A New General Pathway for Synthesis of Reference Compounds of N-Terminal Valine−Isocyanate Adducts

Ronnie Davies,[†] Per Rydberg,*[,†] Emelie Westberg,[†] Hitesh V. Motwani,[†] Erik Johnstone,[‡] and Margareta Törnqvist[†]

Department of Materials and Environmental Chemistry and Department of Organic Chemistry, Stockholm University, SE-106 91 Stockholm, Sweden

Received August 11, 2009

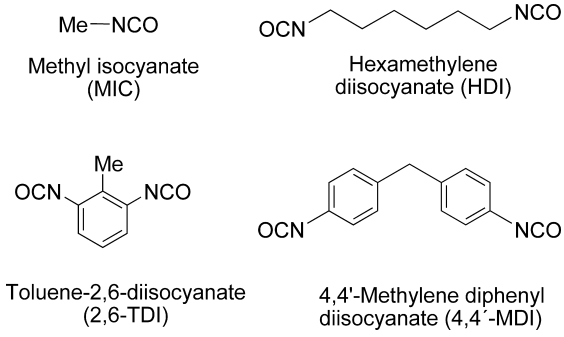
Adducts to Hb could be used as biomarkers to monitor exposure to isocyanates. Particularly useful is the measurement of carbamoylation of N-terminal valines in Hb, after detachment as hydantoins. The synthesis of references from the reactive isocyanates, especially diisocyanates, has been problematic due to side reactions and polymerization of the isocyanate starting material. A simpler, safer, and more general method for the synthesis of valine adducts of isocyanates has been developed using *N*-[(4-nitrophenyl)-carbamate]valine methylamide (NPCVMA) as the key precursor to adducts of various mono- and diisocyanates of interest. By reacting NPCVMA with a range of isocyanate-related amines, carbamoylated valines are formed without the use of the reactive isocyanates. The carbamoylated products synthesized here were cyclized with good yields of the formed hydantoins. The carbamoylated derivative from phenyl isocyanate also showed quantitative yield in a test with cyclization under the conditions used in blood. This new pathway for the preparation of *N*-carbamoylated model compounds overcomes the above-mentioned problems in the synthesis and is a general and simplified approach, which could make such reference compounds of adducts to N-terminal valine from isocyanates accessible for biomonitoring purposes. The synthesized hydantoins corresponding to adducts from isocyanic acid, methyl isocyanate, phenyl isocyanate, and 2,6-toluene diisocyanate were characterized by LC-MS analysis. The background level of the hydantoin from isocyanic acid in human blood was analyzed with the LC-MS conditions developed.

Introduction

Organic mono-, di-, and polyisocyanates are common industrial chemicals (Scheme 1). Monoisocyanates, such as phenyl isocyanate (PIC) and methyl isocyanate (MIC), have been used as synthetic intermediates in the chemical industry with MIC in particular being used for the synthesis of various carbamate pesticides. MIC is infamously known as the toxic agent in the tragic chemical disaster in Bhopal, India, 1984, in which over 3000 persons died (*1*). Diisocyanates constitute the monomeric subunits of polyurethanes (PURs). PURs have a wide range of applications from flexible and rigid foams, elastomers, and coatings to textiles (*2, 3*). PUR production constituted 12 million metric tons worldwide in 2007 and is currently growing at the rate of 5% each year due to the high demand of PUR products (*3*). Diisocyanates used in the industry include toluene diisocyanate (TDI; a mixture of the 2,4- and 2,6-isomers), 4,4′-methylene diphenyl diisocyanate (MDI, both isomers and homologues), and 1,6-hexamethylene diisocyanate (HDI) (Scheme 1).

The reactivity of mono- and diisocyanates, which makes them useful for many purposes, stems from the highly electrophilic carbon atom in the isocyanate group. In vivo, this reactivity can lead to carbamoylation reactions with proteins, etc. Furthermore, the corresponding amines are formed through hydrolysis, and exposure to, for instance, TDI and MDI thus also leads to internal exposure to the corresponding aromatic amines (*4, 5*).

Scheme 1. Common Commercial Isocyanates



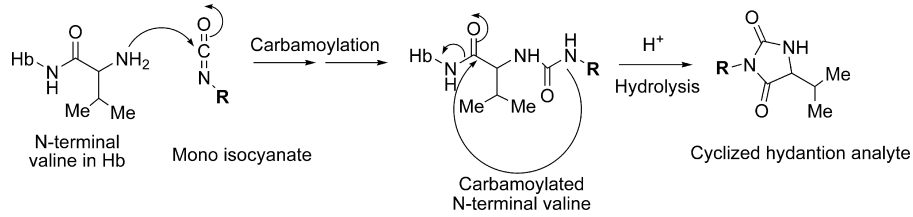
It was established after the Bhopal disaster that adducts to N-terminal valine in hemoglobin (Hb) can be used for biomonitoring of isocyanate exposure (*6, 7*). Investigators were able to measure MIC exposure through detachment of the carbamoylated N-terminal valine of Hb by acid hydrolysis, to produce 3-methyl-5-isopropylhydantoin (MVH) (Scheme 2). Carbamoylation of other amino acids was, as well, detected in preserved tissue samples (*1, 7*). Since then, carbamoylation of N termini in Hb has also been used to identify and quantify internal exposure to dimethylformamide (which metabolizes to MIC) (*8, 9*) and TDI in humans (*10*) and to MDI, so far only in rats (*11*).

Besides the occupational exposure to isocyanates, there is a general endogenous exposure to isocyanic acid, which is

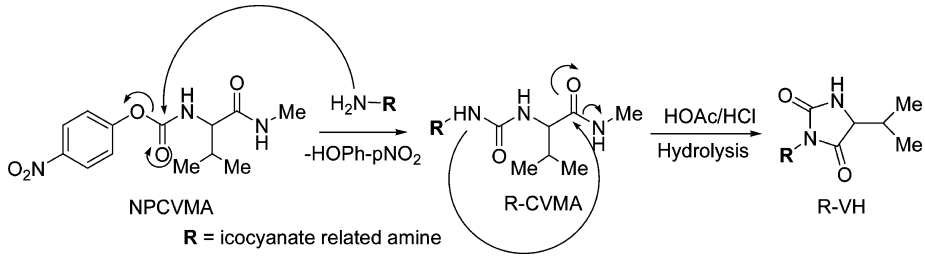
* To whom correspondence should be addressed. Tel: +46-8-163769. Fax: +46-8-163979. E-mail: per.rydberg@mk.su.se.
[†] Department of Materials and Environmental Chemistry.
[‡] Department of Organic Chemistry.

10.1021/tx900278p
Published on Web 01/19/2010

Scheme 2. Carbamoylation and Hydrolysis of N-Terminal Valine



Scheme 3. Synthetically Produced Carbamoylation Using Amine and a Carbamate Group



produced in the spontaneous breakdown of urea (*12*). In patients with kidney failure, urea levels have been monitored through increased carbamoylation of N-terminal valine in Hb (*13*). The measurement of carbamoylation of N termini in Hb was already pioneered in 1973 by Manning et al. in the treatment of sickle cell disease by cyanate (*14*).

For the measurement of hydantoins formed from *N*-carbamoylated terminal valine in Hb as a biomarker of internal exposure to isocyanates, it is useful to have access to reference compounds that mimic the adduct to N termini in Hb. The references should be useful in studies of yield in the acid-catalyzed cyclization reaction and of the extraction of the hydantoins, detached from Hb or from isolated globin (*15*). An established pathway to synthesize *N*-carbamoylated reference compounds and corresponding internal standards for analysis of adducts from isocyanates is to react a model compound of N-terminal valine with the selected isocyanates (*8, 10, 11, 16, 17*). The formed carbamoylated compound is then cyclized to produce the hydantoin (Scheme 2). However, this route using isocyanates as starting materials is problematic due to side reactions of the mono- and diisocyanate with water, producing symmetrical urea compounds. When using diisocyanates, polymerization side reactions are a major problem, which lead to very low yields of the desired products (our unpublished work). Furthermore, the use of volatile and toxic isocyanates in the preparation of reference compounds should be avoided. A strategy to overcome such polymerization side reactions when preparing reference standards from 2,4-TDI with single amino acids was introduced by Sabbioni et al. in 2001 (*10*). Instead of using TDI, one of the isocyanate group was masked by a nitro group. After the carbamoylation reaction, this group was reduced to an amine. This was an innovative solution, and improved yields could be obtained as compared to the earlier methods. However, a major drawback with this approach is that it includes several synthetic steps with rather specific starting materials for the preparation of the analytes of different isocyanates. In summary, there is a need for a new simple and general synthetic route to prepare *N*-carbamoylated reference compounds.

The basic idea to attack the problem with low yields, etc., was to "invert" the chemistry for synthesis of *N*-carbamoylated reference compounds. Instead of using electrophilically reactive isocyanates, nucleophilic aliphatic or aromatic amines/diamines were reacted with an electrophilically activated carbamate precursor. The studied precursors were synthesized from valine methylamide reacted with 4-nitrophenylchloroformate (see Scheme 3). The approach to use such precursors is analogous by the chemistry used in solid and liquid phase chemistry to synthesize ureas from carbamates and amines (*18−20*). This strategy could be especially useful for the synthesis of carbamoylvalines, as the "activated precursor" only reacts once. This means that the risk for polymerization side reactions is eliminated. Another great benefit will be that chemical libraries can be build from a single activated carbamate precursor.

The reference substances of PIC synthesized in this work were used to test the procedure to form hydantoin in human blood. Conditions were established for LC-MS/MS analysis of the hydantoins and were used for the analysis of carbamoylated N-terminal valine in human Hb from the naturally occurring isocyanic acid.

Materials and Methods

Caution: *Chloroformates and aromatic amines are toxic and were handled with protective clothing in an efficient hood.*

Chemicals. Ethyldi-isopropylamine was purchased from Aldrich (Steinheim, Germany). Valine amide hydrochloride (VA-HCl) was obtained from Novabiochem AG (Läufelflingen, Germany). Aniline and ammoniumsulphate were obtained from Merck (Darmstadt, Germany). NaOH was obtained from EKA Chemicals AB (Bohus, Sweden). Methylamine (40%), L-valine methyl ester hydrochloride (L-VME-HCl) (99%), and 4-nitrophenyl chloroformate (97%) were obtained from Acros (Geel, Belgium). $KMnO_4$ and 2,6-diaminotoluene (97%) and isopropylhydantoin (VH) were obtained from Aldrich. The hemolysate used for study of yield in cyclization reaction was obtained from commercial human blood from Karolinska Hospital (Stockholm, Sweden). All other chemicals and solvents used were of analytical grade.

Analytical Methods. All ^{1}H NMR and ^{13}C NMR (300 MHz) were recorded on a Varian Mercury 400 spectrometer in CD_3OD, $CDCl_3$, DMSO-d_6, or $(CD_3)_2CO$ solutions (50 mg/mL) at 25 °C with TMS as the internal standard. ^{1}H NMR and ^{13}C NMR spectra of compounds **2**, **3**, and **5**−**10** are also available as Supporting Information.

Melting points were determined on a Büchi 353 apparatus. TLC was performed using silica gel 60 F-254 from Merck, and R_f values were obtained by the application of 10 μg aliquots of the reaction mixtures. Spots were visualized with UV (254 nm), ninhydrin, and $KMnO_4$.

The LC-MS/MS system consisted of a Shimadzu Prominence LC-system coupled to an API 3200 Q trap instrument (Applied Biosystems). The mass spectrometer was operated using an

electrospray ionization source in the positive ion mode (ESI^+). Acquisition and data processing were done with the Analyst software version 1.5 (Applied Biosystems). The separation was performed with an C_{18} column (5 μm, 1.0 mm × 150 mm, Ace). The mobile phase system consisted of A, 0.1% formic acid (FA) in water:acetonitrile (95:5, v/v), and B, 0.1% FA in water:ACN (5:95,v/v). The gradient was 0% B for 1 min, followed by a linear increase to 50% B in 1 min, and 50−100% B in 5 min followed by isocratic 100% B in 3 min, with a flow rate of 75 μL/min. The MS/MS was operated in product ion scan (PIS) mode and multiple reaction monitoring (MRM) mode. The instrumental settings used for the MRM were as follows: ion source temperature, 500 °C; declustering potential, 30 V; collision cell exit potential, 25 V; ion spray voltage, 5000 V; curtain gas (N_2), 20; collision gas (N_2), 5; ion source gas (N_2), 55; and turbo gas (N_2), 70 (latter four are arbitrary units from the Analyst software). Varied collision energy (15−25 V), entrance potential (5−10 V), collision cell entrance potential (7−15), and collision cell exit potential (1−5) were applied for the different transitions used in MRM.

Initially, a less optimized LC-MS system, without column separation, was used for the characterization of synthesized compounds and for studies of yield in the cyclization reaction. Samples from the cyclization studies were also analyzed with the optimized system as described above.

Synthesis

Valine methyl amide (VMA) (1). L-VME-HCl (57 mmol, 9.5 g) was added to methylamine (40% w/v, 150 mL) while stirring in an ice bath. The solution was left for 2 days at 22 °C. The solvent was evaporated, and dry tetrahydrofuran (THF) (60 mL) was added to the solid residue. The white suspension was ultrasonicated for 30 min to obtain dissolution. After the solution was stored at +4 °C overnight, valine and methylamine HCl were filtered off, and the eluate was evaporated to yield the desired product **1** (4.4 g, 34 mmol, 60%) as an oil. Analysis: ^{1}H NMR ($CDCl_3$, 25 °C): δ 0.82, 0.99 [2d, 6H, J = 6.8 Hz, C$H_3(\gamma,\gamma')$], 1.4 [s, 2H, NH_2], 2.13 [m, 1H J = 3.7 Hz, C$H(\beta)$], 2.82, 2.83 [dd, 3H, J = 5.2 Hz, NH-CH_3], 3.24 [d, 1 H, J = 3.7 Hz, C$H(\alpha)$].

***N*-[(4-Nitrophenyl)carbamate]valinmethylamide (NPCVMA) (2).** To a solution of 4-nitrophenyl chloroformate (0.57 g, 2.8 mmol) in dry THF (15 mL), VMA (0.74 g, 5.7 mmol) in THF (15 mL) was dropped over a period of 10 min while stirring on an ice bath under inert atmosphere (N_2). The mixture was slowly heated to ambient temperature while following the progress by TLC [ethyl acetate/dichloromethane (EtOAc/DCM), 3:1]. After 2.5 h, the solvent was evaporated, and the solid product was crystallized from ethanol (EtOH)/ACN (1:5, 75 mL) to yield **2** (0.59 g, 2.0 mmol, 71%) as white crystals. Analysis: mp 182−184 °C, R_f, TLC 0.4, EtOAc/DCM (3:1). ^{1}H NMR ($CDCl_3$, 25 °C): δ 1.0, 1.21 [2d, 6H, J = 6.8 Hz, C$H_3(\gamma,\gamma')$], 2.13 [m, 1H J = 6.8 Hz, C$H(\beta)$], 2.87 [d, 1H, J = 4.8 Hz, CONHCH_3], 3.94, 3.97 [dd, 1H, J = 6.4 Hz, CH (α)], 5.79 [d 1H, J = 8.8, CONHCH], 5.89 [d, 1H, CONH CH_3], 7.31 [d, 2H, J = 6.8 Hz, arom], 8.24 [d, 2H J = 6.8 Hz, arom.]. ^{13}C NMR ($CDCl_3$, 50 °C): δ 18.30, 19.37 [$CH_3(\gamma,\gamma')$], 26.50 [CONH-CH_3], 31.75 [$CH(\beta)$], 61.15 [$CH(\alpha)$], 115.9 122.1, 125.3, 126.4, 145.3, 153.6 [arom.], 156.05 [OCONH], 171.45 [CONHCH$_3$].

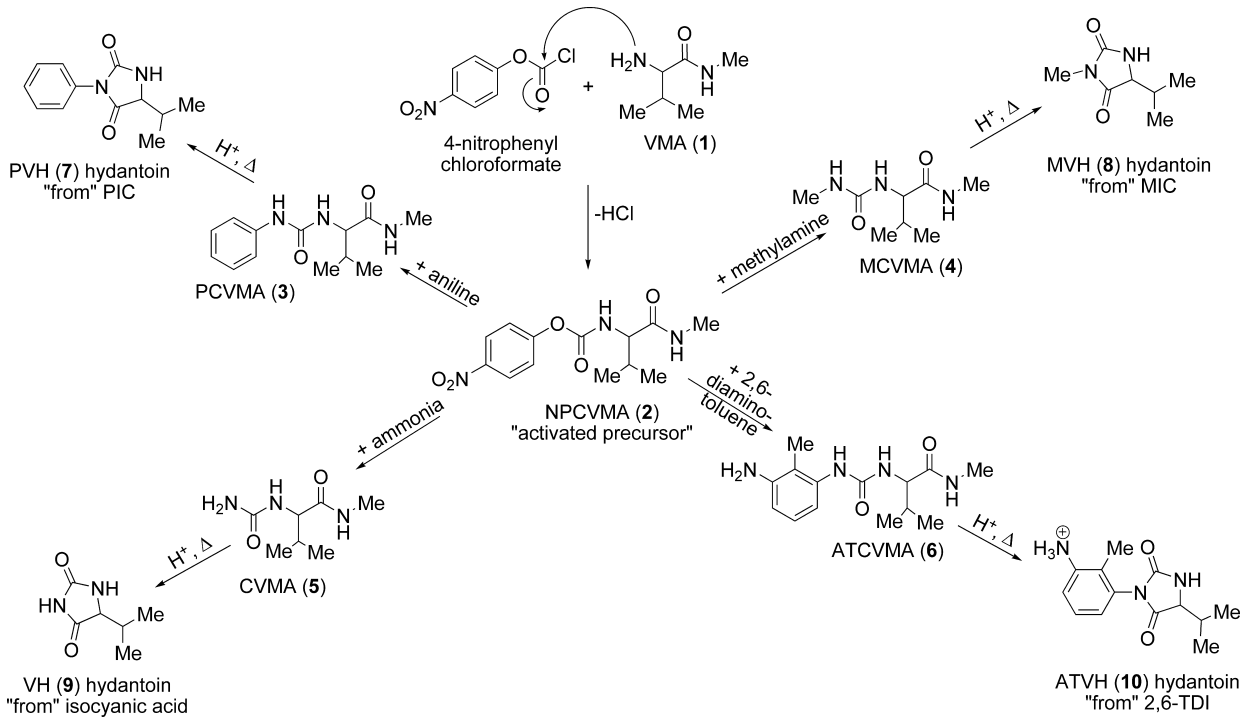
Phenylcarbamoyl-valinmethylamide (PCVMA) (3). NPCVMA (0.68 mmol, 0.20 g) and freshly distilled aniline (4 mL) were mixed together and stirred at 60 °C for 18 h. The reaction was followed by TLC with EtOAc/DCM (1:1) for visualizing the product with UV (R_f 0.3), NPCVMA (R_f 0.4), aniline (R_f 0.8), and the byproduct *p*-nitrophenol (R_f 0.9). The reaction was stopped when all NPCVMA was depleted. Water (30 mL) was added to the reaction mixture, and the pH was adjusted to 9 by dropwise addition of 1 M NaOH (0.1 mL). The product and aniline were extracted with EtOAc (2 × 30 mL), and the organic phase was washed with 0.1 M NaOH (5 × 30 mL). Aniline was extracted from the organic phase by vigorous shaking with 0.1 M HCl (5 × 30 mL). After the organic solvent was evaporated, the product was crystallized from EtOH/ACN (1:1, 10 mL) at 60 °C to yield **3** (42 mg, 0.17 mmol, 25%) as white crystals. Analysis: mp 275−276 °C, R_f, 0.3, EtOAc/DCM (1:1). ^{1}H NMR (DMSO-d_6, 25 °C): δ 0.81, 0.84 [2d, 6H, J = 6.8 Hz, C$H_3(\gamma,\gamma')$], 1.90 [m, 1H, J = 6.4, 5.9 Hz, C$H(\beta)$], 2.58 [d, 3H, J = 4.4 Hz, CONHCH_3], 4.01, 4.37 [dd, 1H, J = 6.0 Hz, C$H(\alpha)$], 6.31 [d, 1H, J = 8.8 Hz, CONHCH], 6.86 [t, 1H, J = 7.2 Hz, para-arom], 7.19 [2t, 2H, J = 7.2 Hz, meta-arom], 7.33 [2t, 2H, J = 7.2 Hz, ortho-arom], 7.97 [d, 1H, J = 4.0 Hz, CONHCH$_3$], 8.63 [s, 1H, arom-NHCO]. ^{13}C NMR (DMSO-d_6, 25 °C): δ 18.24, 19.71 [CH_3 (γ,γ')], 25.85 [NH-CH_3], 31.61 [$CH(\beta)$], 58.05 [$CH(\alpha)$], 117.86, 121.51, 121.51, 129.15, 129.15, 140.9 [arom.], 155.36 [$CO(NH)_2$], 172.47 [CONH$_2$]. LC-MS (ESI^+): m/z 250 {[M + H]$^+$, 10%}, 219 {[M − 31 (−CH_3NH_2)]$^+$, 20%}, 191 {[M − 59 (−CONHCH$_3$)]$^+$, 20%}, 157 {[M − 92 (−C_6H_5NH)]$^+$, 5%}, 131 {[M − 119 (−C_6H_5NHCO)]$^+$, 100%}.

Methylcarbamoyl-valinmethylamide (MCVMA) (4). Dry methylamine hydrochloride ($MeNH_3Cl$) was prepared from 40% aqueous methylamine (40% w/v, 50 mL) with 3 M HCl (300 mL) followed by evaporation. This salt (10 g) was alkalized dropwise with 12 M KOH (15 mL) at 45 °C. The generated $MeNH_2$(g) was dried ($MgSO_4$) and bubbled into a connected flask containing NPCVMA (200 mg, 0.68 mmol) dissolved in EtOH (10 mL), and nitrogen was used as the carrier gas. The reaction was followed by TLC with EtOAc/DCM/acetic acid (HOAc) (3:1:0.1), NPCVMA (R_f 0.5), the deprotonated *p*-nitrophenolate (R_f 0.9), and pH paper for the generation of $MeNH_2$(g). After 6 h the reaction mixture was sealed and left to stand overnight after which all NPCVMA was depleted. The solvent was evaporated, and the residue was dissolved in 0.1% HOAc (50 mL) and extracted with EtOAc (2 × 30 mL) to remove the *p*-nitrophenol side product. The aqueous phase was evaporated, and the product was crystallized from EtOH/ACN 1:1 (4 mL) to yield **4** (26 mg, 0.14 mmol, 21%) as white crystals. Analysis: mp 265−269 °C. LC-MS (ESI^+): m/z 188 {[M + H]$^+$, 10%}, 157 {[M − 31 (−CH_3NH_2)]$^+$, 10%}, 131 {[M − 57 + H]$^+$, 100%}.

Carbamoyl-valinmethylamide (CVMA) (5). Ammonium chloride (10 g) was alkalized dropwise with 12 M KOH (15 mL) at 45 °C as described above to generate dry NH_3(g), which was bubbled into a solution of NPCVMA (200 mg, 0.68 mmol) in EtOH (10 mL). The reaction conditions and purification procedure were exactly as described above. The solid product was crystallized from EtOH/ACN 1:1 (4 mL) to yield **5** (20 mg, 0.12 mmol, 17%) as white crystals. Analysis: mp 235−238 °C. ^{1}H NMR (DMSO-d_6, 25 °C): δ 0.77−0.84 [4d, 6H, J = 7.2 Hz, C$H_3(\gamma,\gamma')$], 1.87 [m, 1H J = 6.4, Hz, C$H(\beta)$], 2.56 [d, 3H, J = 4.4 Hz, CONHCH_3], 3.91, 3.93, [dd, 1H, J = 6.0 Hz, C$H(\alpha)$], 6.26, 6.33 [dd, 1H J = 8.8 Hz, NHCONH$_2$], 6.93, 8.11 [dd, 1H J = 8.8 Hz, NHCONH_2], 7.79, 7.84 [dd, 1H J = 4.4 Hz, CONHCH$_3$]. ^{13}C NMR (DMSO-d_6, 25 °C): δ 18.15, 19.70 [2 × $CH_3(\gamma,\gamma')$], 25.81 [NH-CH_3], 31.54 [$CH(\beta)$], 58.41 [$CH(\alpha)$], 158.01 [$CO(NH)_2$], 172.87 [CONH]. LC-MS (ESI^+): m/z 174 [M + H]$^+$.

***N*-[(3-Amino-4-methylphenyl)carbamoyl]valinmethylamide (ATCVMA) (6).** NPCVMA (0.21 g, 0.71 mmol) in dry THF (4 mL) was added to a solution of 2,6-diaminotoluene (85 mg, 0.70 mmol) (freshly purified in a silica gel column, mobile

Scheme 4. Reference Compounds Synthesized by the New Method from an Activated Carbamate Precursor



phase ACN, R_f 0.8) in dry THF (0.5 mL). The reaction was performed at 60 °C for 4 days with magnetic stirring, and the progress was followed by TLC with EtOAc/DCM (1:1, v/v, R_f values were as follows: NPCVMA, 0.4; *p*-nitrophenol, 0.9; and 2,6-diaminotoluene, 0.3). The desired product precipitated as gray/white crystals and was purified by evaporation of the solvent and crystallization from ACN/EtOH 5:1 (100 mL) to yield **6** (114 mg, 0.41 mmol, 58%) as white crystals. Analysis: mp 279−280 °C, R_f, TLC 0.5, EtOAc/DCM (1:1). ^{1}H NMR (DMSO-d_6, 25 °C): δ 0.84, 0.86 [2 × d 6H, J = 8.2 Hz, CH_3(γ,γ')], 1.89 [m, 1H, CH(β)], 1.89 [s, 3H, arom-CH_3], 2.60 [d, 3H, J = 6.4, CONH-CH_3], 4.03 [dd, 1H, J = 3.6 Hz, CH(α)], 4.78 [s, 2H, arom-NH_2], 6.33 [d, 1H, J = 10.8 Hz arom], 6.57 [d, 1H, CONHCH] 6.75 [t, 1H, J = 10.8 Hz, arom.], 6.90 [d, 1H J = 10.8, Hz, arom], 7.74 [s, 1H, arom-NH-CO], 7.95 [d, 1H, J = 6.0, CONH CH$_3$]. ^{13}C NMR (DMSO-d_6, 25 °C): δ 11.32 [arom-CH$_3$], 17.97, 19.28 [2 × CH_3(γ,γ')], 25.40 [NH-CH$_3$], 31.28 [CH(β)], 57.85 [CH (α)], 109.53, 110.98, 112.29, 125.45, 138.04, 146.84 [arom.], 155.43 [CO(NH)$_2$], 172.25 [CO]. LC-MS (ESI$^+$): m/z 279 [M + H]$^+$.

Hydrolysis and Cyclization of Carbamoylated Compounds To Form Hydantoins: 3-Phenyl-5-isopropylhydantoin (PVH) (7), MVH (8), 5-Isopropylhydantoin (VH) (9), 3-(3-Amino-2-methyl)phenyl-5-isopropylhydantoin (ATVH) (10). The urea compounds were cyclized according to Mraz with minor changes (*16*). The respective compounds **3** (40 mg, 0.17 mmol), **4** (40 mg, 0.23 mmol), **5** (20 mg, 0.12 mmol), and **6** (40 mg, 0.14 mmol) were separately dissolved in concentrated HOAc and concentrated HCl (1:2, v/v, 3 mL) and heated at 70 °C for 18 h (see Scheme 4). The reactions were followed by TLC with EtOAc/toluene (1:1) using UV for aromatic hydantoins and KMnO$_4$ for aliphatic compounds. After the mixtures were cooled, ammonium sulfate (1.5 g) and 10 M NaOH were slowly added to obtain a pH of 7 (approximately 5 mL). The solutions were extracted with EtOAc (4 mL), the organic phase was evaporated, and the solid remains were crystallized as follows: PVH (**7**) from acetone/heptane 1:4 (3 mL) as white needle crystals (mp 128−130 °C) with a yield of 25 mg (0.11 mmol, 65%) from compound **3**. MVH (**8**) was crystallized from CHCl$_3$/pentane 1:4 (3 mL) as white needle crystals (mp 121−122 °C) to yield 18 mg (0.12 mmol, 52%) from compound **4**, and VH (**9**) was crystallized from CHCl$_3$/pentane 1:2 (6 mL) to yield 10 mg (0.070 mmol, 62%) from compound **5** as white crystals (mp 137−140 °C). ATVH (**10**) was purified on a silica gel column eluted with EtOAc to yield 11 mg (0.044 mmol, 32%) from compound **6** as a white residue. Analysis: Compound **7**: ^{1}H NMR (CDCl$_3$, 25 °C): δ 1.00, 1.08 [2d, 6H, J = 6.9, 7.2, CH_3(γ,γ')], 2.32 [m, 1H, J = 7.2, 3.6 Hz, CH(β)], 4.05, 4.06 [dd, 1H, J = 1.6, 1.2 Hz, CH(α)], 6.8 [s, 1H, NH], 7.37 [2t, 1 H, J = 7.4 Hz, para-arom], 7.38 [2t, 2H, J = 7.4 Hz, meta-arom], 7.40 [d, 2H, J = 7.4 Hz, ortho-arom]. ^{13}C NMR (CDCl$_3$, 25 °C): δ 16.03, 18.66 [2 × CH_3(γ,γ')], 30.63 [CH(β)], 62.19 [CH(α)], 126.22, 126.22, 128.29, 128.29, 129.13, 129.13, 131.45 [arom.], 157.3 [NHCONH],172.5 [CO-CH]. Compound **8**: ^{1}H NMR (CD$_3$OD, 25 °C): δ 0.89, 1.05 [2d, 6H, J = 6.8, CH_3(γ,γ')], 2.17 [m, 1H, J = 3.6,Hz, CH(β)], 2.95 [s, 1H, CH_3, NCH_3], 3.99 [d, 1H, CH, J = 3.6 Hz, CH(α)]. ^{13}C NMR (CD$_3$OD, 25 °C): δ 14.89, 17.53 [2 × CH_3(γ,γ')], 22.97 [NCH$_3$], 30.03 [CH(β)], 62.29 [CH(α)], 158.55 [CO-CH], 174.71 [NH-CONH]. Compound **9**: ^{1}H NMR [(CD$_3$)$_2$CO, 25 °C]: δ 0.93, 1.05 [2d, 6H, J = 6.8 Hz, CH_3(γ,γ')], 2.15 [m, 1H, J = 3.6 Hz, CH(β)], 4.03, 4.03 [dd, 1H, CH, J = 3.6 Hz, CH(α)], 7.11 [s, 1H, CONH], 9.60 [s, 1H, (CO)$_2$NH]. ^{13}C NMR [(CD$_3$)$_2$CO, 25 °C]: δ 15.49, 17.98 [2 × CH_3(γ,γ')], 30.05 [CH$_3$], 30.1 [CH(β)], 63.28 [CH(α)], 157.50 [NHCONH], 174.57 [CO-CH]. Compound **10**: ^{1}H NMR (DMSO-d_6, 25 °C): δ 1.03, 1.05, 1.08, 1.10 [2 × dd, 6H, J = 6.8, 7.7 Hz, CH_3(γ,γ')], 1.96, 1.97 [2s, 3H, arom-CH_3], 2.33 [m, 1H, J = 3.6 Hz, CH(β)], 4.09, 4.10, 4.11, 4.12 [2 × dd, 1H, J = 3.6 Hz, CH(α)], 6.22 [d, 1H, J = 13.6 Hz, CONH], 6.53, 6.61 [2 × dd, 1H, J = 6.8 Hz, para-arom], 6.74 [2d, 1H, J = 8.0 Hz, ortho-arom.], 7.10 [2 × dt, 1H, J = 4.0 Hz, meta-arom]. ^{13}C NMR (DMSO-d_6): δ 12.15, 12.16 [arom-CH$_3$], 15.96, 16.45, 18.67, 18.93 [2 × CH_3(γ,γ')], 30.23, 30,64 [CH(β)], 62.33, 62,76 [CH(α)], 116.07,116.10, 118.17, 118.47, 120.52, 120.76, 127.06, 127.07, 130.77, 130.80 [arom], 145.96, 146.01 [arom-C-NH$_2$], 157.24, 157.28 [NHCONH],

Table 1. Fragmentation of the Studied Hydantoins Using LC-MS (ESI^+) with Collision Energy 15−20 V

analyte	PVH (**7**)	MVH (**8**)	VH (**9**)	ATVH (**10**)
precursor	PIC	MIC	isocyanic acid	2,6-TDI
LC-MS/MS (ESI^+, PIS)	219 $[M + H]^+$, 100% 72 $[M + H - 147, -adduct]^+$, 99% 120 $[M + H - 99, -valine]^+$, 32% 191 $[M + H - 28, -CO]^+$, 23% 176 $[M + H - 43, -propyl]^+$, 10%	157 $[M + H]^+$, 100% 72 $[M + H - 85, -adduct]^+$, 57% 129 $[M + H - 28, -CO]^+$, 19%	143 $[M + H]^+$, 20% 72 $[M + H - 71, -adduct]^+$, 100% 115 $[M + H - 28, -CO]^+$, 37%	248 $[M + H]^+$, 33% 149 $[M + H - 99, -valine]^+$, 100% 72 $[M + H - 176, -adduct]^+$, 25% 205 $[M + H - 43, -propyl]^+$, 17%
retention time (min)	9.00	8.41	8.02	8.40 and 8.48 (double peak)

172.51, 172.61 [*C*O-CH]. Because of steric hindrance between the methyl group in the toluene ring and the 2-carbonyl group in the hydantoin system, compound **10** occurred in two conformers. This was observed as all signals measured by ^1H NMR and ^{13}C NMR were in duplicates with only slightly different chemical shifts.

Test of Acid-Catalyzed Conversion of PCVMA (3) to PVH (7) in Blood Samples. A dilution series of PCVMA (**3**) ($n = 5$) in hemolysate was prepared by adding 100 μL of 90−1000 μM PCVMA in water to samples of hemolysate (0.5 mL) ($n = 5$). The samples were then hydrolyzed and purified according to Kwan et al. (*21*) with minor modifications. Concentrated HCl:HOAc (1:1, v/v, 2 mL) was added to each sample, which was heated for 18 h at 90 °C. After the tubes were cooled, the pH was adjusted to 4 by 10 M NaOH (∼2 mL) (added dropwise at 0 °C). The samples were then extracted with EtOAc (4 mL) by rocking the tubes for 10 min. The organic phases were isolated after centrifugation (2000 rpm) and then washed with 1 M $NaHCO_3$ (2 mL) followed by H_2O (2 mL). The organic phases were transferred to new vessels, evaporated, and redissolved in methanol (2 mL per sample). Calibration samples ($n = 5$) were prepared by addition of the corresponding concentrations of the hydantoin PVH (**7**) to the washed extract from blood samples hydrolyzed as described above. In addition, a reproducibility test was done with the addition of 100 μL of 500 μM PCVMA ($n = 5$) to samples of hemolysate, which were treated as described above, to give a final concentration of 25 μM. The samples in these tests were analyzed by LC-MS/MS by double injections for each sample.

As a further test of the method, the background level of VH formed from isocyanic acid was analyzed in control Hb samples. These analyses were done with the improved LC-MS/MS conditions with separation and with more careful sample preparation, with preisolation of globin (according to Mraz et al., ref. *15*). Globin was isolated by precipitation from hemolysate (*22*) and worked up after hydrolysis for 1 h at 100 °C according to Mraz et al. (*15*) with a few modifications. Globin (50 mg) was dissolved in concentrated HCl/HOAc (2:1, v/v, 2.5 mL) and heated for 1 h at 100 °C in a Pyrex tube with screw cap (8 mL). Ammonium sulfate (1.5 g) was then added. The mixture was neutralized by addition of 10 M NaOH (pH 7.0−7.5) (approximately 3 mL). The solution was extracted with EtOAc (4 mL), vortex-mixed, and then centrifuged at 3000 rpm. The organic phase was isolated and evaporated at 60 °C under a gentle stream of nitrogen. The obtained residue was dissolved, and 1/5 was transferred to a vial H_2O:ACN (9:1, 100 μL) and analyzed (5 μL, corresponding to 0.5 mg globin) by LC-MS/MS.

Results and Discussion

Synthesis. The use of VMA (**1**) as a model compound for N-terminal valines in Hb is the more suitable choice than valine or valine amide. The amide bond in VMA is expected to be quite similar to a peptide bond during hydrolysis. The synthesis of VMA was a modification of the earlier protocol (*23*) with exclusion of the time-consuming ion exchange step. The modified synthesis allows the methylamine hydrochloride salt and the hydrolyzed L-VME-HCl (valine) to fully precipitate at +4 °C overnight to give sufficient yields (60%) of neutralized VMA after the precipitate is filtered off.

The next step to improve the procedure for the synthesis of the desired compounds was to synthesize a key precursor that can be utilized for the synthesis of a wide range of valine adducts of isocyanates by a reaction with amines instead of isocyanate precursors. This was achieved by synthesis of an activated valyl-carbamate, which was formed from carbamoylating VMA with phenyl chloroformate derivatives (data not shown). Ultimately chosen was 4-nitrophenyl chloroformate to form NPCVMA (**2**), as the nitro group enhances the reactivity of the precursor. This compound (**2**) was synthesized with 70% yield.

In the synthesis of PCVMA (**3**), freshly distilled aniline was used in excess as the reactant and solvent, giving a faster rate of reaction and minimizing side reactions. The formation of **7** and disappearance of **2** could be followed easily by TLC using UV and by the formation of *p*-nitrophenol with its deep yellow color. The desired product could easily be purified from side products by extractions. A yield of 25% was obtained.

In the synthesis of MCVMA (**4**) from **2**, various synthetic routes were tested. The best result was obtained by reacting **2** with anhydrous methylamine dissolved in dry EtOH instead of a 40% aqueous solution. By this route, the hydrolysis of **2** could be avoided. The solution turned yellow within an hour showing that *p*-nitrophenol was released from **2**. Methylamine was periodically added during the next 6 h to saturate the solution, which finally was left to stand overnight, and **2** was depleted. Although a few attempts were made to improve this reaction, the yield did not exceed 30%.

In the synthesis of the *N*-[(3-amino-2-methylphenyl)carbamoyl]valinmethylamide (ATCVMA) (**6**), 2,6-diaminotoluene was reacted with **2** in THF at 60 °C for 4 days. This synthesis gave a higher yield (57%) with little side reactions such as cross-linking. It was found to be imperative to purify the 2,6-diaminotoluene directly before use. In the synthesis of monoiso-

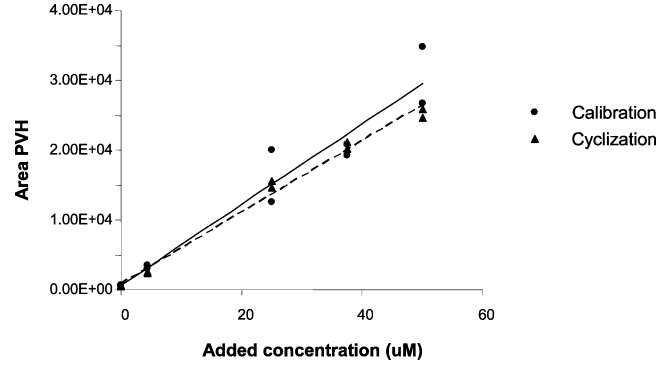


Figure 1. Calibration line with different concentrations of PVH (upper line). Cyclization to PVH with different concentrations of PCVMA (dashed line).

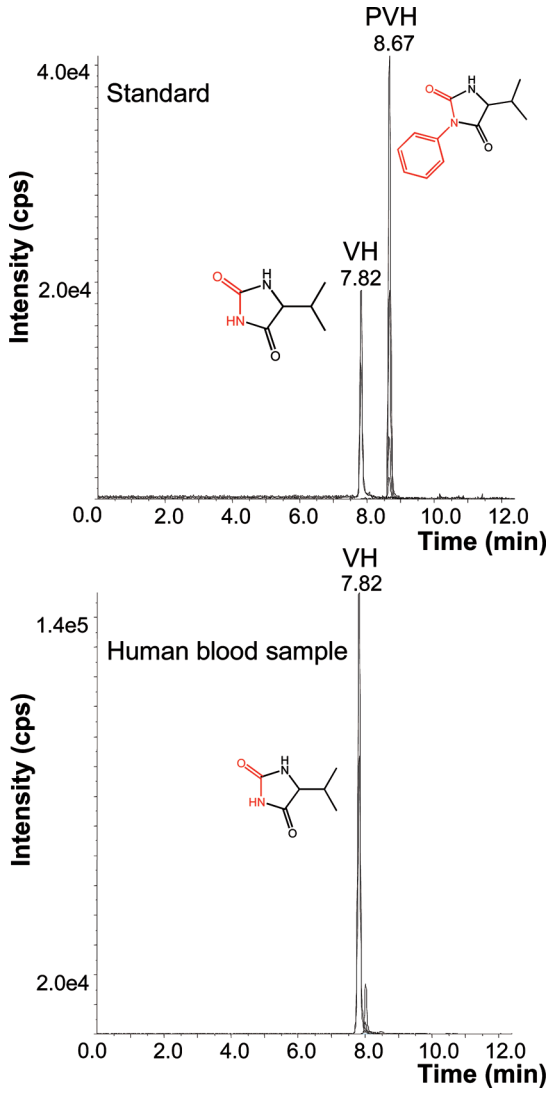


Figure 2. LC-MS/MS (ESI$^+$) MRM chromatograms of standard hydantoins (VH and PVH) and analysis of hydrolyzed control blood in which only VH was formed.

cyanate reference standards, a longer time in the reaction chamber could increase the yields, as the use of UV in the disappearance of NPCVMA could not be an adequate indicator.

Acid-catalyzed cyclization of the *N*-carbamoylated standards (**3**−**6**) to form PVH (**7**), MVH (**8**), VH (**9**), and ATVH (**10**) was performed in HOAc/HCl (1:2) and gave yields between 30 and 75% after recrystallization. The ring-closed hydantoins were characterized with LC-MS, ^{1}H NMR, or ^{13}C NMR. Data from LC-MS/MS fragmentation for the hydantoins are summarized in Table 1. The hydantoins were also characterized by GC-MS/MS (data not shown).

Analysis. In samples of hemolysate that were hydrolyzed and extracted, the yield of PVH formed through cyclization of added PCVMA was studied. The obtained levels corresponded to a quantitative yield of PVH (see Figure 1). Calibration lines of the PVH added to extracts from blood gave r^2 values of 0.93 in the analysis with the initially used conditions for LC-MS analysis. In a reproducibility test of the method by Kwan et al. (*21*) for hydrolysis and cyclization, a quantitative yield was obtained, with a relative standard deviation of 17% ($n = 2 \times 5$). Considering that this was a pilot study of the reproducibility in the analysis of carbamoyl adducts as hydantoins, using a preliminary analytical method, it was found acceptable with the potential of improvements.

A few control blood samples, without spiking with the standard VH, were analyzed with improved conditions. The LC-MS/MS conditions were optimized with regard to the separation and the measured ions, and the blood was processed according to Mraz et al. (*15*), using hydrolysis of isolated globin. Reference standards (VH and PVH) were analyzed (see Figure 2). A background level of VH was present as expected, and PVH was not observed in the blood samples (see Figure 2). The observed level of VH was in the range of earlier observed levels in human Hb of about 200 nmol/g Hb (*13*). In the LC-MS analysis of the adduct of VH detached from globin, using an injected volume to 5 μL (corresponding to 0.5 mg of globin), a s/n $\sim$ 1000 was obtained for the VH peak.

Future Applications of the New Synthesis Route. The aim of this work was to develop a new and versatile synthesis pathway for reference substances for adducts from isocyanates to valine, useful for quantification of exposure to isocyanates. Workup of human blood samples spiked with PCVMA gave high yields of the formed hydantoin, PVH. The background of VH formed from urea could be observed in a few samples of human blood analyzed by LC-MS/MS. The developed synthetic methods presented here could be used as a general route for the synthesis of reference substances for accurate biomonitoring of isocyanates and urea. Although not tested here, the more important commercial diisocyanates, 4,4′-MDI, 2,4-TDI and isophorone diisocyanate (IPDI), could also be synthesized from their corresponding amines reacted with the activated precursor.

This approach to activate the nitrogen in valine methylamide by 4-nitrophenyl chloroformate could relatively easily be extended to include peptides. These "activated" peptide precursors could then be reacted with selected amines for building chemical libraries of *N*-carbamoylated reference peptide compounds. Such reference compounds would be optimal for the measurement of Hb adducts as the yields of detached hydantoin might vary depending on the nature of the adduct. Pure *N*-carbamoylated reference peptides, with and without isotope substitution at the N-terminal amino acid, would possibly even be a more ultimate choice for synthetic references and internal standards.

As the focus of this work was to develop a new synthetic route, less effort was done to optimize the analytical procedure/conditions. The high adduct levels of 10^2 nmol/g of VH (*13*) or MVH (*9*) earlier observed in persons with renal failure or dimethylformamide exposure, respectively, would be measurable with the current procedure. This was also shown in our analysis, with a high s/n ratio, of background VH in human blood. The analysis of adducts from diisocyanates in exposed persons would most probably need further development of the analysis. In workers exposed to TDI, a corresponding adduct level of 70 pmol/g has been observed (*10*). This would probably be below the LOQ of the method used here.

Acknowledgment. Economical support from the Sw. Council for Working Life and Social Research (FAS), the Swedish Research Council Formas, and the Swedish Cancer and Allergy Foundation are acknowledged. Maria Anthanasiadou, Ph.D., Hans von Stedingk, M.Sc., and Carl Axel Wachtmeister, Prof. Em., are gratefully acknowledged for excellent viewpoints. Björn Åkermark, Prof. Em., is acknowledged for support with NMR analyses.

Supporting Information Available: ^{1}H NMR and ^{13}C NMR spectra of compounds **2**, **3**, and **5**−**10**. This material is available free of charge via the Internet at http://pubs.acs.org.

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TX900278P