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Synthetic Approaches To Obtain Amino Acid Adducts of 4,4′-Methylenediphenyl Diisocyanate

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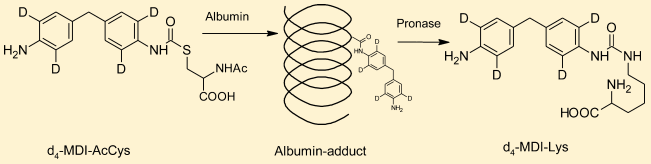
Supporting Information

ABSTRACT: 4,4′-Methylenediphenyl diisocyanate (MDI) is the most important isocyanate used in the chemical industry. Lung sensitization and asthma are the main types of damage after exposure to MDI. Albumin adducts of MDI might be involved in the etiology of sensitization reactions. It is therefore necessary to have sensitive and specific biomarkers such as blood protein adducts to monitor people exposed to isocyanates. For the discovery of new isocyanate adducts with blood proteins present in vivo, new synthetic standards are needed. To achieve this, we developed five methods to obtain amino acid adducts of MDI. We synthesized and isolated MDI adducts of aspartic acid, glutamic acid, cysteine, and valine. The new adducts were characterized by LC-MS/MS and NMR. We synthesized the corresponding isotope-labeled MDI adducts to develop analytical methods using LC-MS/MS. Glutathione adducts of isocyanates are an important way of transportation of the reactive isocyanates to distant sites from the original site of exposure. Therefore, we used *N*-acetyl-cysteine adducts of MDI as reactants: *N*-acetyl-*S*-[[4-(4-aminobenzyl)phenyl]carbamoyl]-cysteine (MDI-AcCys) and *N*-acetyl-*S*-[[4-(4-acetylaminobenzyl)phenyl]carbamoyl]-cysteine (AcMDI-AcCys). MDI-AcCys or AcMDI-AcCys formed adducts with albumin, N_α-acetyl lysine, and valine. Isotope-labeled albumin adducts (= d_4-MDI-albumin) were synthesized from d_4-MDI-AcCys and albumin. d_4-MDI-albumin can be used as an internal standard to analyze biological samples. Such an internal standard will not correct only for the extraction recovery of the adducts but also for the potential variation of the enzymatic digestions used in the procedure to analyze albumin adducts of MDI. The synthetic procedures described in this manuscript will be applicable to the synthesis of amino acid adducts from other isocyanates.



INTRODUCTION

Isocyanates are highly reactive compounds that have a variety of commercial applications. Diisocyanates such as 4,4′-methylenediphenyl diisocyanate (MDI) are used for manufacturing polyurethane foam, elastomers, paints, adhesives, coatings, insecticides, consolidation of loose rock zones in coal mining or tunneling, and many other products.[1] The high chemical reactivity of diisocyanates makes them toxic. Adverse effects at the cellular and subcellular level have been reported, such as irritative and immunological reactions. Inhalation of diisocyanate vapors is associated with various pulmonary ailments, such as eosinophilic airway inflammation, airway hyper-reactivity, early and late-onset asthma, exogenous allergic alveolitis, and direct toxic responses.[2−6] Diisocyanates are of great concern for occupational health, being considered one of the main causes of occupational asthma.[2,7,8] The steady rise in asthma over the past decades points to the potential relationship between isocyanates in consumer products and increasing prevalence of asthma in the general population, especially children.[9] The prevalence and incidence of diisocyanate-induced disorders depend on the degree of exposure. Occupational exposure to diisocyanates may take place during their production and application in the production of polyurethane foam and other products containing monomeric or polymeric diisocyanates. The main route of occupational exposure is through inhalation.

Arylisocyanates lead to cytotoxic and genotoxic effects.[10,11] Arylisocyanates react directly with biomolecules and/or hydrolyze to arylamines (Figure 1). Protein adducts of arylisocyanates are believed to be involved in the etiology of sensitization reactions.[12,13] Important vehicles for isocyanates are their reaction products with glutathione.[14,15] The glutathione adducts release the isocyanate moiety to react with other nucleophiles, for example, proteins. Therefore, glutathione adducts are thought to be responsible for the transport of isocyanate to reactive sites away from the site of isocyanate uptake. Isocyanate-specific adducts of MDI with the N-terminal valine of hemoglobin in rats[16] and isocyanate-specific adducts of MDI with lysine present in albumin have been identified in rats and humans exposed to MDI.[17,18] Albumin adducts have a half-life of 20−25 days. [19] Therefore, albumin adducts are a marker of exposure and a marker that is related to the mechanism of sensitization caused by isocyanates. Reaction products with other amino acids than lysine are possible in albumin such as the N-terminal adducts with aspartic acid (humans) and glutamic acids (rats), serine, tyrosine, histidine, and tryptophan (Figure 1).

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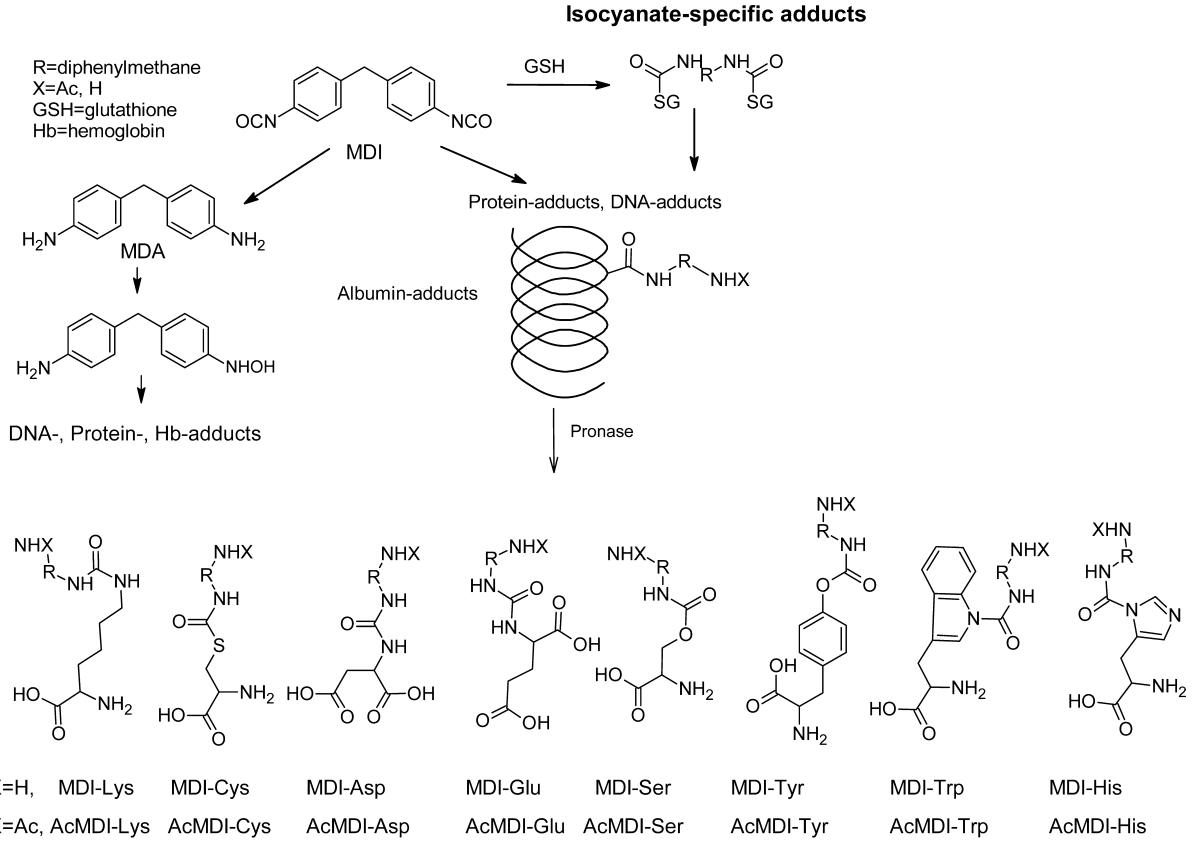

Figure 1. Putative reaction products of MDI with biomolecules.

The following adducts of MDI and *N*-[4-(4-isocyanatobenzyl)phenyl]acetamide (AcMDI) with amino acids have been synthesized up to date. In vitro reactions of MDI with cysteine and *N*-acetyl-cysteine yielded the bis-thiocarbamate adduct, methylene-bis-(*S*-{[(4-phenyl)amino]-carbonyl}cysteine),[20] and methylene-bis-(*S*-{[(4-phenyl)-amino]carbonyl}*N*-acetyl-cysteine),[21,22] respectively. Glutathione and MDI reacted in vitro to the monothiocarbamate, *S*-({[4-(4′-aminobenzyl)phenyl]amino}carbonyl)-glutathione, and to the bis-thiocarbamate product, methylene-bis-(*S*-{[(4-phenyl)amino]carbonyl}glutathione).[23] AcMDI reacted with lysine and with valine to yield N^6-[[4-[4-acetylaminobenzyl]-phenyl]carbamoyl]-lysine (AcMDI-Lys)[17] and *N*-[[4-[4-acetylaminobenzyl]phenyl]carbamoyl]-valine (AcMDI-Val),[16] respectively.

To identify further reaction products of MDI with proteins, additional synthetic standards of MDI-amino acid adducts are needed. Therefore, for the present work, we developed methods to synthesize more amino acid adducts of MDI to be used for the identification of new amino acid adducts in proteins. In addition, we present a method to modify albumin in vitro, which yields potentially the same products present in vivo.

MATERIALS AND METHODS

Caution: *The aromatic amines used in this work are potentially carcinogenic. Isocyanates are strong irritants. Triphosgene is severely irritating to the eyes and skin. Avoid contact and inhalation. All of these compounds should be handled with protective clothing in a well-ventilated fume hood.*

Chemicals. Methanol (A454-4) for sample preparation and methanol (LC/MS Optima grade, A456-4) for LC-MS/MS were obtained from Fisher Scientific (New Jersey). Pronase E (protease, type XIV bacterial, from *Streptomyces griseus*) (#P5147), acylase I from porcine kidney (#3010), ammonium formate (#17843), sodium hydroxide, sodium phosphate monobasic monohydrate, human serum albumin (# A1653), water for LC-MS/MS (Chromasolv; #39253), triphosgene, trifluoroacetic acid, L-aspartic acid dimethyl ester hydrochloride, anhydrous 1,4-dioxane (#296309), all of the *N*-α Boc-protected amino acids, and all of the isotope-labeled amino acids were purchased from Sigma-Aldrich (St. Louis, MO). *S*-(−)-2-Isocyanatoglutaricacid diethyl ester and methyl-*S*-(−)-2-isocyanato 3-methylbutyrate ester were purchased from TCI America (Portland, OR). Coomassie plus protein assay reagent for protein determination was acquired from Thermo Scientific (Rockford, IL). The Strata-X-33u (8B-S100-FBJ) polymeric reversed-phase solid-phase extraction cartridges (200 mg/3 mL) were purchased from Phenomenex Inc. (Torrance CA). *N*1-[4-(4-Aminobenzyl)phenyl]acetamide (AcMDA),[24] *tert*-butyl [4-(4-aminobenzyl)phenyl]carbamate (BocMDA),[17] *tert*-butyl [4-(4-isocyanatobenzyl)phenyl]carbamate (BocMDI),[17] *N*-acetyl-4′-amino-4-nitrodiphenylmethane (4-AcNO_2MDA),[25] 4,4′-methylene-bis(2,6-dideuteroaniline) (d_4-MDA),[26] *N*1-[4-(4-amino-(3,5-dideuterobenzyl))-(2,6-dideuterophenyl)]acetamide (d_4-AcMDA),[26] *N*-acetyl-*O*-[(4-methylphenyl)carbamoyl]-tyrosine (4MPI-AcTyr),[29] *N*-acetyl-*O*-[(4-methylphenyl)carbamoyl]-serine (4MPI-AcSer),[29] N^2-acetyl-N^6-[(4-methylphenyl)carbamoyl]-lysine (4MPI-AcLys),[29] and *N*-[(4-methylphenyl)carbamoyl]-aspartic acid (4MPI-Asp)[29] were synthesized previously in our laboratory. Deacetylation with acylase[28,29] of 4MPI-AcTyr, 4MPI-AcSer, and 4MPI-AcLys yielded N^6-[(4-methylphenyl)carbamoyl]-lysine (4MPI-Lys), *O*-[(4-methylphenyl)carbamoyl]-serine (4MPI-Ser), and *O*-[(4-methylphenyl)carbamoyl]-tyrosine (4MPI-Tyr), respectively.

Instrumentation and Analytical Methods. NMR spectra were recorded on a Bruker AC 500 instrument with d_6-DMSO as the solvent and as the internal standard. The degree of substitution of the C atoms was determined using the distortionless enhancement by polarization transfer method. The raw NMR data were processed with

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the program MestRe-C (Cobas, J. C., Cruces, J., and Sordina, F. J. Magnetic Resonance Companion, Departamente de Quimica Organica, Universidad de Santiago de Compostela, 15706 Santjago de Compostela, Spain) or the ACD/NMR processor Academic edition, Version 12.01 (Advanced Chemistry Development, Inc., Toronto, Canada). An API 4000Q Trap (ABSciex, Foster City, CA) mass spectrometer interfaced to an HPLC (Shimadzu Prominence 20AD) was used in the LC-MS/MS analyses (software Analyst 1.4.2). A UV-1800 Spectrophotometer from Shimadzu was used for protein determination. HPLC monitoring of the reactions was performed with an Agilent 1100 equipped with a photodiode detector: HPLC-column = Lichrospher 100 RP18 (125 mm × 4 mm, 5 μm), flow rate of 1.0 mL/min, and λ = 250 nm. Method 1: solvent A, 0.1% formic acid; solvent B, methanol. The analyses were performed with a linear gradient of 15−80% B in 20 min. Method 2: solvent A, 10 mM ammonium acetate; B, methanol. The analyses were done with a linear gradient of 30−80% B in 20 min. The lipophilicity of the synthesized products was estimated with ACD/LogD v8.02 (Advanced Chemistry Development, Inc., Toronto, Canada, www.acdlabs.com) and/or Marvin Beans 5.10.0 (ChemAxon, Budapest, Hungary, www.chemaxon.com).

Analysis of Amino Acid Adducts Using LC-MS/MS. Shimadzu Prominance 20AD interfaced to an API 4000Q Trap LC-MS/MS (ABSciex, Foster City, CA) mass spectrometer system was used for all of the quantitative analysis. The MS parameters were optimized in the electrospray ionization mode (ESI). Parameter optimization was carried out by infusing 100 pg/μL solution of analyte with the flow rate of 10 μL/min in the negative ionization mode. MDI-Val, AcMDI-Val, MDI-Glu, AcMDI-Glu, MDI-Asp, AcMDI-Asp, MDI-Lys, and AcMDI-Lys were showing corresponding deprotonated $[M - H]^-$ ions at m/z 340.1, 382.1, 370.1, 412.1, 356.1, 398.1, 369.1, and 411.1, respectively. The quantitative optimization mode was used to optimize the multiple reaction monitoring signals and set the maximum declustering potential and collision energy parameters for the compounds. For better resolution and sensitivity of the analyte, quadrupole mass analyzers (Q1 and Q3) were set to 0.7 ± 0.1 amu resolution window. The mass spectrometer was operated in negative (or positive) ionization mode and with an electrospray voltage −4500 V (+4500 V) and a source temperature of 500 °C. The same parameters were used for positive ESI-MS analyses. The positive ESI-MS data were only used for the characterization of the synthesized MDI adducts. The negative ESI-MS runs were used for quantification of the adducts. Nitrogen was used as the ion spray (GS1), drying (GS2), and curtain gas at 40, 45, and 10 arbitrary units, respectively. The declustering potential and collision energy were optimized for all compounds. The entrance potential for all compounds was −10 V. All data were processed using Analyst software 1.4.2 (Applied Biosystems/MDS Sciex).

The MDI adducts were separated on a reversed phase C_{18} column and analyzed with a LC-MS/MS Instrument. [Luna C18(2) (100 Å, 150 mm × 2.0 mm, 3 μm) (Phenomenex Inc., Torrance, CA) protected by a C_{18} guard column (AJO-4287; 4 mm L × 3.0 mm i.d.), using a gradient system with solvent A (5 mM ammonium formate) and solvent B (methanol): 0.2 mL/min, 0−3 min: 10% B, 3−12 min, 10−90% B linear gradient, 12−15 min 90% B. LC-MS/MS analyses of the standard compounds yielded the following peaks (Figure 5 in the Supporting Information): MDI-Asp t_R = 12.1 min (m/z 356.1 → 132.1, 88.1), MDI-Glu t_R = 12.2 min (m/z 370.1 → 146.1, 128.1), MDI-Lys t_R = 13.2 min (m/z 369.1 → 145.0, 171.3), and MDI-Val t_R = 14.3 (m/z 340.1 → 116.0). MDI-Lys and AcMDI-Lys was quantified using the internal standards MDI-[$^{13}C_6{}^{15}N_2$]Lys and d_4-AcMDI-Lys as published previously.[17,18]

Method 1: Synthesis of Amino Acid Adducts Using AcMDI. Synthesis of Ureas from AcMDI and Amino Acids. General Procedure 1. Triphosgene (367 mg, 1.25 mmol) was added to a stirred solution of AcMDA (243 mg, 1 mmol) in dioxane (20 mL) and then heated to 80 °C. The reaction was monitored by HPLC. After 3 h of reaction time, the reaction mixture was added dropwise to a stirred solution of an amino acid (1 mmol) or a peptide (1 mmol) in 0.25 M $NaHCO_3$ (20 mL). $NaHCO_3$ was added to maintain the pH at ca. 8.0. After 2 h of reaction time, the reaction mixture was cooled on ice. The precipitate was filtered off and washed with water (20 mL) and ethanol (1 mL). The filtrate was acidified with 2 M HCl to pH 2 and extracted with ethyl acetate (3 × 50 mL). The organic layer was extracted twice with saturated $NaHCO_3$ solution (20 mL). The aqueous phase was separated, acidified, and extracted with ethyl acetate (3 × 50 mL). After it was dried over $MgSO_4$, the organic phase was evaporated at reduced pressure. The residue was recrystallized with ethanol/water.

N-[[4-(4-Acetylaminobenzyl)phenyl]carbamoyl]-aspartic Acid (AcMDI-Asp). AcMDI-Asp was synthesized following general procedure 1 with aspartic acid (200 mg, 1.50 mmol). Crystallization from EtOH/H_2O yielded a brown solid (104 mg, 17.3%). ^{1}H NMR (d_6-DMSO): 12.6 (s, broad, 2 COO*H*), 9.82 (s, 1 H, N*H*-Ac), 8.74 (s, 1 H, N*H*-CO-NHCH), 7.46 (d, J = 8.4, 2H, Ar−H), 7.28 (d, J = 8.5, 2H, Ar−H), 7.10 (d, J = 8.4, 2H, Ar−H), 7.05 (d, J = 8.5, 2H, Ar−H), 6.47 (d, J = 8.4, CONHCH), 4.49 (m_c, C*H*CH$_2$), 3.78 (*s*, Ar−C*H*$_2$-Ar), 2.76 (dd, J = 16.8, 5.6, 1 H, CHC*H*$_2$), 2.68 (dd, J = 16.8, 5.0, 1 H, CHC*H*$_2$), 2.01 (s, 3 H, Ph-NHCOMe). ^{13}C NMR (d_6-DMSO): 173.0 (CO), 172.1 (CO), 168.0 (CO), 154.7 (NHCONH), 138.1 (Ar *C*), 137.2 (Ar *C*), 136.2 (Ar *C*), 134.2 (Ar *C*), 128.8 (Ar *C*H), 128.7 (Ar *C*H), 119.1 (Ar *C*H), 117.7 (Ar *C*H), 48.7 (*C*HCH$_2$), 40.4 (Ar-*C*H$_2$−Ar), 36.8 (CH*C*H$_2$), 23.9 (Ph-NHCOMe). ESI-MS (positive) m/z: 400.2 $[M + H]^+$, MS/MS of 400.2 → 132.0. ESI-MS (negative) m/z: 398.1 $[M - H]^-$, MS/MS of 398.0 → 132.0

N-[[4-[4-Acetylaminobenzyl]phenyl]carbamoyl]-glutamic Acid (AcMDI-Glu). AcMDI-Glu was synthesized according to general procedure 1 with L-glutamic acid (221 mg, 1.5 mmol). Crystallization from EtOH/H_2O yielded a beige solid (124 mg, 20.3%). ^{1}H NMR (d_6-DMSO): 12.4 (s, broad, 2 H, COO*H*), 9.81 (s, 1 H, N*H*), 8.50 (s, 1 H, N*H*), 7.46 (d, J = 8.4, 2 H, Ar−H), 7.27 (d, J = 8.5, 2 H, Ar−H), 7.09 (d, J = 8.4, 2 H, Ar−H), 7.05 (d, J = 8.5, 2 H, Ar−H), 6.39 (d, J = 8.0, 1 H, N*H*CH), 4.19 (dd, J = 8.0, 13.2, 1 H, C*H*CH$_2$), 3.78 (s, 2 H, Ar−C*H*$_2$-Ar), 2.28 (m_c, 2 H, CHCH$_2$C*H*$_2$), 2.00 (s, 3 H, PhNHCOC*H*$_3$), 2.00 (m_c, 1 H, CHC*H*$_2$), 1.79 (m_c, 1 H, CHC*H*$_2$). ^{13}C NMR (d_6-DMSO): 173.9 (*C*O), 173.6 (*C*O), 168.0 (*C*O), 154.8 (NHCONH), 138.1, 137.2 (Ar *C*), 136.2 (Ar *C*), 134.2 (Ar *C*), 128.8 (Ar *C*H), 128.7 (Ar *C*H), 119.1 (Ar *C*H), 117.8 (Ar *C*H), 51.5 (*C*HCH$_2$CH$_2$), 40.4 (Ar-*C*H$_2$−Ar), 29.9 (CH*C*H$_2$CH$_2$), 27.2 (CHCH$_2$*C*H$_2$), 23.9 (PhNHCO*C*H$_3$). ESI-MS (positive) m/z: 414.0 $[M + H]^+$, 241.0. ESI-MS (negative) m/z: 412.3 $[M - H]^-$, MS/MS of 412.3 → 146.1, 128.0.

N-Acetyl-S-[[4-(4-acetylaminobenzyl)phenyl]carbamoyl]-cysteine (AcMDI-AcCys). AcMDI-AcCys was synthesized according to general procedure 1 with *N*-acetyl-L-cysteine (256 mg, 1.57 mmol). Crystallization from ethanol yielded a yellow solid (175 mg, 26%). ^{1}H NMR (d_6-DMSO): 10.29 (s, N*H*COS), 9.87 (*s*, N*H*COCH$_3$), 8.31 (d, J = 8.1, CHN*H*), 7.47 (d, J = 8.4, 2 H, Ar−H), 7.40 (d, J = 8.5, 2 H, Ar−H), 7.13 (d, J = 8.3, 2 H, Ar−H), 7.11 (d, J = 8.3, 2 H, Ar−H), 4.35 (ddd, J = 8.5, 8.4, 5.0, NHC*H*), 3.81 (s, 2 H, Ar−C*H*$_2$-Ar), 3.38 (dd, J = 13.8, 5.0, 1 H, SC*H*$_2$), 3.05 (dd, J = 13.7, 8.8, 1 H, SC*H*$_2$), 2.01 (s, 3 H, Ph-NHCO*Me*), 1.85 (s, 3 H, CHNHCO*Me*). ^{13}C NMR (d_6-DMSO): 171.8 (COOH), 169.3, 168.0 (Ph-NHCOMe, CHNHCOMe), 163.7 (NHCOS), 137.2 (Ar *C*), 136.7 (Ar *C*H), 136.5 (Ar *C*), 135.8 (Ar *C*), 128.9 (Ar *C*H), 128.7 (Ar *C*H), 119.1 (Ar *C*), 52.1 (NHCH), 40.4 (Ar-CH$_2$−Ar), 30.5 (SCH$_2$), 23.8 (CHNHCO*Me*), 22.3 (Ph-NHCO*Me*). ESI-MS (positive) m/z: 430.0 $[M + H]^+$, MS/MS of 430.0 → 132.2. ESI-MS (negative) m/z: 428.2 $[M - H]^-$, MS/MS of 428.2 → 162.1, 84.2.

N^2-Acetyl-N^6-[[4-(4-acetylaminobenzyl)phenyl]carbamoyl]-lysine (AcMDI-AcLys). AcMDI-AcLys was synthesized according to general procedure 1 with N_α-acetyl-L-lysine (231 mg, 1.23 mmol). Crystallization from EtOH/H_2O yielded a pale brown solid (65 mg, 11.6%). ^{1}H NMR (d_6-DMSO): 12.5 (s, COO*H*), 9.90 (s, N*H*COCH$_3$), 8.35 (s, Ar−N*H*), 8.12 (d, J = 7.9, CHN*H*), 7.48 (d, J = 8.1, 2 H, Ar−H), 7.28 (d, J = 8.0, 2 H, Ar−H), 7.13 (d, J = 8.1, 2 H, Ar−H), 7.05 (d, J = 8.0, 2 H, Ar−H), 6.08 (t, J = 5.6, N*H*CH$_2$), 4.12 (m_c, NHC*H*), 3.78 (s, C*H*$_2$), 3.06 (m_c, NHC*H*$_2$), 2.02 (s, COC*H*$_3$), 1.85 (s, COC*H*$_3$), 1.62 (m_c, C*H*$_2$), 1.35 (m_c, 2 CH$_2$). ^{13}C NMR (d_6-DMSO): 173.7 (COOH), 169.2, 168.0 (NHCOCH$_3$), 155.2 (NHCONH), 138.4 (Ar *C*), 137.1 (Ar *C*), 136.2 (Ar *C*), 133.8 (Ar

C), 128.7 (Ar CH), 119.0 (Ar CH), 117.7 (Ar CH), 51.7 (NHCH), 39.8 (Ar-CH_2–Ar), 38.7 ($NHCH_2CH_2$), 30.7 ($CHCH_2CH_2$), 29.4 ($NHCH_2CH_2$), 23.8 ($COCH_3$), 22.8 ($CHCH_2CH_2$), 22.2 ($CHNHCOCH_3$). ESI-MS (positive) *m/z*: 455.0 $[M + H]^+$, MS/MS of 453.0 → 241.0. ESI-MS (negative) *m/z*: 453.0 $[M + H]^-$, MS/MS of 453.0 → 187.0, 146.2.

Method 2: Synthesis of Amino Acid Adducts Using BocMDI. N-Acetyl-S-[[4-(4-aminobenzyl)phenyl]carbamoyl]-cysteine (MDI-AcCys). Freshly prepared hot BocMDI in 1,4-dioxane was added dropwise to a solution of *N*-acetyl-L-cysteine (50 mg, 0.226 mmol) in 0.25 M sodium bicarbonate (5 mL, pH 8.3). The reaction temperature was kept at 25 °C. Ultrasonication was applied for 3 min for every 10 min interval. After 2 h, the reaction mixture was cooled with ice and then filtered. The filtrate was evaporated to a final volume of approximately 5.0 mL and then extracted with ethyl acetate (3 × 10 mL). The ethyl acetate phase was dried with anhydrous sodium sulfate, filtered, and evaporated on a rotoevaporator. The crude product (BocMDI-AcCys, purity = 81.7%, 28.8 mg) was treated with trifluoroacetic acid (300 μL) for 20–25 min. After it was evaporated under reduced pressure and dried with a high vacuum pump, MDI-AcCys was recrystallized from ethyl acetate yielding yellow crystals with a purity of 90% (HPLC method 2, t_R = 6.5 min) (44.2 mg, yield 77%). ^{1}H NMR (d_6-DMSO): 8.27 (d, 1 H, *J* = 8.0, N*H*-CO-NHCH), 7.39 (d, *J* = 8.6, 2 H), 7.14 (d, *J* = 8.2, 2 H), 7.13 (d, *J* = 8.6, 2 H), 6.96 (d, *J* = 8.3, 2 H), 6.45 (d, *J* = 7.4, CONHCH), 4.42 (dt, *J* = 5.9, 8.6, C*H*CH_2), 3.83 (s, Ar–CH_2-Ar), 2.76 (dd, *J* = 16.3, 4.8, 1 H, CHCH_2), 2.68 (dd, *J* = 16.3, 5.8, 1 H, CHCH_2), 1.84 (s, 3H, NHCOCH_3). ESI-MS (positive) *m/z*: 388.1 $[M + H]^+$.

N-Acetyl-S-[(4-{4-amino-3,5-dideuterobenzyl}2,6-dideuterophenyl)carbamoyl]-cysteine (d_4-MDI-AcCys). Boc-d_4-MDA (2 mg, 66 μmol) was added to a solution of triphosgene (1.95 mg, 66 μmol) and triethylamine (667 μg, 66 μmol) in anhydrous 1,4-dioxane (1 mL). The reaction mixture was stirred for 3 h at 80 °C under nitrogen and then added slowly to a solution of *N*-acetyl-L-cysteine (1.5 mg) in 0.25 M sodium bicarbonate (2.5 mL, pH 8.2) at room temperature. After every 10 min, the mixture was sonicated for 3 min. The reaction was monitored by HPLC. After 2 h, the reaction mixture was cooled with ice and then filtered. The filtrate was evaporated to a final volume of approximately 1 mL and then extracted with ethyl acetate (3 × 2 mL). The ethyl acetate phase was dried with anhydrous sodium sulfate, filtered, and evaporated on a rotoevaporator. The crude product was treated with trifluoroacetic acid (100 μL) for 20–25 min. After it was evaporated under reduced pressure and dried with a high vacuum pump, d_4-MDI-AcCys was obtained with a purity of 84% (HPLC method 2, t_R = 6.8 min). ESI-MS (positive) *m/z:* 392.1 $[M + H]^+$, MS/MS of 392.1 → 164.1.

MDI-[$^{13}C_5{}^{15}N$]Val. L-[$^{13}C_5{}^{15}N$]Val (10 mg, 81.3 μmol) in 0.25 M sodium bicarbonate (2.5 mL, pH 8.2) was reacted with BocMDI (81.3 μmol). After the final hydrolysis with trifluoroacetic acid, MDI-[$^{13}C_4{}^{15}N$]Val (61%) and the corresponding hydantoin 3-[4-(4-aminobenzyl)phenyl]-5-[($^{13}C_3$)propan-2-yl](4,5-$^{13}C_2$,1-^{15}N)-imidazolidine-2,4-dione (MDI-[$^{13}C_5{}^{15}N$]Val-Hyd) (31%) were obtained. MDI-[$^{13}C_4{}^{15}N$]Val was purified by HPLC (purity 90%, HPLC method 2, t_R = 8.0 min) with solvent A (10 mM ammonium acetate) and solvent B (methanol): 20 min 30–80% B linear gradient. t_R = 8.0 min for MDI-[$^{13}C_5{}^{15}N$]Val and 12.2 min for MDI-[$^{13}C_5{}^{15}N$]Val-Hyd. ESI-MS (positive) *m/z*: $[M + H]^+$ 348.3, 124.0 and $[M + H]^+$ 330.2, respectively.

MDI-[$^{13}C_4{}^{15}N$]Asp. L-[$^{13}C_4{}^{15}N$]Asp (10 mg, 72.5 μmol) dissolved in 0.25 M sodium bicarbonate (2 mL, pH 8.2) was reacted with freshly prepared BocMDI (72.5 μmol) dissolved in dry 1,4-dioxane (1 mL). After the final hydrolysis with trifluoroacetic acid and evaporation, MDI-[$^{13}C_4{}^{15}N$]Asp (2.2 mg, 8% yield) was obtained (93% HPLC purity, method 1, t_R = 10.7 min). ESI-MS (negative) *m/z*: $[M - H]^-$ 361, MS/MS of 361.1 → 137.0. Acid hydrolysis (2 M HCl, 30 min, 100 °C) yielded [1-[4-(4-aminobenzyl)phenyl]-2,5-dioxo-(4,5-^{13}C2,3-^{15}N)imidazolidin-4-yl](^{13}C2)acetic acid (MDI-[$^{13}C_4{}^{15}N$]Asp-Hyd). ESI-MS (negative) *m/z:* 343.0 $[M - H]^-$, MS/MS of 343.0 → 299.2, 254.0.

Method 3: Synthesis of Amino Acid Adducts by Reaction of 2-Isocyanato Amino Acid Esters with MDA. N-([4-(4-Aminobenzyl)-phenyl]carbamoyl)-valine (MDI-Val). MDA (1237.5 mg, 6.25 mmol) dissolved in dichloromethane (25 mL) was added slowly to methyl-*S*-(−)-2-isocyanato 3-methylbutyrate ester (118.9 mg, 1.25 mmol) dissolved in dichloromethane (2 mL). After it was stirred for 2 h at room temperature, the reaction was stopped. According to HPLC analyses, most of the isocyanate reacted with MDA yielding the MDI-methyl-3-methylbutyrate ester (mono, 94%) and the dimer (6%). *n*-Hexane (60 mL) was added to the reaction mixture and mixed well. The solution was washed with acid (pH 2, 4 × 15 mL). The pH of the aqueous phase was adjusted to 8 with $NaHCO_3$ and extracted with ethyl acetate (4 × 10 mL). The ethyl acetate layer was dried with anhydrous sodium sulfate, filtrated, and concentrated with a rotoevaporator to yield the crude MDI-methyl-3-methylbutyrate ester. The crude ester was dissolved in tetrahydrofuran (2 mL). The solution was diluted with water (16 mL), and 0.25 M NaOH (7 mL) was added dropwise at 0 °C. After 2 h, the reaction mixture was washed with ethyl acetate (3 × 25 mL). The organic phase was discarded. The pH of the water phase was adjusted to pH 4 with 2 M HCl (the log *D* = 2.39 of MDI-Val is highest at pH 4) and extracted with ethyl acetate (3 × 20 mL). The organic extract was dried on anhydrous sodium sulfate and concentrated with a rotoevaporator at room temperature to avoid formation of the corresponding hydantoin, 3-[4-(4-aminobenzyl)phenyl]-5-(propan-2-yl)imidazolidine-2,4-dione (MDI-Val-Hyd).[16] The residue was recrystallization from ethyl acetate, yielding yellowish crystals of MDI-Val (42.8 mg, yield 80.6%, HPLC method 2, purity = 97.7%, t_R = 8.15 min). ^{1}H NMR (d_6-DMSO): 8.46 (s, 1 H, N*H*-CO-NHCH), 7.25 (d, *J* = 8.4, 2 H), 7.01 (d, *J* = 8.4, 2 H), 6.83 (d, *J* = 8.1, 2 H), 6.48 (d, *J* = 8.1, 2 H), 6.27 (d, *J* = 8.7, 1 H, N*H*CH), 4.12 (dd, 1 H, NC*H*CH), 3.66 (s, 2 H, Ar–CH_2-Ar), 2.08 (m_c, 2 H, CH(CH_3)$_2$), 0.92 (d, *J* = 6.9, 3H, CH_3), 0.88 (d, *J* = 6.9, 3H, CH_3). ^{13}C NMR (d_6-DMSO): 173.5 (COOH), 154.9 (NHCONH), 146.3 (Ar *C*), 137.8 (Ar *C*), 134.9 (Ar *C*), 128.8 128.5 (Ar CH), 128.6 (Ar *C*), 117.5 (Ar CH), 113.9 (Ar CH), 57.1 ($CHCHCH_3$), 40.4 Ar-CH_2–Ar), 30.1 (CH(CH_3)$_2$), 18.9 (CH_3), 17.4 (CH_3). ESI-MS (positive) *m/z*: 342.2 $[M + H]^+$, MS/MS of 342.2 → 118.3, 72.2. ESI-MS (negative) *m/z*: 340.1 $[M - H]^-$, MS/MS of 340.1 → 116.0.

N-([4-{4-Amino-3,5-dideuterobenzyl}2,6-dideuterophenyl]-carbamoyl)-valine (d_4-MDI-Val). d_4-MDI-Val was synthesized according to the synthesis of MDI-Val. Methyl-*S*-(−)-2-isocyanato 3-methylbutyrate ester (1.51 mg, 10 μmol) in 3.5 mL of dichloromethane was added to d_4-MDA (10.8 mg, 50 μmol) in dichloromethane (2 mL). After hydrolysis with NaOH, 2.5 mg (72%) of d_4-MDI-Val was obtained (HPLC method 2, t_R = 8.3 min, 93% purity). ESI-MS (positive) *m/z*: 346.1 $[M + H]^+$, 118.1. ESI-MS (negative) *m/z*: 344.0 $[M - H]^-$, 116.0.

N-([4-(4-Aminobenzyl)phenyl]carbamoyl)-glutamic Acid (MDI-Glu). MDA (495 mg, 2.5 mmol) dissolved in dichloromethane (10 mL) was added slowly to *S*-(−)-2-isocyanatoglutaricacid diethyl ester (114 mg, 0.5 mmol) dissolved in dichloromethane (2 mL) at room temperature. After it was stirred for 2 h, the reaction was stopped. According to HPLC analysis, most of the isocyanate reacted with MDA, yielding the MDI-glutaric acid diethyl ester (mono, 94%) and dimer (6.0%). *n*-Hexane (60 mL) was added to the reaction mixture and mixed well. The solution was washed with acid (pH 2, 4 × 15 mL). The pH of the aqueous phase was adjusted to 8 with $NaHCO_3$ and extracted with ethyl acetate (4 × 10 mL). The ethyl acetate layer was dried with anhydrous sodium sulfate, filtrated, and concentrated with a rotoevaporator to yield the crude MDI-glutamic acid diethyl ester. The crude ester was dissolved in tetrahydrofuran (2 mL). Water (5 mL) was added, and the reaction mixture was cooled on ice to 0 °C. NaOH (0.25 M, 6 mL) was added dropwise. After 2 h, the reaction mixture was washed with ethyl acetate (3 × 25 mL). The pH of the aqueous phase was adjusted to 4 with 2 M HCl (log *D* = 0.7 of MDI-Glu at pH 4.0). While adjusting the pH of the reaction mixture from 8 to 4, a white solid precipitated. The white precipitate was collected (HPLC analysis = MDI-Glu, 97.6% purity). The aqueous phase was extracted with ethyl acetate (3 × 20 mL). The organic phase was dried with anhydrous sodium sulfate, filtered, and concentrated in a

rotoevaporator. After this was dried at the high vacuum pump, we obtained yellowish white crystals of MDI-Glu (97.6% HPLC purity, method 1, t_R = 10.7 min): total yield (precipitate + extract) = 80.6%. ^{1}H NMR (d_6-DMSO): 8.59 (s, 1 H, N*H*-CO-NHCH), 7.25 (d, *J* = 8.4, 2 H), 7.01 (d, *J* = 8.4, 2 H), 6.82 (d, *J* = 8.3, 2 H), 6.48 (d, *J* = 8.3, 2 H), 6.44 (d, *J* = 7.5, 1 H, NHCH), 4.19 (m_c, 1 H, C*H*CH$_2$), 3.66 (s, 2 H, Ar−C*H*$_2$-Ar), 2.28 (m_c, 2 H, CHCH$_2$C*H*$_2$), 1.97 (m_c, 1 H, CHC*H*$_2$), 1.81 (m_c, 1 H, CHC*H*$_2$). ^{13}C NMR (d_6-DMSO): 173.7 (COOH), 173.4 (COOH), 154.8 (NHCONH), 146.3 (Ar C), 137.9 (Ar C), 134.9 (Ar C), 128.8 (Ar *C*H), 128.4 (Ar *C*H), 128.6 (Ar *C*), 117.6 (Ar *C*H), 113.9 (Ar *C*H), 51.5 (*C*HCH$_2$CH$_2$), 40.4(Ar-CH$_2$−Ar), 29.9 (CHCH$_2$CH$_2$), 27.3 (CHCH$_2$CH$_2$). ESI-MS (positive) *m*/*z*: 372.1 [M + H]$^+$, MS/MS of 372.1 → 148.2, 84.0. ESI-MS (negative) *m*/*z*: 370.1 [M − H]$^-$, MS/MS of 370.1 → 146.1, 128.1.

N-[(4-{4-Acetylamino-3,5-dideuterobenzyl}2,6-dideuterophenyl)-carbamoyl]-glutamic Acid (d_4-AcMDI-Glu). Synthesized from d_4-AcMDI (10 mg, 41 μmol) and *S*-(−)-2-isocyanatoglutaricacid diethyl ester (9 mg, 41 μmol) yielded 15.2 mg (88%) of d_4-AcMDI-Glu as a gray solid. The HPLC purity was 97% (method 1, t_R = 12.9). ESI-MS (negative) *m*/*z*: 416.0 [M − H]$^-$, MS/MS of 416.0 → 146.0.

N-([4-{4-Amino-3,5-dideuterobenzyl}2,6-dideuterophenyl]-carbamoyl)-glutamic Acid (d_4-MDI-Glu). *S*-(−)-2-Isocyanatoglutaricacid diethyl ester (2.3 mg, 0.01 mmol) in dichloromethane (1 mL) was added slowly to d_4-MDA (10 mg, 0.05 mmol) in dichloromethane (2 mL) according to the procedure described for MDI-Glu. After hydrolysis and workup, 2.8 mg (7.4% yield) with a purity of 93.1% (HPLC method 1, t_R = 10.7 min) was obtained. ESI-MS (negative) *m*/*z*: 374.1 [M − H]$^-$.

N-[[4-(4-Aminobenzyl)phenyl]carbamoyl]-aspartic Acid (MDI-Asp). A mixture of L-aspartic acid dimethyl ester hydrochloride (197.6 mg,1 mmol) and triethylamine (223.6 mg, 2.2 mmol) dissolved in dichloromethane (4 mL) was added dropwise to triphosgene (109.5 mg, 0.37 mmol) dissolved in dichloromethane (2 mL) over the period of 30 min. After 1 h, the reaction mixture was added slowly at room temperature to MDA (990 mg, 5 mmol) dissolved in dichloromethane (10 mL). After 2 h, the reaction was stopped based on the HPLC results, indicating that most of the starting material reacted with MDA and formed MDI-aspartic acid dimethyl ester (mono, 94%) and dimer (5−6.0%). The workup procedure was the same as described in section 1. The ethyl acetate phase dried the over anhydrous sodium sulfate and concentrated on the rotoevaporator yielded a crude MDI-aspartic acid dimethyl ester. The crude ester was taken into round-bottom flask (50 mL), dissolved in tetrahydrofuran (2 mL), and mixed well until it dissolved. Then, water (10 mL) was added, and the solution was cooled to 0 °C with an ice-cold water bath. NaOH (7 mL, 0.25 M) was added dropwise. After 2 h, the reaction was stopped and washed with ethyl acetate (3 × 25 mL). The pH of the aqueous phase was adjusted to 4 with 2 M HCl (the log *D* of MDI-Asp is highest at pH 4). While the pH of the reaction mixture was adjusted from 8 to 4, a white solid precipitated. The mixture was centrifuged. The supernatant was eliminated, and the remaining white solid was dried under high vacuum overnight. White crystals of MDI-Asp were obtained (HPLC method 1, purity 94.8%, t_R = 10.9 min yield 85.9%). ^{1}H NMR (d_6-DMSO): 8.72 (s, 1 H, N*H*-CO-NHCH), 7.46 (d, *J* = 8.4, 2 H), 7.28 (d, *J* = 8.5, 2 H), 7.10 (d, *J* = 8.4, 2 H), 7.05 (d, *J* = 8.5, 2 H), 6.45 (d, *J* = 7.4, CONHCH), 4.42 (m_c, C*H*CH$_2$), 3.66 (s, Ar−C*H*$_2$-Ar), 2.76 (dd, *J* = 16.3, 4.8, 1 H, CHC*H*$_2$), 2.68 (dd, *J* = 16.3, 5.8, 1 H, CHC*H*$_2$). ^{13}C NMR (d_6-DMSO): 172.9 (COOH), 171.8 (COOH), 154.6 (NHCONH), 146.2 (Ar *C*), 137.9 (Ar *C*), 134.9 (Ar *C*), 128.6 (Ar *C*), 128.8 (Ar *C*H), 128.4 (Ar *C*H), 117.6 (Ar *C*H), 114.0 (Ar CH), 48.8 (CHCH$_2$), 39.6 (Ar-CH$_2$−Ar), 37.4 (CHCH$_2$). ESI-MS (positive) *m*/*z*: [M + H]$^+$ 358.3, MS/MS of 358.3 → 134.0, 74.1. ESI-MS (negative) *m*/*z*: 356.2 [M − H]$^-$; MS/MS of 356.2 → 132.0, 88.0. Hydrolysis of MDI-Asp in 2 M HCl for 2 h yielded {1-[4-(4-aminobenzyl)phenyl]-2,5-dioxoimidazolidin-4-yl}acetic acid (MDI-Asp-Hyd). ESI-MS (negative) m/*z*: 338.0 [M − H]$^-$, MS/MS of 338.0 → 295.0, 251.2.

N-([4-{4-Amino-3,5-dideuterobenzyl}2,6-dideuterophenyl]-carbamoyl)-aspartic Acid (d_4-MDI-Asp). The procedure used to synthesize MDI-Asp was applied. d_4-MDA (10 mg) was taken instead of MDA. The small amount of d_4-MDI-Asp did not precipitate. Therefore, the water phase (pH 4) was applied to a solid-phase column (RP18). d_4-MDI-Asp was eluted with methanol (6 mL). After evaporation, d_4-MDI-Asp was obtained with 93% purity (HPLC method 1: t_R = 11.0 min). ESI-MS (positive) *m*/*z*: 362.0 [M + H]$^+$, 229.2, 134.1. ESI (negative) *m*/*z*: 360.0 [M − H]$^-$, 132.2.

N-[(4-{4-Acetylamino-3,5-dideuterobenzyl}2,6-dideuterophenyl)-carbamoyl]-aspartic Acid (d_4-AcMDI-Asp). The procedure to synthesize d_4-MDI-Asp was applied. d_4-AcMDA was taken instead of d_4-MDA. d_4-AcMDI-Asp had the following MS properties. ESI-MS (positive) *m*/*z*: 404.3 [M + H]$^+$, MS/MS of 404.3 → 245.2, 134.2. ESI-MS (negative) *m*/*z*: 402.0 [M − H]$^-$, MS/MS of 402.0 → 132.0.

Method 4: Synthesis of Amino Adducts Using 4′-Isocyanate-4-nitrodiphenyl Methane (NO_2MDI). 4′-Amino-4-nitrodiphenylmethane (NO_2MDA). A 6 M HCl solution (46 mL) was added to a solution of NO_2AcMDA (3.66 mmol) in ethanol (95 mL). The reaction mixture was kept under reflux for 3 h. A saturated Na_2CO_3 solution (50 mL) was added carefully. The mixture was extracted with dichloromethane (5 × 20 mL). The organic layer was dried over $MgSO_4$, filtered, and evaporated under reduced pressure. Crystallization from EtOH yielded 750 mg (90%) of NO_2MDA as a yellow solid.

N-[[4-(4-Nitrobenzyl)phenyl]carbamoyl]valylglycyl-glycine (NO_2MDI-Val-GlyGly). NO_2MDI-Val-GlyGly was obtained according general procedure 1 using NO_2MDA (360 mg, 1.58 mmol), phosgene (325 mg, 1.1 mmol), and L-valine-glycine-glycine (235 mg, 1.1 mmol). Yield (245 mg, 46%) colorless solid. ^{1}H NMR (d_6-DMSO): 8.65 (*s*, Ar−N*H*), 8.35 (*t*, broad, NHCH$_2$), 8.15−8.14 (m, Ar-*H*, Ar-*H*, NHCH$_2$, 3H), 7.47 (d, *J* = 8.8, 2 H), 7.30 (d, *J* = 8.5, 2 H), 7.12 (d, *J* = 8.5, 2 H), 6.35 (d, *J* = 8.5, NHCH), 4.00 (s, CH$_2$), 3.77−3.75 (m, 5 H, NHC*H*, 2 NHC*H*$_2$), 1.98 (m_c, C*H*Me$_2$), 0.88 (d, *J* = 6.8, Me), 0.84 (d, *J* = 6.8, Me). ^{13}C NMR (d_6-DMSO): 172.2, 171.3, 169.3 (COOH, 2 CONH), 155.3 (NHCONH), 150.2 (Ar *C*), 146.0 (Ar *C*), 138.9 (Ar *C*), 132.6 (Ar *C*), 130.0 (Ar *C*H), 129.4 (Ar *C*H), 123.8 (Ar *C*H), 118.0 (Ar *C*H), 57.9 (NHCH), 41.8 (CH$_2$), 40.7 (NHCH$_2$), 40.0 (NHCH$_2$), 31.1 (CHMe$_2$), 19.5 (Me), 17.9 (Me). ESI-MS (positive) *m*/*z*: 486.1 [M + H]$^+$, 354.0.

N-([4-(4-Aminobenzyl)phenyl]carbamoyl)-valylglycyl-glycine (MDI-Val-GlyGly). Pd−C and ammonium formate were added at room temperature to a stirred solution of NO_2MDI-Val-GlyGly in dry methanol under nitrogen. After 3 h, the reaction mixture was filtered over Celite. The solvent was evaporated in vacuum, and the residue was dried. ^{1}H NMR (d_6-DMSO): 8.66 (s, NH), 8.36 (t, *J* = 5.9, 1 H, NHCH), 8.07 (t, *J* = 5.5, 1 H, NHCH), 7.25 (d, *J* = 8.3, 2 H, Ar−H), 7.01 (d, *J* = 8.2, 2 H, Ar−H), 6.83 (d, *J* = 8.2, 2 H, Ar−H), 6.47 (d, *J* = 8.3, 2 H, Ar−H), 4.10 (m_c, 1 H, NHC*H*), 3.80−3.69 (m, 4 H, 2 × NHC*H*$_2$), 3.65 (s, CH$_2$), 1.97 (m_c, C*H*Me$_2$), 0.89 (d, *J* = 6.8, Me), 0.85 (d, *J* = 6.8, Me). ^{13}C NMR (d_6-DMSO): 172.0 171.1 168.8 (CO), 155.1 (NHCONH), 146.5 (Ar *C*), 138.0 (Ar *C*), 134.9 (Ar *C*), 128.9 (Ar *C*H), 128.6 (Ar *C*H), 117.5 (Ar *C*H), 113.9 (Ar *C*H), 57.7 (CH), 41.6 (CH$_2$), 40.9 (CH$_2$), 39.8 (CH$_2$), 30.8 (CH), 19.2 (CH$_3$), 17.6 (CH$_3$). ESI-MS (positive) *m*/*z*: 456.1 [M + H]$^+$.

Method 5: Synthesis of Amino Acid Adducts Using MDI-AcCys and AcMDI-AcCys. MDI-Val. L-Valine (6 mg, 51 μmol) and MDI-AcCys (2 mg, 5.1 μmol) were stirred at 37 °C in 0.25 M sodium phosphate (2 mL, pH 7.4). The product composition was analyzed by HPLC. MDI-AcCys, MDI-Val, and the 1,3-bis({4-[4-aminobenzyl]-phenyl}urea (MDI-MDA) eluted at 6.8, 8.6, and 14.5 min, respectively. After 5 h, the ratio of the products was 1:16:8 for MDI-AcCys, MDI-Val, and MDI-MDA, respectively. Pure MDI-Val has been synthesized previously with method 3 (see above).

N^2-Acetyl-N^6-[[4-(4-aminobenzyl)phenyl]carbamoyl]-lysine (MDI-AcLys). L-*N*α-Acetyl-L-lysine (9.6 mg, 51 μmol) and MDI-AcCys (0.2 mg, 0.51 μmol) were stirred at 37 °C in 0.25 M sodium phosphate (2 mL, pH 7.4). After 1 h, no MDI-AcCys was present but mainly MDA and MDI-AcLys in the ratio 1:1. Pure MDI-AcLys has been synthesized previously with method 2. [17]

AcMDI-AcLys. L-*N*α-Acetyl-lysine (9.6 mg, 51 μmol) and AcMDI-AcCys (219 μg, 0.51 μmol) were stirred at 37 °C in 0.25 M sodium phosphate (2 mL, pH 7.4). After 30 min, no AcMDI-AcCys was present but mainly AcMDA and AcMDI-AcLys in the ratio 1:1. The

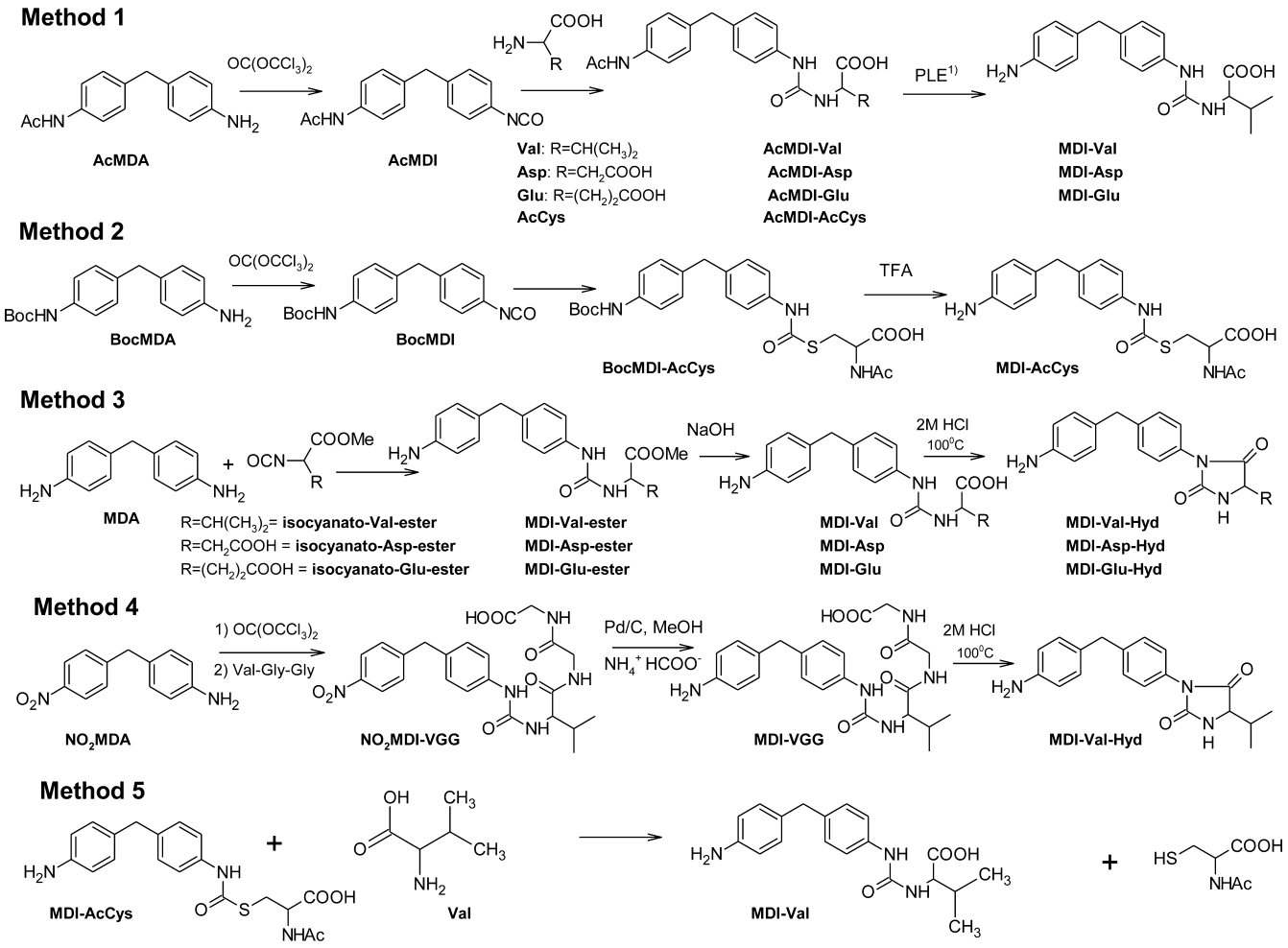

Figure 2. Methods to synthesize amino acid adducts of MDI. [1)]PLE = pig liver esterase.

product composition was analyzed by HPLC: A, 0.1% formic acid; B, methanol; linear gradient from 10 to 70% B in 19 min. Pure AcMDI-AcLys has been synthesized with method 1; see above.

In Vitro Modification of Human Serum Albumin with MDI-AcCys, AcMDI-AcCys, and d_4-MDI-AcCys. MDI-AcCys (0.15 μmol, 1.0 mg/mL) dissolved in sodium phosphate buffer (50 mM, pH 7.4) was added dropwise to a solution (3 mL) of albumin (10 mg, 0.15 μmol) in sodium phosphate buffer (50 mM, pH 7.4) at 37 °C. After 16 h, the reaction mixture was centrifuged to separate off the white precipitate. The supernatant was washed with ethyl acetate (2 × 3 mL). Sodium chloride was added to separate the organic and aqueous layers clearly. The protein solution (3 mL) was transferred into a 15 cm dialysis tube (Fisher brand, 11000−14000 molecular weight cutoff, 0.32 mL/cm, 0.2 mm thickness), which was presoaked in deionized water for 20 min. The dialysis was carried out against deionized water (2 L) at 4 °C. After 4 h, the dialysis was stopped. The concentration of the obtained albumin solution (4 mL) was determined using the Coomassie plus protein assay reagent (#23236) for protein determination from Thermo Scientific (Rockford, IL). Using the same method, albumin was modified with MDI-AcCys at two different levels, 1:1 and 1:10 mol/mol, using the above procedure. Albumin was modified with d_4-MDI-AcCys in the same manner (100:1 mol/mol).

Deacetylation of AcMDI Derivatives. Pig liver esterase (PLE) (50 μL) was added to AcMDI-Lys (2.5 mg) dissolved in 50 mM sodium phosphate buffer (pH 7.4) following a procedure by Schutze et al.[25] After incubation at 37 °C in a shaking bath for 1 day, the deacetylated product MDI-Lys was obtained. According to HPLC analyses, the parent compound was converted to the final product. The same reaction conditions were applied to the compounds AcMDI-Val, AcMDI-Asp, and AcMDI-Glu. According to HPLC analyses, these products were only partially converted to the deacetylated products MDI-Val, MDI-Asp, and MDI-Glu, respectively.

Digestion of in Vitro-Modified Human Serum Albumin. Unmodified albumin (5 mg), 100 μg of d_4-MDI-albumin (modified with d_4-MDI-AcCys/albumin = 1/100), and MDI-[$^{13}C_6{}^{15}N_2$]Lys (13.25 pmol) in 50 mM ammonium bicarbonate buffer (2 mL, pH 8.9 adjusted with NaOH) were incubated for 15 h at 37 °C with Pronase E (1.7 mg) following a procedure published previously for the analysis of albumin from rats exposed to MDI.[17] The digest was acidified to pH 4.0 with 2 M hydrochloric acid and purified with solid-phase extraction (Strata-X-33u, polymeric reversed phase columns, 200 mg). The columns were activated with 3 mL of methanol and then equilibrated with 3 mL of 0.1% formic acid (pH 4.0). The samples were applied on the columns and subsequently washed with 3 mL fractions of 0, 10, and 20% methanol in 0.1% formic acid. d_4-MDI-Lys and MDI-[$^{13}C_6{}^{15}N_2$]Lys were eluted with 6 mL of 80% methanol in 0.1% formic acid. The eluates were concentrated to approximately 1 mL in a speed evaporator at 45 °C and analyzed with LC-MS/MS. The compounds were separated on a C-18 reversed phase column (Luna C18(2), 100 Å, 150 mm × 2.0 mm, 3 μm) using a gradient elution system with solvent A (10 mM ammonium formate) and solvent B (methanol) at a flow rate of 0.2 mL/min: 0 (B 20%), 3 (B 20%), 16 (B 90%), and 20 min (B 90%). Quantitation of d_4-MDI-Lys was performed using the calibration line obtained from MDI-Lys and MDI-[$^{13}C_6{}^{15}N_2$]Lys as described previously.[17,18] d_4-MDI-Lys was present at 9.93 ± 0.42 pmol (4.2% relative standard deviation of 5 experiments) per mg modified albumin. The same procedure was used for the digestion of albumin modified in vitro with MDI-AcCys and AcMDI-AcCys, respectively. The quantitation of MDI-Lys (Figure 3) and AcMDI-Lys was performed as described previously.[17,18]

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Determination of the N-Terminal Adduct in Human Albumin Modified in Vitro with MDI-AcCys at a Molar Ratio of 1:1. (a) Control albumin, (b) in vitro-modified albumin (0.5 mg), and (c) albumin (0.5 mg) with MDI-Asp (69.6 pmol) were hydrolyzed with 2 M HCl for 2 h in the presence of the internal standard MDI-[$^{13}C_4{}^{15}N$]Asp (68.4 pmol). The digests were purified by solid-phase extraction as described for the analysis of MDI-Lys[18] and analyzed with LC-MS/MS. The compounds were separated on a C-18 reversed phase column (Luna C18(2), 100 Å, 150 mm × 2.0 mm, 3 μm), using a gradient elution system with solvent A (10 mM ammonium formate) and solvent B (methanol) at a flow rate of 0.2 mL/min: 0 (B 20%), 3 (B 20%), 16 (B 90%), and 20 min (B 90%). LC-MS/MS analyses showed the formation of the corresponding hydantoin MDI-Asp-Hyd (MS/MS of m/z 338 $[M - H]^- \rightarrow$ 295.0, 251.2) with the same retention time 12.9 min as the internal standard MDI-[$^{13}C_4{}^{15}N$]-Asp-Hyd (MS/MS of m/z 343 $[M - H]^- \rightarrow$ 299.2, 254.0. For quantitation of experiments a and b, the peak ratio of MDI-Asp-Hyd/MDI-[$^{13}C_4{}^{15}N$]-Asp-Hyd = 0.99 was taken from experiment c.

Degradation of MDI-AcCys and AcMDI-AcCys in Buffer Solutions. MDI-AcCys (1.4 mg) was dissolved in 50 mM sodium phosphate (2.0 mL, pH 7.4) and stirred at 37 °C. The progress of the reaction was monitored by HPLC. A 10 μL aliquot of the reaction mixture was diluted with methanol (90 μL) and then analyzed by HPLC (25 μL) using method 2. This was repeated after 1, 3, 5, and 20 h. The logarithmic values of the obtained peak areas were plotted against the time (r^2 = 0.99).

The same approach was used to determine the stability kinetics of AcMDI-AcCys. In this case, aliquots of 10 μL were measured after 0, 1, 4, 7, 22, and 26 h. The logarithmic values of the obtained peak areas were plotted against the time (r^2 = 0.96). AcMDI-AcCys decomposed to AcMDA (t_R = 9.7 min, identical with standard AcMDA, t_R = 9.7 min) and to *N*-[4-({4-[({4-[4-acetamidobenzyl]phenyl}carbamoyl)-amino]phenyl}methyl)phenyl]acetamide (AcMDI-AcMDA) (t_R = 18.1 min). After 26 h, AcMDA and AcMDI-AcMDA are the main products formed in the ratio 3:4. ESI-MS (positive) of AcMDI-AcMDA: m/z 507 $[M + H]^+$; ESI-MS (negative) m/z: 505 $[M - H]^-$.

The same experiment was performed with AcMDI-AcCys in 50 mM ammonium bicarbonate buffer adjusted to pH 8.9. The half-life of the products was 15 min. AcMDA eluting at t_R = 8.9 min was the major product after 3 h. AcMDI-AcMDA was a minor product (AcMDA:AcMDI-AcMDA = 20:1).

RESULTS AND DISCUSSION

For the analysis of in vivo-modified proteins, especially albumin and hemoglobin, we synthesized different potential reaction products of MDI with amino acids. The most reactive centers are the N-terminal acids such as valine in hemoglobin, glutamic acid in rat albumin, aspartic acid in human albumin, the ε amino groups of lysine, the hydroxyl group in serine and tyrosine, the nitrogen in histidine and tryptophan, and sulfur of cysteine. We applied several synthetic strategies (methods 1−5, Figure 2). The adducts obtained in vivo can be acetylated on the free amino group of MDA, yielding, for example, AcMDI-Lys, as shown previously in rat albumin.[17] Therefore, the corresponding adducts derived from AcMDI had to be synthesized.

For the first method, we synthesized AcMDI from AcMDA and triphosgene (method 1). The α-amino groups of the amino acids were protected with a Boc group or with an acetyl group, except for valine, glutamic acid, and aspartic acid. The Boc group can be cleaved with trifluoroacetic acid at room temperature. The acetyl group can be cleaved enzymatically with acylase. AcMDI was reacted with N_α-Boc-Lys and Val to yield AcMDI-Lys[17] and AcMDI-Val,[16] respectively. The same procedure was applied to the present work with glutamic acid, aspartic acid, *N*-acetyl cysteine, and N_α-acetyl-lysine yielding AcMDI-Glu, AcMDI-Asp, AcMDI-AcCys, and AcMDI-AcLys, respectively (Figure 2). The reaction of AcMDI with *N*-acetyl-serine, N_α-acetyl-tyrosine, N_α-acetyl tryptophan, and N_α-acetyl histidine was not successful with this method (data not shown). This is partially in contrast to reactions of 4-methylphenyl isocyanate (4MPI) published previously where adducts with *N*-acetyl-serine and N_α-acetyl-tyrosine could be accomplished.[27] For the synthesis of the nonacetylated products, the AcMDI adducts obtained with method 1 were incubated with pig liver esterase to yield the corresponding MDI-amino acid adducts. This method worked for AcMDI-Lys but only partially for the deacetylation of AcMDI-Val, AcMDI-Asp, and AcMDI-Glu. Therefore, another approach was taken to synthesize MDI-Val, MDI-Asp, and MDI-Glu.

We applied method 2 (Figure 2) starting from BocMDA, a MDA derivative with one amino group protected with Boc. BocMDI was generated from BocMDA and triphosgene. MDI-Lys[17] was obtained after reaction of BocMDI with N_α-Boc-Lys and subsequent hydrolysis with trifluoroacetic acid. This method was applied to the synthesis of the new cysteine adduct MDI-AcCys. For the adducts with the N-terminal acids (aspartic acid in human albumin, glutamic acid in rat albumin, and valine in hemoglobin), the acidic conditions (TFA at room temperature) used to cleave the Boc group yielded also partially the corresponding hydantoins, for example, MDI-Val-Hyd (see Figure 2). In this case, we had to purify the compounds with HPLC. For a complete transformation to MDI-Val-Hyd, boiling in 2 M HCl is necessary as shown previously. [16]

Therefore, method 3 was applied to synthesize MDI-Val, MDI-Asp, and MDI-Glu (Figure 2). The isocyanate function was introduced on the α-amino group of the amino acids. 2-Isocyanato aspartic acid dimethyl ester was synthesized from L-aspartic acid dimethyl ester hydrochloride and triphosgene following a method used for the synthesis of the corresponding glutaric acid isocyanate.[30] The isocyanates of valine and glutaric acid, isocyanato 3-methylbutyrate ester, and 2-isocyanato glutaric acid diethyl ester, respectively, were commercially available. To avoid the reaction of both amino groups of MDA with the amino acid isocyanate, 1-mol equiv of amino acid isocyanate was added to 5 mol equiv of MDA. MDI-Glu, MDI-Asp, and MDI-Val were obtained with more than 80% yield (Figure 2). The corresponding deuterated standards were synthesized following the same procedure but using d_4-MDA instead of MDA. d_4-MDI-Glu, d_4-MDI-Asp, and d_4-MDI-Val were obtained. Method 3 has great potential for the synthesis of urea compounds deriving from amino acid adducts and aromatic or aliphatic isocyanates.

Method 4 (Figure 2) was used to synthesize adducts of MDI, which are not N-acetylated. For the analysis of N-terminal adducts of isocyanates with hemoglobin, the isocyanate-modified tripeptide AcMDI-Val-Gly-Gly, and the isotope-labeled d_4-AcMDI-Val-Gly-Gly were used to generate the calibration line for the quantitation of the adducts present in rats.[16] However, in vivo the major adduct with the N-terminal amino acid—valine—will be the nonacetylated MDI derivative according to the results obtained for the albumin adducts MDI-Lys and AcMDI-Lys.[17] Therefore, for the present work, we propose the synthesis of the nonacetylated product, MDI-Val-Gly-Gly (Figure 2). 4-Amino-4-nitrodiphenylmethane (NO_2MDA) was reacted with triphosgene to obtain NO_2MDI, which was used for the reaction with Val-GlyGly to obtain NO_2MDI-Val-Gly-Gly. The nitro group of NO_2MDI-Val-Gly-Gly was reduced with ammonium formate and Pd on carbon to yield MDI-Val-Gly-Gly.

All final products synthesized with methods 1−4 had to be extracted from the water phase. To establish the pH to obtain the best yields for the extractions into organic solvent, the log *D* values of the adducts were calculated using software by ACDlabs and/or Chemaxon. In most cases, the products were most lipophilic (=highest log *D* − value) at pH 4, except for the hydantoins—MDI-Asp-Hyd and MDI-Val-Hyd. The obtained products were characterized with ^{1}H NMR, ^{13}C NMR, and ESI-MS.

Important vehicles for isocyanates are their reaction products with glutathione.[14,15] The glutathione adducts release the isocyanate moiety to react with other nucleophiles, for example, proteins. Therefore, for method 5 (Figure 2), we investigated the reactions of cysteine adducts of MDI (MDI-AcCys and AcMDI-AcCys) with amino acids and then with albumin. MDI-AcCys was reacted at pH 7.4 with valine and *Nα*-acetyl-lysine to obtain MDI-Val and MDI-AcLys, respectively. A large excess (100:1) of amino acid leads to less side products and shorter reaction times as demonstrated with the synthesis of MDI-AcLys. AcMDI-AcCys was used to synthesize AcMDI-AcLys at pH 9. *Nα*-Acetyl-lysine was used in large excess, 100:1. The HPLC profile of the reaction mixture did not change after 30 min. Therefore, for syntheses with MDI-AcCys derivatives, high excess of amino acids should be used. These reactions were all performed at room temperature. Therefore, because the synthesis of AcMDI-AcLys and MDI-Val was successful, also reactions with proteins should be successful under mild conditions. The harsh reaction conditions described in methods 1−4 are not recommended for reactions with proteins.

The stability of MDI-AcCys and AcMDI-AcCys was studied at pH 7.4. The half-life (=3.1 h) of MDI-AcCys was calculated from the equation of the linear regression curve. The half-life of AcMDI-AcCys was estimated to be 7.1 h. The main decomposition products of AcMDI-AcCys were AcMDA and AcMDI-AcMDA according to the performed HPLC analyses. The same experiment was performed with AcMDI-AcCys at pH 8.9. The half-life of the products was 15 min. AcMDA was the major product.

Reactions with Albumin. Albumin was reacted at pH 7.4 using different molar ratios with MDI-AcCys and/or AcMDI-AcCys. Albumin was digested with Pronase and analyzed with LC-MS/MS (Figure 3). MDI-Lys and AcMDI-Lys were quantified as described previously.[17] In the experiments with a molar ratio of 1:1 and 10:1 of MDI-AcCys to albumin, 4432 and 34324 pmol of MDI-Lys were found per mg of albumin. In the experiments with a molar ratio of 1:1 and 10:1 of AcMDI-AcCys to albumin, 3446 and 24757 pmol of AcMDI-Lys were found per mg of albumin. Albumin was reacted with MDI-AcCys at the ratio of 1:1 at pH 7.4 and at pH 9. The adduct levels for MDI-Lys were 1.3 larger at pH 9 than at pH 7.4.

For the analysis of protein adducts of xenobiotics, enzymatic digestions have been used.[31] Ideally, all methods using enzymatic digestion procedures should take into account the variation of the digestion yields. Therefore, we synthesized albumin adducts of d_4-MDI, which can be used as internal standard for the analysis of biological standards. For this purpose, we synthesized first d_4-MDI-AcCys. d_4-MDI-AcCys was reacted with albumin at pH 7.4 at a molar ratio of 1:100 (d_4-MDI-AcCys/albumin). After enzymatic digestion of albumin with Pronase, we obtained 99.3 pmol of d_4-MDI-Lys/mg albumin (Figure 3). To test the precision of the method, we repeated the digestion of d_4-MDI-AcCys-modified albumin five

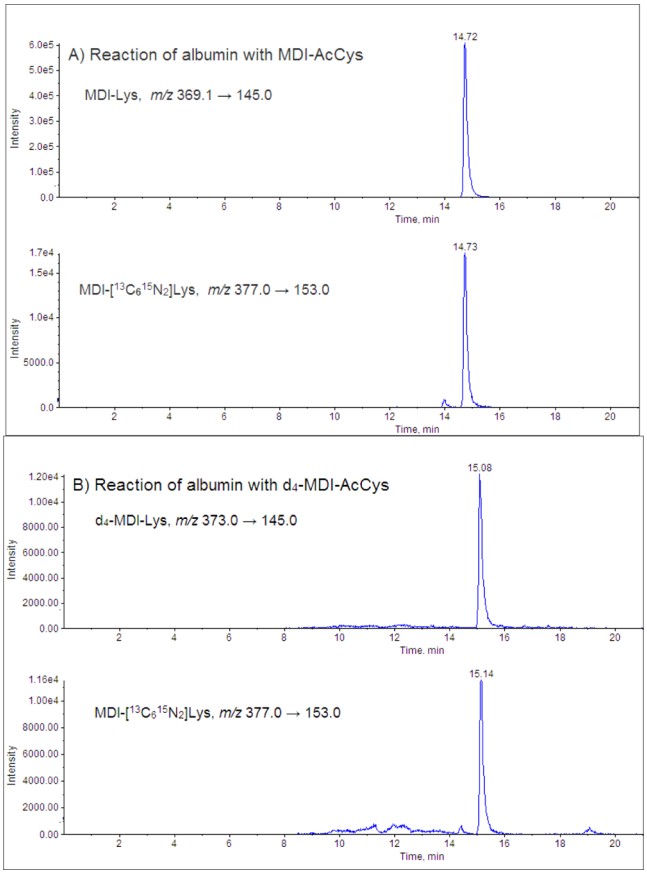

Figure 3. Main reaction product obtained from albumin modified in vitro with (A) MDI-AcCys at a molar ratio of 1:1 and with (B) d_4-MDI-AcCys at a molar ratio of 1:100 (d_4-MDI-AcCys/albumin). Albumin was digested with Pronase in the presence of the internal standard MDI-[$^{13}C_6$ $^{15}N_2$]-Lys (13.25 pmol). The digest was purified by solid phase extraction and analyzed with LC-MS/MS.

times. The variation coefficient was ±4.2% (=relative standard deviation).

Albumin adducts of MDI have been in found in vivo[17] and in vitro[32] with lysine. We investigated the presence of other adducts such as the adduct with the N-terminal amino acid of human albumin, which is aspartic acid. To achieve this, we looked at proteins modified in vitro with MDI-AcCys. After digestion of albumin with Pronase, we found only MDI-Lys. We compared the digests of albumin to the LC-MS/MS chromatograms of the synthetic standards (Figure 5 in the Supporting Information). We monitored also the mass fragment ions for the adducts that could not be synthesized and characterized by NMR: MDI-His, MDI-Ser, MDI-Trp, and MDI-Tyr, respectively. The following MS/MS transitions were monitored: 378.2 → 154.2, 328.3 → 104.1, 427.3 → 203.2, and 404.4 → 180.1 for MDI-His, MDI-Ser, MDI-Trp, and MDI-Tyr, respectively. These product ions correspond to the mass of the parent amino acid. This is the main product ion seen in the MS/MS analyses of the synthesized MDI-amino acid adducts. According to the calculated lipophilic properties (calculated with program by ChemAxon) of the compounds in a 10 mM ammonium formate solution, the following elution time increase was expected (=increasing lipophilicity): MDI-Asp, MDI-His, MDI-Ser, MDI-Lys, MDI-Val, MDI-Trp, and MDI-Tyr. Only MDI-Lys was found unambiguously in albumin digested enzymatically with Pronase. This is contrast to results

obtained with albumin modified in vitro with 4MPI (see below and the Supporting Information). Other digestion procedures as described for the analysis of nitrotyrosine[33] might release some of the other adducts. We applied acid hydrolysis to release the N-terminal amino acid of albumin, a method that has been successfully used for years for the analysis of N-terminal adducts of environmental pollutants.[34] The N-terminal adduct with aspartic acid was detected in in vitro-modified albumin after acid hydrolysis (Figure 4). The hydrolysis and the

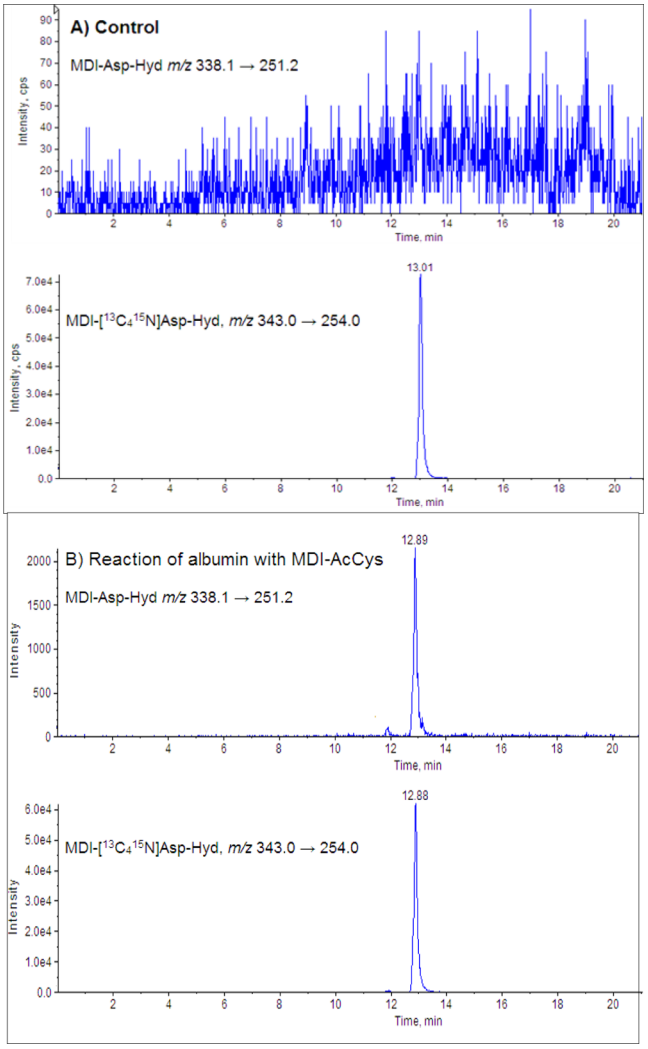

Figure 4. N-terminal adduct of MDI found in human albumin modified in vitro with MDI-AcCys at a molar ratio of 1:1. (A) Control albumin and (B) in vitro modified albumin (0.5 mg) were hydrolyzed with 2 M HCl for 2 h in the presence of the internal standard MDI-[$^{13}C_4{}^{15}N$]Asp (68.4 pmol). LC-MS/MS analyses showed the formation of the corresponding hydantoin MDI-Asp-Hyd with the same retention time as the internal standard MDI-[$^{13}C_4{}^{15}N$]-Asp-Hyd.

workup were controlled using an isotope-labeled standard, MDI-[$^{13}C_4{}^{15}N$]Asp. The levels of MDI-Asp-Hyd (5 pmol/mg) are approximately 900 times lower than the MDI-Lys (4432 pmol/mg albumin) levels measured in the same samples after enzymatic digestion with Pronase.

The reactions of MDI derivatives obtained with albumin in vitro (see above) were compared with the results obtained with 4MPI. Albumin was reacted with 4MPI and then digested with Pronase. The standards were obtained as described in the literature.[27] The α-amino acetyl group of the adducts 4MPI-AcLys, 4MPI-AcSer, and 4MPI-AcTyr was cleaved with acylase following a procedure applied successfully for aflatoxin adducts of N_α-Ac-Lysine.[28] The obtained products were compared to the digest of 4MPI-modified albumin. 4MPI-Lys appears to be the major product, since the relative ionization and dissociation efficiencies of such different amino acid adducts are comparable. However, the efficiency of Pronase to release these different 4MPI adducts from albumin might be different. Other adducts such as 4MPI-Asp, 4MPI-Ser, 4MPI-Lys, and 4MPI-Tyr were present (Figure 6 in the Supporting Information). In control albumin, these adducts were not found. Therefore, more products with amino acids could be found in albumin modified with 4MPI than in albumin modified with MDI derivatives. Possibly the larger MDI adducts might hinder the digestion of albumin to the single amino acids.

The methods developed for the synthesis of amino acid adducts of MDI should be applicable to all aromatic and aliphatic isocyanates. Blood protein adducts can be synthesized under physiological conditions using MDI-AcCys, and AcMDI-AcCys. This will enable us to optimize the synthesis of standard antigens, which can be used in the immunoassays for the quantitation of antibodies in exposed workers. The immunological results obtained in the past have been controversial and topic of critical reviews.[35,36] In future studies, it will be important to produce antigens according to the adducts found in vivo. Then, the immunological tests, the disease status, and the albumin adduct levels can be compared to confirm the relationship between the two biomarkers and the disease.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figures of LC-MS/MS (negative ESI) analyses of the standard compounds and reaction products. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

4MPI, 4-methylphenyl isocyanate; 4MPI-AcLys, N^2-acetyl-N^6-[(4-methylphenyl)carbamoyl]-lysine; 4MPI-AcSer, *N*-acetyl-*O*-[(4-methylphenyl)carbamoyl]-serine; 4MPI-AcTyr, *N*-acetyl-*O*-[(4-methylphenyl)carbamoyl]-tyrosine; 4MPI-Asp, *N*-[(4-methylphenyl)carbamoyl]-aspartic acid; 4MPI-Lys, N^6-[(4-methylphenyl)carbamoyl]-lysine; 4MPI-Ser, *O*-[(4-methylphenyl)carbamoyl]-serine; 4MPI-Tyr, *O*-[(4-methylphenyl)carbamoyl]-tyrosine; AcMDA, *N*1-[4-(4-aminobenzyl)phenyl]acetamide; AcMDI, *N*-[4-(4-isocyanatobenzyl)phenyl]acetamide; AcMDI-AcCys, *N*-acetyl-*S*-[[4-(4-acetylaminobenzyl)phenyl]carbamoyl]-cysteine; AcMDI-Asp, *N*-[[4-(4-acetylaminobenzyl)phenyl]carbamoyl]-aspartic acid; AcMDI-AcLys, N^2-acetyl-N^6-[[4-(4-acetylaminobenzyl)phenyl]carbamoyl]-lysine; AcMDI-

AcMDA, *N*-[4-({4-[({4-[4-acetamidobenzyl]phenyl}-carbamoyl)amino]phenyl}methyl)phenyl]acetamide; AcMDI-Glu, *N*-[[4-[4-acetylaminobenzyl]phenyl]carbamoyl]-glutamic acid; AcMDI-Lys, N^6-[[4-[4-acetylaminobenzyl]phenyl]-carbamoyl]-lysine; AcMDI-Val, *N*-[[4-[4-acetylaminobenzyl]-phenyl]carbamoyl]-valine; $AcNO_2MDA$, *N*-acetyl-4′-amino-4-nitrodiphenylmethane; BocMDA, *tert*-butyl [4-(4-aminobenzyl)phenyl]carbamate; BocMDI, *tert*-butyl [4-(4-isocyanatobenzyl)phenyl]carbamate; d_4-AcMDA, *N*1-[4-(4-amino-(3,5-dideuterobenzyl))-(2,6-dideuterophenyl)]-acetamide; d_4-AcMDI-Asp, *N*-[(4-{4-acetylamino-3,5-dideuterobenzyl}2,6-dideuterophenyl)carbamoyl]-aspartic acid; d_4-AcMDI-Glu, *N*-[(4-{4-acetylamino-3,5-dideuterobenzyl}2,6-dideuterophenyl)carbamoyl]-glutamic acid; d_4-MDA, 4,4′-methylene-bis(2,6-dideuteroaniline); d_4-MDI-AcCys, *N*-acetyl-*S*-[(4-{4-amino-3,5-dideuterobenzyl}2,6-dideuterophenyl)carbamoyl]-cysteine; d_4-MDI-Asp, *N*-([4-{4-amino-3,5-dideuterobenzyl}2,6-dideuterophenyl]carbamoyl)-aspartic acid; d_4-MDI-Glu, *N*-([4-{4-amino-3,5-dideuterobenzyl}2,6-dideuterophenyl]carbamoyl)-glutamic acid; d_4-MDI-Val, *N*-([4-{4-amino-3,5-dideuterobenzyl}2,6-dideuterophenyl]carbamoyl)-valine; MDI, 4,4′-methylenediphenyl diisocyanate; MDI-AcCys, *N*-acetyl-*S*-[[4-(4-aminobenzyl)phenyl]carbamoyl]-cysteine; MDI-AcLys, N^2-acetyl-N^6-[[4-(4-aminobenzyl)phenyl]carbamoyl]-lysine; MDI-Asp, *N*-[[4-(4-aminobenzyl)phenyl]carbamoyl]-aspartic acid; MDI-Asp-Hyd, {1-[4-(4-aminobenzyl)phenyl]-2,5-dioxoimidazolidin-4-yl}acetic acid; MDI-[$^{13}C_5{}^{15}N$]Asp-Hyd, [1-[4-(4-aminobenzyl)phenyl]-2,5-dioxo(4,5-^{13}C2,3-^{15}N)imidazolidin-4-yl](^{13}C2)acetic acid; MDI-Lys, N^{ϵ}-([4-(4-aminobenzyl)-phenyl]carbamoyl)-lysine; MDI-Glu, *N*-([4-(4-aminobenzyl)-phenyl]carbamoyl)-glutamic acid; MDI-MDA, 1,3-bis({4-(4-aminobenzyl)methyl]phenyl}urea; MDI-Val, *N*-([4-(4-aminobenzyl)phenyl]carbamoyl)-valine; MDI-Val-GlyGly, *N*-([4-(4-aminobenzyl)phenyl]carbamoyl)-valylglycyl-glycine; MDI-Val-Hyd, 3-[4-(4-aminobenzyl)phenyl]-5-(propan-2-yl)-imidazolidine-2,4-dione; MDI-[$^{13}C_5{}^{15}N$]Val-Hyd, 3-[4-(4-aminobenzyl)phenyl]-5-[($^{13}C_3$)propan-2-yl](4,5-$^{13}C_2$,1-^{15}N)-imidazolidine-2,4-dione; NO_2MDA, 4′-amino-4-nitrodiphenyl-methane; NO_2MDI-Val-GlyGly, *N*-[[4-(4-nitrobenzyl)phenyl]-carbamoyl]valylglycyl-glycine; PLE, pig liver esterase

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