOH), 155.6 (NH—*C*O—NH—Ph), 138.5 (C-1), 130.8 (C-4), 130.0 (C-3/5), 118.5 (C-2/6), 49.6 (*C*H—COOH), 37.7 (*C*H_2—COOH), 21.2 (CH_3—Ph).

FAB-MS: *m*/*z* (%): 267 (100, $[M+1]^+$), 266 (13, M^+), 134 (40), 106 (11), 88 (13).

N_α-Acetyl-*N*-(4-methylphenyl-carbamoyl)-ε-lysine (4MPI-AcLys)

N_α-Acetyl-L-lysine (188 mg, 1 mmol) was transformed according to procedure 1. Crystallization from ethanol yielded 4MPI-AcLys (153.2 mg, 47.6 %) as a white solid.

^{1}H-NMR ([D_6]DMSO, 250 MHz): δ = 13.00 (broad s, 1H, COOH), 8.30 (s, 1H, NH—Ph), 8.14 (d, *J* = 7.9 Hz, 1H, N*H*—CO—CH_3), 7.27 (d, *J* = 8.5 Hz, 2H, H-2/6), 7.07 (d, *J* = 8.5 Hz, 2H, H-3/5), 6.09 (t, *J* = 5.6 Hz, 1H, N*H*-ε-CH_2), 4.15 (m, 1H, C*H*—COOH), 3.05 (m, 2H, ε-CH_2), 2.22 (s, 3H, CH_3—Ph), 1.85 (s, 3H, CH_3—CO), 1.64 (m, 4H, γ-CH_2/δ-CH_2), 1.37 (m, 2H, β-CH_2).

^{13}C-NMR ([D_6]DMSO, 63 MHz): δ = 173.8 (COOH), 169.3 (*C*O—CH_3), 155.2 (NH—*C*O—NH), 137.9 (C-1), 129.5 (C-4), 128.9 (C-3/5), 117.6 (C-2/6), 51.8 (*C*H—COOH), 39.0 (ε-CH_2) 30.7 (β-CH_2), 29.4 (δ-CH_2), 22.9 (γ-CH_2), 22.2 (*C*H_3—CO), 20.2 (*C*H_3—Ph).

FAB-MS: *m*/*z* (%): 322 (100, $[M+1]^+$), 321 (12, M^+), 215 (18), 108 (44), 107 (42), 106 (15), 84 (21).

Synthesis of carbamic acid-S-*esters*

N_α-Acetyl-*S*-(4-methylphenyl-carbamoyl)-L-cysteine (4MPI-AcCys)

4MPI (292.6 mg, 2.2 mmol) was added to a solution of N_α-acetyl-L-cysteine (179 mg, 1.10 mmol) dissolved in 0.25 M $NaHCO_3$ (10 ml). The reaction mixture was treated with ultrasound for 3 min in 10 min intervals at 25°C for 1 h. Work up was performed as described in procedure 1. Crystallization from ethanol/water yielded 4MPI-AcCys (154.1 mg, 47.3%) as yellowish-brown crystals.

^{1}H-NMR ([D_6]DMSO, 250 MHz): δ = 10.26 (s, 1H, NH—Ph), 8.31 (d, *J* = 8.7 Hz, 1H, N*H*—CO—CH_3), 7.37 (d, *J* = 8.4 Hz, 2H, H-2/6), 7.10 (d, *J* = 8.4 Hz, 2H, H-3/5), 4.36 (m, 1H, C*H*—COOH), 3.41 (dd, *J* = 13.2 Hz, *J* = 5.0 Hz, 1H, CH_2—S), 3.05 (dd, *J* = 13.2 Hz, *J* = 8.7 Hz, 1H, CH_2—S), 2.24 (s, 3H, CH_3—Ph), 1.85 (s, 3H, NH—CO—CH_3). The signal for COOH was not visible.

^{13}C-NMR ([D_6]DMSO, 63 MHz): δ = 171.8 (COOH), 169.2 (*C*O—CH_3), 163.6 (NH—CO—S), 136.3 (C-1), 132.3 (C-4), 129.2 (C-3/5), 118.9 (C-2/6), 52.1 (*C*H—COOH), 30.5 (S—CH_2), 22.3 (*C*H_3—CO), 20.3 (CH_3—Ph).

FAB-MS: *m*/*z* (%): 297 (100, $[M+1]^+$), 296 (6, M^+), 164 (53), 122 (18), 107 (18), 106 (14).

Synthesis of carbamates

Procedure 2: Amino acid (0.5 mmol) was dissolved in 3 ml dry pyridine. After addition of 4MPI (0.55 mmol) the solution was stirred and heated to 80°C for 2 h. The solvent was evaporated *in vacuo* and the residue was taken up in 0.5M $NaHCO_3$ (15 ml). The insoluble residue was separated by centrifugation. The water phase was washed twice with ethyl acetate (15 ml). The water phase was acidified with HCl t

with ethyl acetate (3×30 ml). The organic phase was dried over magnesium sulphate, filtered and evaporated *in vacuo*. The residue was recrystallized with ethanol/water.

N_α-Acetyl-*O*-(4-methylphenyl-carbamoyl)-tyrosine (4MPI-AcTyr)

N_α-Acetyl-L-tyrosine (111.6 mg, 0.5 mmol) was dissolved in dry pyridine (3 ml). After addition of 4MPI (0.55 mmol) the solution was stirred overnight at room temperature. The work up was performed according to procedure 2. Crystallization from ethanol yielded 4MPI-AcTyr (34.6 mg, 19.4%) as a white solid.

^{1}H-NMR ([D_6]DMSO, 250 MHz): δ = 12.7 (s, 1H, COOH), 10.12 (s, 1H, NH—Ph), 8.27 (d, *J*= 7.9 Hz, 1H, N*H*—CO—CH_3), 7.39 (d, *J*= 8.5 Hz, 2H), 7.27 (d, *J*= 8.5 Hz, 2H), 7.13 (d, *J*= 8.5 Hz, 4H), 4.41 (m, 1H, C*H*—COOH), 3.06 (dd, *J*= 14.0 Hz, *J*= 4.7 Hz, 1H, Ph—CH_2—CH), 2.86 (dd, *J*= 14.0 Hz, *J*= 9.4 Hz, 1H, Ph—CH_2—CH), 2.25 (s, 3H, CH_3—Ph), 1.79 (s, 3H, NH—CO—CH_3).

^{13}C-NMR ([D_6]DMSO, 63 MHz): δ = 173.1 (COOH), 169.2 (NH—*C*O—CH_3), 151.6 (C), 149.0 (C), 136.1 (C), 134.7 (C), 131.7 (C), 129.9 (CH), 129.2 (CH), 121.6 (CH), 118.3 (CH), 53.4 (*C*H—COOH), 36.0 (Ph´—*C*H_2—CH), 22.3 (*C*H_3—CO), 20.3 (CH_3—Ph).

FAB-MS: *m*/*z* (%): 357 (100, [M+1]$^+$), 356 (4, M$^+$), 315 (19), 269 (15), 164 (21), 136 (17), 107 (20).

N_α-Acetyl-*O*-(4-methylphenyl-carbamoyl)-D,L-serine (4MPI-AcSer)

N_α-Acetyl D,L-serine (73.6 mg, 0.5 mmol) was transformed and worked up according to procedure 2. 4MPI-AcSer (87.0 mg, 52%) was obtained as yellowish crystals.

^{1}H-NMR ([D_6]DMSO, 250 MHz): δ = 12.60 (broad s, IH, COOH), 9.60 (s, 1H, N*H*—Ph), 8.32 (d, *J*= 7.8 Hz, 1H, N*H*—CO—CH_3), 7.35 (d, *J*= 8.3 Hz, 2H, H-2/6), 7.07 (d, *J*= 8.3 Hz, 2H, H-3/5), 4.52 (m, 1H, C*H*—COOH), 4.39 (dd, *J*= 11.0 Hz, *J* =4.4 Hz, NH—CO—OCH_2), 4.18 (dd, *J*= 11.0 Hz, *J*= 6.7 Hz, NH—CO—OCH_2), 2.22, (s, 3H, CH_3—Ph), 1.87 (s, 3H, NH—CO—CH_3).

^{13}C-NMR ([D_6]DMSO, 63 MHz): δ = 170.9 (COOH), 169.4 (*C*O—CH_3), 153.0 (NH—*C*O-O), 136.3 (C-1), 131.2 (C-4), 129.0 (C-3/5), 118.2 (C-2/6), 63.2 (O—CH_2). 51.5 (*C*H—COOH), 22.3 (*C*H_3—CO), 20.2 (CH_3–Ph).

FAB-MS: *m*/*z* (%): 281 (90, [M+1]$^+$), 356 (5, M$^+$), 148 (16), 130 (100), 106 (18).

Synthesis and analysis of hydantoins

Procedure 3: Urea derivatives (0.2 mmol) were dissolved in 10 ml dioxane and 3 ml conc. HCl and heated for 1h at 80–90°C. The reaction mixture was evaporated to dryness, dissolved in ethanol, filtered and then water was added drop-wise until precipitation occurs. After cooling, the precipitate was filtered off and dried.

3-(4-Methylphenyl)-5-isopropyl-1,3-imidazoline-2,4-dione (Val-Hyd)

4MPI-Val (50 mg, 0.2 mmol) was transformed according procedure 3. Crystallization from ethanol yielded Val-Hyd (30 mg, 64.6%) as white needles.

^{1}H-NMR ([D_6]DMSO, 250 MHz): 8.50 (s, 1H, CO—NH), 7.27 (d, *J*= 8.3 Hz, 2H, H-2/6), 7.18 (d, *J*= 8.3 Hz, 2H, H-3/5), 4.12 (dd, *J*= 3.5 Hz, J = 1.3 Hz, 1H, NH—C*H*), 2.33 (s, 3H, CH_3—Ph), 2.11 (m, 1H, C*H*—CH_3), 1.01 (d, *J*= 6.9 Hz, 3H, CH—CH_3), 0.87 (d, *J*= 6.9 Hz, 3H, CH—CH_3).

^{13}C-NMR ([D_6]DMSO, 63 MHz): δ = 172.8 (CO—N), 156.1 (N—CO—NH), 137.2 (C-4), 129.4 (C-1), 129.2 (C-3/5), 126.4 (C-2/6), 61.2 (*C*H—COOH), 29.9 (*C*H—CH_3), 20.6 (CH_3—Ph), 18.4 (CH—*C*H_3), 15.8 (CH—*C*H_3).

MS, EI (70 eV) *m*/*z*(%) 233 (7, [M+1]$^+$), 232 (45, M$^+$), 191 (5), 190 (47), 134 (22), 133 (100), 132 (18), 105 (9), 104 (14), 72 (42), 57 (7), 55 (11).

2-(1-(4-Methylphenyl)-2,5-dioxoperhydro-4-imidazolyl) acetic acid (Asp-Hyd)

4MPI-Asp (51 mg, 0.2 mmol) was transformed according to procedure 3. Crystallization yielded Asp-Hyd (37 mg, 75%).

^{1}H-NMR ([D_6]-DMSO, 250 MHz): 12.70 (broad s, 1H, COOH) 8.47 (s, 1H, NH), 7.27 (d, J = 8.4 Hz, 2H, H-2/6), 7.18 (d, J =8.4 Hz, 2H, H-3/5), 4.40 (m, 1H, C*H*—CH_2—COOH), 2.83 (dd, *J*= 17.2 Hz, *J*= 5.1 Hz, 1H, CH—CH_2—COOH, 2.74, (dd, *J*= 17.2 Hz, *J*= 4.4 Hz, 1H, CH—CH_2—COOH), 2.34 (s, 3H, CH_3—Ph).

^{13}C-NMR ([D_6]DMSO, 63 MHz): δ = 173.9, 171.9 (*C*OOH, *C*O—N), 157.0 (N—*C*O—NH), 138.1 (C-4), 130.7 (C-1), 130.0 (C-3/5), 127.4 (C-2/6), 53.9 (*C*H—CH_2—COOH), 36.3 (CH—*C*H_2—COOH), 21.6 (CH_3—Ph).

FAB-MS: *m*/*z*(%): 249 (100, [M+1]$^+$), 248 (20, M$^+$), 231 (10), 189 (17), 102 (12).

Gas chromatographic analyses of the hydantoin Val-Hyd

4MPI-Val-Gly-Gly (1, 0.1 and 0.01 μg) and *N*-(4-chlorophenyl-carbamoyl)-L-valyl-glycyl-glycine (0.1 μg) (M. Möller *et al.* unpublished results) in methanol (100 μl) was added to 6M HCl (1 ml) and refluxed for 1 h. The hydrolysate was basified to pH 9 with NaOH, and then extracted with methylene chloride (3 ml). The organic phase was evaporated under a stream of nitrogen. The residue was taken up in ethyl acetate (15 μl). Aliquots of 1 μl were analysed by splitless injection on a Rtx-5MS (15 m×0.25 mm, 0.5 μm). The injector and the transfer line temperature was set at 260°C. The oven temperature was kept for 1 min at 50°C and then increased at a rate of 50°C per min to 260°C.

Hydrolyses of the adducts 4MPI-Val, 4MPI-Asp, 4MPI-AcCys, 4MPI-AcLys, 4MPI-AcSer, 4MPI-AcTyr and HPLC analyses

The isocyanate adducts were hydrolysed under three different conditions: adducts (20 μg) in 20 μl methanol were added to 1 ml: (1) 6 M HCl at 100°C, (2) 0.1M NaOH 25°C; and (3) 0.3 M NaOH at 100°C. After 1h, 3h and 24h 10 μl of a 0.2% 4-chloroaniline solution in methanol (or 4-fluoroaniline for the hydrolysate of 4MPI-AcTyr) was added to the hydrolysate. A separate vial was used for each reaction and time point. An aliquot of the hydrolysate (10 μl) was added to ammonium formate (3M) in an Eppendorf cup and 50 μl were analyzed by HPLC on an LiChrospher 100 RP 18 (125×4 mm, 5 μm) (Merck, Darmstadt, Germany) eluting with 4

ammonium formate at 1 ml min^{-1}. The peaks were detected with a UV detector (λ = 235 nm) and with a fluorimeter (emission at 356 nm, excitation at 253 nm). The released 4MA was quantified against a calibration curve obtained with three levels of 4MA and one level of the internal standard. Under the given conditions 4MPI-Val, 4MPI-AcLys, 4MPI-AcCys, 4MPI-AcTyr, 4MPI-AcSer, 4CA, 4FA and 4MA eluted at 3.1, 3.2, 3.4, 7.5, 3.3, 6.9, 3.0 and 5.0 min.

Results and discussion

Synthesis of isocyanate adducts

The urea derivatives with the free amino group of valine, aspartic acid and N_α-acetyl-lysine were synthesized according to Figure 2. The compounds 4MPI-Val, 4MPI-Asp and 4MPI-AcLys were synthesized by adding 4MPI to the corresponding amino acids in carbonate buffer at 80°C. The products were obtained in satisfactory yields (40–70%) and were fully characterized (see paragraph about spectroscopic properties). The yields were lower than similar reactions in organic solvents because of the concurrent hydrolysis of the isocyanate in aqueous solution to the unstable carbamic acid, which decarboxylates and then reacts with another molecule of 4MPI. 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 reaction conditions close to the physiological pH. Considering, that locally the pK_a of amino acids in proteins change up to 2 pK_a units, the choice of a reaction pH, 2 units above the physiological pH is a good compromise to mimic physiological conditions. Raising the temperature to 80°C increased the yield by a factor of 2 compared to the reaction at 37°C. For the amino acids with functional groups, the α-amino group was protected with an acetyl group.

Hydantoin formation was achieved by heating the adducts (4MPI-Val, 4MPI-Asp) in 6M hydrochloric acid at 90°C for 1 h. The hydantoins (Val-Hyd, Asp-Hyd) were obtained in ca 70% yield. The hydantoin Val-Hyd was analysed by GC–MS on a non-polar capillary column. The major single ions at *m/z* 133, 190, and 232 were monitored by MS after electron impact ionization. Injections of 20 pg yielded a signal to noise ratio better than 10:1 monitoring the ion at *m/z* 232. The same hydantoin should be released from 4MPI adducts with the N-terminal valine of the α-globin chain of haemoglobin. In order to develop a method for the quantitation of *in vivo* material, we synthesized a tripeptide (Val-Gly-Gly) with the N-terminal valine modified with 4MPI, yielding 4MPI-Val-Gly-Gly (Figure 3). In addition the tripeptide Val-Gly-Gly was reacted with 4-chlorophenyl isocyanate, yielding *N*-(4-chlorophenyl-carbamoyl-L-valyl-glycyl-glycine (4CPI-Val-Gly-Gly) (this synthesis will be published elsewhere, M. Möller *et al.* unpublished results). For 4MPI-exposed workers, the 4CPI-Val-Gly-Gly can be taken as internal standard. In a preliminary experiment 100 ng of the internal standard and 1, 10 and 100 ng of the 4MPI-Val-Gly-Gly was added in methanol to the HCl solution. After 1 h at 100°C the hydrolysate was extracted with methylene chloride, evaporated to dryness and analysed by GC–MS. Both hydantoins could be monitored at 5.2 min (Val-Hyd) and 5.4 min (3-(4-chlorophenyl)-5-isopropyl-1,3-imidazoline-2,4-dione)) using GC–MS with single ion monitoring in the EI-mode. The method is not sensitive enough to detect the lowest concentration of 4MPI-Val-Gly-Gly.

The reactions of 4MPI with functional groups other than the

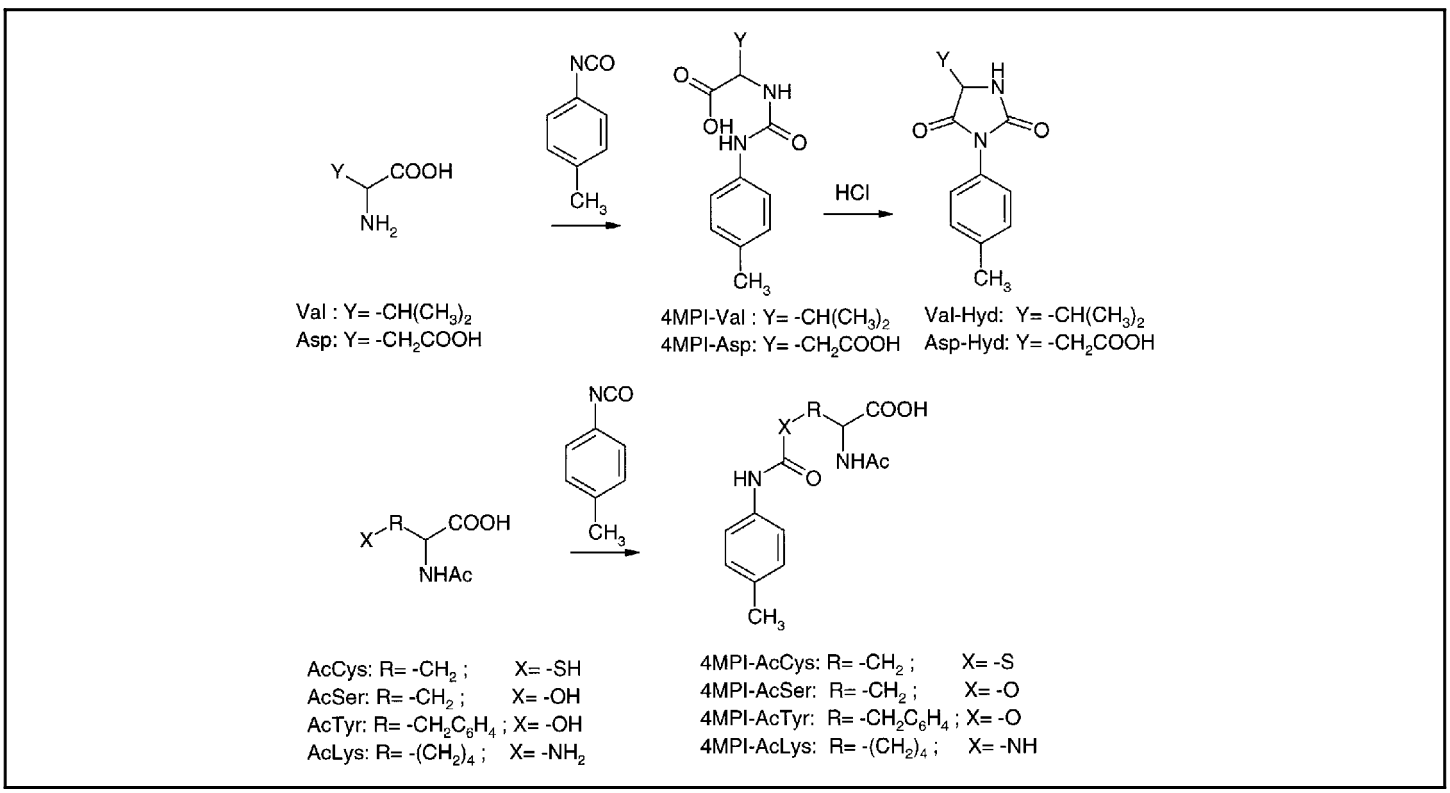

Figure 2. Reactions of 4MPI with amino acids.

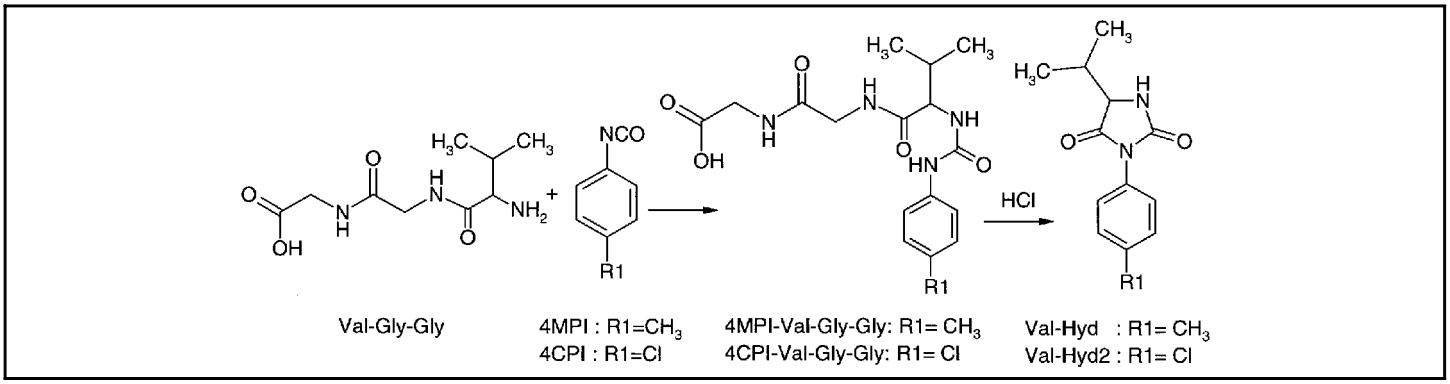

Figure 3. Reaction of 4MPI with Val-Gly-Gly.

α-amino group were done under various conditions. The synthesis of carbamoyl-adducts from 4MPI with thiol groups was performed with N_α-acetyl-cysteine and 4MPI at 30°C in carbonate buffer. The carbamic acid *S*-ester 4MPI-AcCys was achieved with an acceptable yield of 47%. Increasing the reaction temperature to 80°C results in 4MA being the main product. N_α-Acetyl-serine, N_α-acetyl-tyrosine, N_α-acetyl-histidine and N_α-acetyl-tryptophan did not react with 4MPI in the 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 (Brown and Wold 1973, Brown 1975, Brown *et al.* 1982). Therefore, the reactions of N_α-acetyl-serine, and N_α-acetyl-tyrosine with 4MPI were performed in pyridine. The carbamates were obtained in good yields (40–70%). Attempts to synthesize the adducts N_α-acetyl tryptophan or N_α-acetyl-histidine with 4MPI under the same conditions were not successful.

Spectroscopic characterization of the products

All products were characterized with MS, ^{1}H-NMR and ^{13}C-NMR. For all compounds except for Val-Hyd, FAB-MS were generated. The signals are listed in the experimental section. The $(M+1)^+$ ion was the base peak for all compounds, except for the serine adduct 4MPI-AcSer. The major fragments for 4MPI-Val resulted from the cleavage of the urea bond yielding the fragments for the amine (*m*/*z* 107) and valine (*m*/*z* 118). For the lysine adduct 4MPI-AcLys, the urea bond is mainly cleaved yielding 4-methylaniline (*m*/*z* 108) (Figure 4). For the aspartate adduct, 4MPI-Asp, the urea bond is mainly cleaved between the N_α of aspartate and the carbamoyl group yielding a fragment for *N*-formyl-4-methylaniline (*m*/*z* 134). In the case of the carbamic *S*-ester, 4MPI-AcCys, the S-carbamoyl bond is cleaved yielding a fragment for N_α-acetyl-cysteine (*m*/*z* 164). For the carbamate adduct of serine 4MPI-AcSer, the main fragments results from the cleavage of the O-alkyl bond yielding a serine fragment at *m*/*z* 130. For the N_α-acetyl tyrosine adduct, 4MPI-AcTyr, all other fragments are only 25% of the base peak $[M+1]^+$.

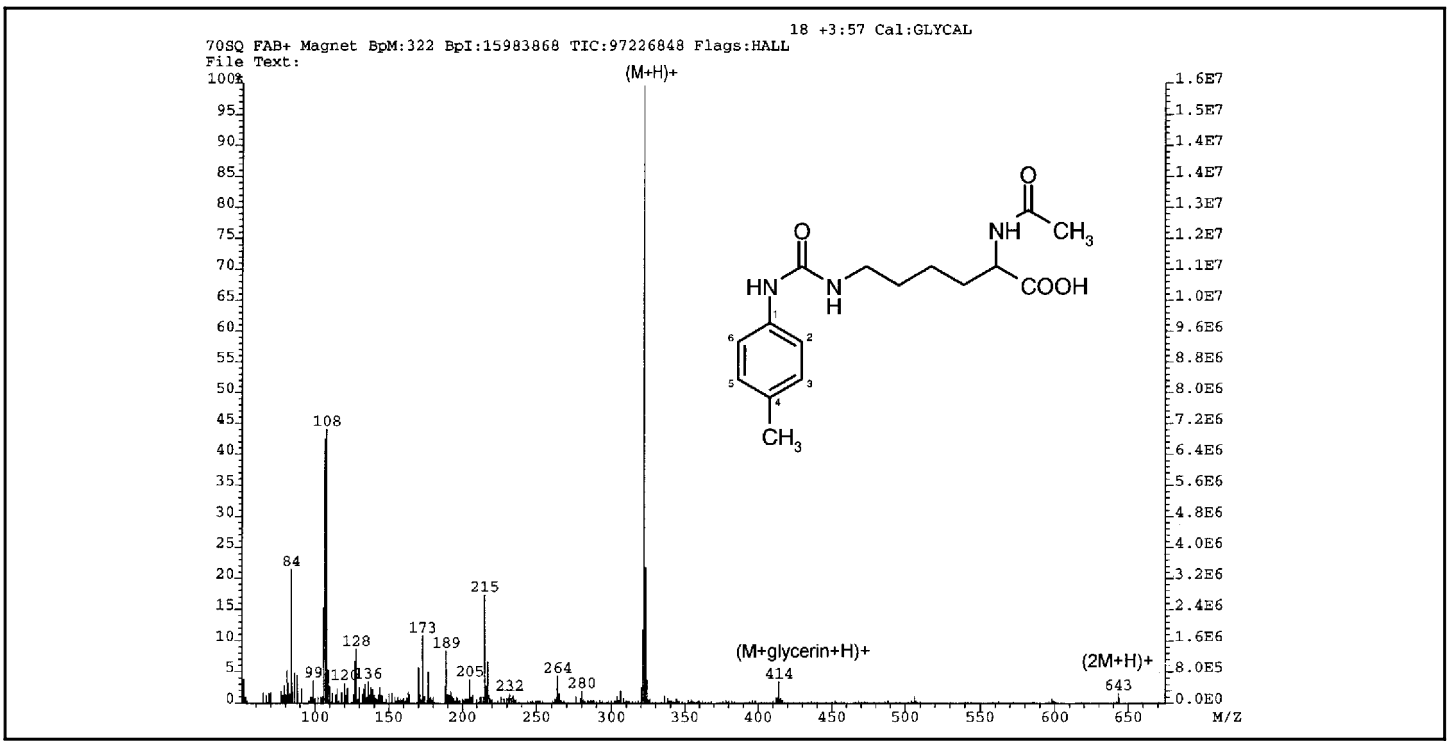

Figure 4. FAB-MS of N_α-acetyl-*N*-(4-methylphenyl-carbamoyl)-ε-lysine (4MPI-AcLys).

The hydantoin synthesized from 4MPI and valine was analysed by GC–MS. The full EI-spectrum of Val-Hyd is listed in the experimental section. The molecular ion is one of the main ions.

The ^{1}H-NMR-spectra were all recorded in [D_6]DMSO at 25°C. The signals of the aromatic protons of the arylamine moiety were estimated with the increment rules (Hesse *et al.* 1984) and assigned accordingly. The chemical shift of H-3/H-5 is upfield compared with the H-2/H-6 protons (Table 1). A good correlation between the calculated and the experimental values was seen. The coupling of these aromatic protons should correspond to an AA′ XX′ -spectrum. Mostly only four lines (2 AB-type doublets) are visible instead of 20. J_{AX} ($J_{A'X'}$) was assigned. $J_{AA'}$ ($J_{XX'}$) and $J_{AX'}$ ($J_{A'X}$) were not elucidated. The protons of the amino acids were assigned according to the literature (Wüthrich 1987). In all cases the protons represented an ABXY-spectrum (Figure 5) except for the valine-adducts with an AXY-spectrum. The obtained coupling constants and the chemical shifts were entered in the NMR-simulation program Raccoon by Paul F. Schatz. The values given in the experimental section, correspond best with the calculated spectra. The N—H protons of the arylamine moiety were found between 8.3 and 8.7 ppm for the urea compounds 4MPI-Val, 4MPI-Asp and 4MPI-AcLys, between 9.6 and 10.1 ppm for the carbamates 4MPI-AcSer and 4MPI-AcTyr and at 10.3 ppm for the carbamic acid *S*-ester 4MPI-AcCys. The amino protons of the amino acids were found at 6.5 when attached to the acetyl group, and at 8.1–8.3 ppm when attached to the carbamoyl group. The vicinal coupling constant of the amino acid NH with the α-CH is around 8 Hz for all compounds. In the case of the hydantoins this coupling is 0 Hz for the valine product Val-Hyd and the aspartate product Asp-Hyd. This can be explained by the change of the dihedral angle CHNH to near 90°. Compared with the protons of the aliphatic N-H group in 4MPI-Val and 4MPI-Asp at 6.4–6.5 ppm, the corresponding NH signal shifts 2 ppm downfield to 8.4–8.5 ppm for the hydantoins Val-Hyd and Asp-Hyd.

	COOH	NH—Ph	H-2/6	H-3/5	NH—COR
4MPI-Val	12.80	8.68	7.26	7.04	6.35[a]
4MPI-Asp	12.50	8.50	7.26	7.03	6.48[a]
4MPI-AcLys	13.00	8.30	7.27	7.07	8.14
4MPI-AcCys	-[b]	10.26	7.37	7.10	8.31
4MPI-AcSer	12.60	9.60	7.35	7.07	8.32
4MPI-AcTyr	12.70	10.12			8.27
Val-Hyd			7.27	7.18	8.50
Asp-Hyd	12.70		7.27	7.18	8.38

Table 1. ^{1}H-NMR data of the 4MPI adducts.

[a] R = NHP.

[b] Not visible.

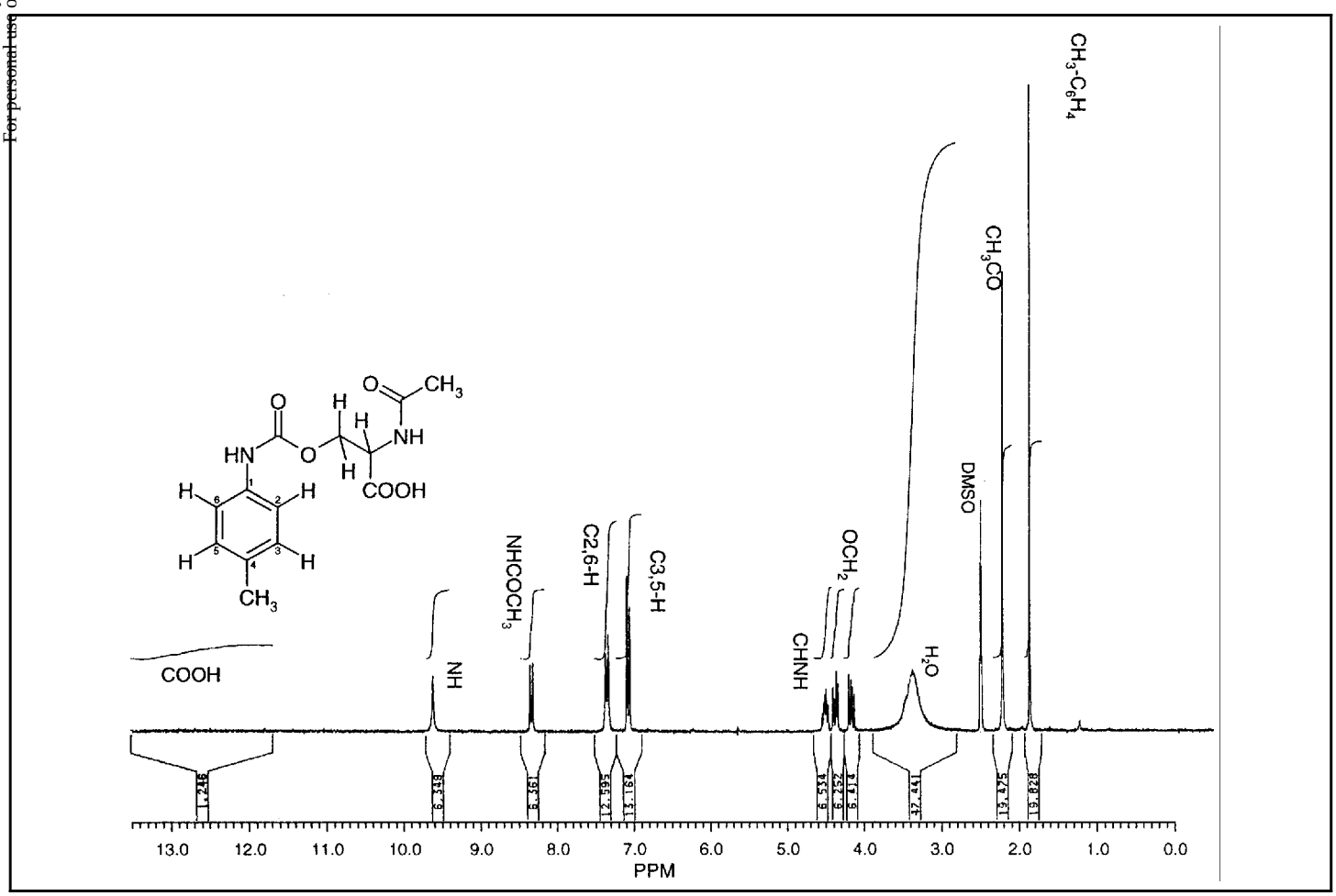

Figure 5. ^{1}H-NMR of N_α-acetyl-*O*-(4-methylphenyl-carbamoyl)-D,L-serine (4MPI-AcSer).

	COOH	NH—CO—R	C-1	C-2/6	C-3/5	C-4	NH—$COCH_3$
4MPI-Val	173.8	155.0	137.6	117.4	129.0	129.8	
4MPI-Asp	174.0; 173.1	155.6	138.5	118.5	130.0	130.8	
4MPI-AcLys	173.8	155.2	137.9	117.6	128.9	129.5	169.3
4MPI-AcCys	171.8	163.6	136.3	118.9	129.2	132.3	169.2
4MPI-AcSer	170.9	153.0	136.3	118.2	129.0	131.2	169.4
Val-Hyd		156.1	129.4	126.4	129.2	137.2	
Asp-Hyd		157.0	130.8	127.4	130.0	138.1	

Table 2. ^{13}C-NMR data of the 4MPI adducts.

The signals of the ^{13}C-NMR-spectra were estimated and assigned using the increment rules published by Kalinowski *et al.* (1984). The degree of C-substitution was determined with a DEPT experiment. In ^{13}C-NMR spectra the signals for the aromatic carbon (C1–C6, C1 is bound to the amino group) are almost identical for all urea derivatives, except for the hydantoins Val-Hyd and Asp-Hyd, where C4 and C2(C6) shift downfield and C1 upfield compared with the corresponding urea compounds 4MPI-Val and 4MPI-Asp (Table 2). The order of chemical shifts is the same for all other compounds: C1 > C4 > C3(C5) > C2(C6). C1 is at the lowest field and C2(C6) at the highest field. The carbamoyl carbon is found around 155 ppm for the urea compounds (4MPI-Val, 4MPI-Asp, 4MPI-AcLys) and hydantoins (Val-Hyd, Asp-Hyd). The same carbon shifts 2 ppm upfield for the carbamate compound (4MPI-AcSer) and 8.6 ppm downfield for the carbamic acid S-ester (4MPI-AcCys) (Figure 6). The carbons of the amino acids were assigned according to the literature (Voelter *et al.* 1971). The carbons of the carboxylic acids and the carbons of the amide are at 170–176 ppm.

Hydrolysis of the adducts

The stability of the adducts was tested under acidic and basic conditions. The isocyanates (1 mg) were dissolved in methanol (1 ml) and hydrolysed for 1h, 3h and 24h in 0.1 M NaOH at room temperature and 6M HCl, following methods applied for the analyses of biological samples of exposed workers (Schütze *et al.* 1995, Sepai *et al.* 1995a, Skarping and Dalene 1995, Skarping *et al.* 1996) and animals (Sepai *et al.* 1995c). In addition, new hydrolysis conditions were tested with 0.3M NaOH at 100°C for 1h, 3h and 24h. The hydrolysates were analysed using HPLC with UV and fluorescence detector, and 4MA quantified against the internal standard, 4-fluoroaniline or 4-chloroaniline. Only the amines were fluorescent under the used conditions. In most cases at the highest yields no starting material was detectable by UV detection. The cysteine adduct 4MPI-AcCys was cleaved in 10 min in 0.1M NaOH. In 6M HCl, 4MPI-AcCys was still present after 1h and 3h. 4MA was obtained with 0%, 2% and 60% yield after 1h, 3h and 24h, respectively. The tyrosine adduct 4MPI-AcTyr was cleaved in base at room temperature with 29%, 55% and 54% yield after 1h, 3h, and 24h. At 100°C the maximum yield was reached after 1h with 60%. Under acidic conditions, the yield of cleaved 4MA increased with increasing hydrolysis time: 14% after 1 h, 33% after 3h, and 55% after 24h. The valine adduct 4MPI-Val and the aspartate adduct 4MPI-Asp were stable in base at room temperature. 4MPI-Val released 4MA after hydrolysis in 0.3M NaOH at 100°C with 5%, 15% and 72%

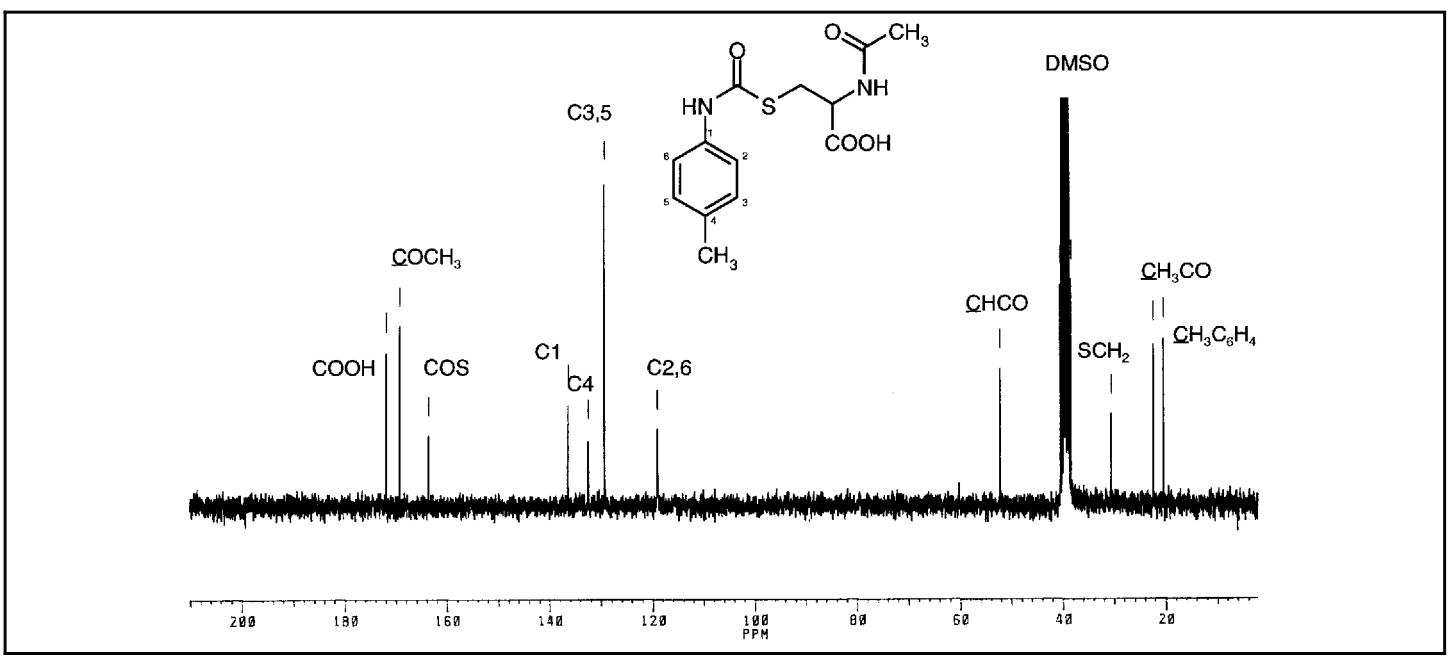

Figure 6. ^{13}C-NMR of N_α-acetyl-*S*-(4-methylphenyl-carbamoyl)-L-cysteine (4MPI-AcCys).

yield, after 1h, 3h and 24h, respectively. The respective hydantoins Val-Hyd and Asp-Hyd were obtained after acid hydrolysis. After 1h no 4MPI-Val was present. Base hydrolysis at room temperature released 4MA slowly from 4MPI-AcSer: 1.8%, 3.3% and 13% 4MA were obtained after 1h, 3h, and 24h. With 0.3M NaOH, the yield of 4MA was already 44% after 1h, and 63% after 24h. Similar levels of 4MA were only obtained by acid hydrolysis at 100°C after 24h: the yield of 4MA was 3.4%, 10.7% and 50% after 1h, 3h and 24h reaction time. 4MPI-AcLys was stable at room temperature in base. With acid hydrolysis 4MA was obtained with 2%, 7% and 36% after 1h, 3h, and 24h. The best yields from 4MPI-AcLys were obtained using 0.3M NaOH at 100°C: 8%, 24% and 58% 4MA were obtained after 1h, 3h, and 24h. For the future analysis of albumin and haemoglobin of exposed workers, we suggest boiling the samples for 3h in base, in order to obtain the maximum yield of released amine. To distinguish between typical arylamine and isocyanate adducts, we propose to determine the *N*-terminal amino acid adducts with haemoglobin and albumin, which are valine and aspartic acid in humans. Mild base hydrolysis will release arylamines from the typical sulphinic acid amide adducts of arylamines and from the isocyanate adducts with cysteine and tyrosine. Therefore, in the future it will be possible to establish isocyanate exposure by measuring the *N*-terminal adducts, but it will not be possible to distinguish between the isocyanate and arylamine adducts which are cleaved under mild base hydrolysis. In the case of diamines, mild base hydrolysis can result in the release of mono-acetylated diamines (Schütze *et al.* 1995, Sepai *et al.* 1995a, b). This indicates that a sulphinic acid amide adduct was present, since it is unlikely that only one of the two original isocyanate groups was hydrolysed. However, since *N*-acetyltransferases (Risch *et al.* 1996) have been found in erythrocytes, it should be investigated whether the *N*-acetyl group can also be introduced in the diamine when the diamine is bound on the haemoglobin.

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