Original article:

REACTIONS OF N-ACETYLCYSTEINE ADDUCTS OF AROMATIC (DI)ISOCYANATES WITH FUNCTIONAL GROUPS OF ORGANIC MOLECULES: TRANSCARBAMOYLATION REACTIONS IN AQUEOUS BUFFER AND IN AN ORGANIC SOLVENT

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ABSTRACT

Glutathione thiocarbamate conjugates of isocyanates play a key role in transport and final reactions of isocyanates in the human body by transcarbamoylation. N-acetylcysteine is the simplest model for thiocarbamate reactions. Therefore, transcarbamoylation of N-acetylcysteine adducts of *p*-tolylisocyanate (*p*TI-AcCys) and 4,4'-diisocyanatodiphenyl–methane (MDI(AcCys)$_2$) with N-acetylcysteine methyl ester (thiolysis), morpholine (aminolysis), methoxyethanol (alcoholysis), and water (hydrolysis) has been studied in aqueous phosphate buffer solution and in dimethylacetamide (DMAc). Expected reaction products have been synthesised as reference compounds for HPLC-analysis. Concentrations of adducts and of reaction products were monitored by HPLC. Reaction rates and activation energies were determined for *p*TI in both media, reactions of MDI(AcCys)$_2$ were run at one temperature only. Formation of insoluble reaction products and side reactions due to hydrolysis prevented in depth kinetic analysis of the reactions. Two regimes of reaction rate were observed in aqueous buffer, clear second order kinetics resulted in DMAc. In aqueous buffer (pH 7.4) a reactivity thiolysis > aminolysis > hydrolysis was found, while in DMAc aminolysis was faster than thiolysis. This can be explained by formation of thiolate at pH 7.4, which is not possible in anhydrous DMAc. Reactions of (MDI(AcCys)$_2$) are by a factor of 2 to 4 faster than those of *p*TI-AcCys. *p*-Toluidine (*p*TA) was found in the aqueous system due to hydrolysis, while no 4,4'-methylene dianiline (MDA) could be detected. Under physiological conditions hydrolysis should compete with thiolysis under homogeneous conditions while ureas and carbamates should be much more stable against hydrolysis. No free isocyanate groups could be detected in any of the reactions. In conclusion the isocyanate moiety in thiocarbamates is readily transferred to sulfhydryl- and amino groups but not to aliphatic hydroxy groups. Under physiological conditions hydrolysis competes with these transcarbamoylation reactions. Formation of free isocyanate groups in analytical quantities was shown to be highly unlikely.

Keywords: Aromatic isocyanates, N-acetyl cysteine-S-adducts, transcarbamoylation reactions, ureas, urethanes, amines

INTRODUCTION

Aromatic diisocyanates like toluene-diisocyanate (TDI) and diphenylmethane-diisocyanate (MDI) find widespread use in the manufacture of flexible foams for bedding, furniture, cushions, carpet underlay and in transportation seating. They are also used for insulation as rigid foams, for shoe sole systems, elastomers, coatings, adhesives, and sealing compounds as main applications (Oertel, 1993; Saunders and Frisch, 1962). These isocyanates comprise a family of compounds which contain two (or more) functional -N=C=O groups, the key to the chemistry of polyurethanes (PUR). *p*-Toluene isocyanate (*p*TI) and its derivatives, although much smaller in production volume, play an important role in the synthesis of herbicides and pesticides (Brochhagen, 1991). The high reactivity of isocyanates is the reason why they are of concern in toxicology and in occupational hygiene (Bolognesi et al., 2001).

Isocyanate groups readily react with active hydrogen containing nucleophiles in hydroxy-, amino-, and sulfhydryl group containing bio-molecules of living organisms. If an aqueous medium contains reactive biological compounds such as amino acids, peptides or proteins, competing reactions of the aromatic isocyanates with water and functional groups (e. g. NH_2, NH, OH, SH, and COOH) in biomolecules can be assumed (Brown et al., 1987). Acidity (pH), solubility of the isocyanate in aqueous liquids, heterogeneous or homogeneous conditions, temperature, and concentration of the reactants greatly influence the course and the rate of the reactions.

Studies of the physiological activity of isocyanates have revealed that carbamoylation of proteins is the main reaction of isocyanates, which also determines their biological effects. Adducts of isocyanates and mercapto groups of cysteine have been identified as key intermediates (Baillie and Slatter, 1991). These thiocarbamates were shown to be reactive intermediates which can transfer the carbamoyl residue to other active hydrogen containing molecules present in the system, e. g. to amine functions in lysine or N-terminal groups in proteins and peptides, to hydroxy groups in serine or even to water (Pearson et al., 1991). The mechanism of protection and transport is assumed to proceed via formation of thiocarbamates and transcarbamoylation reactions (Han et al., 1989). Mráz and Bousková studied the reaction of 2,4-TDI and hexamethylenediisocyanate with several amino acid residues in vitro under physiological conditions (Mráz and Bouskova, 1999). Pearson and coworkers investigated the transcarbamoylation reaction of S-(N-methylcarbamoyl) glutathione (Pearson et al., 1990). Day and coworkers reported formation, solvolysis, and transcarbamoylation reactions of the bis(S-glutathionyl) adducts of 2,4- and 2,6-TDI (Day et al., 1997). However, neat solvolysis products were not synthesized by these researchers. Reisser et al. reported synthesis, characterisation, and solvolysis of mono- and bis(S-glutathionyl) adducts of MDI (Reisser et al., 2002). Chipinda et al. investigated the hydrolysis of bisthiocarbamates from cysteine methyl ester and MDI, TDI, hexamethylene diisocyanate (HDI) (Chipinda et al., 2006). All three research groups postulated the formation of free isocyanate groups as intermediates in transcarbamoylation reactions.

In the first part of our studies the N-acetyl-(L)-cysteine (AcCys) adducts of *p*TI, 2,4-TDI, and 4,4'-MDI have been made available. Furthermore, the main reaction products formed when these isocyanates react with aqueous solution of AcCys at different pH-conditions and different molar ratios have been identified and quantified (Mormann et al., 2006).

Quantitative data of the reactivity of AcCys adducts with amino-, hydroxy-, and sulfhydryl nucleophiles have not been reported in the literature so far, although these compounds should be ideal models to study the stability of thiocarbamates and their reactivity with nucleophilic groups prone to react with the thiocarbamates in a transcarbamoylation reaction. AcCys adducts of isocyanates provide a basis to gain this information.

In the study presented here the reactivity of AcCys adducts with aqueous and alcoholic hydroxy-, amino- and sulfhydryl groups has been investigated. Apart from quantitative information on the reactivity it will be discussed, whether the transcarbamoylation reactions proceed via free isocyanates as it has been claimed in the literature (Day et al., 1997; Reisser et al., 2002) or by an addition/elimination mechanism. The latter mechanism is also the usual path for reactions of carboxylic acid derivatives with active hydrogen bearing nucleophilic compounds (Anslyn and Dougherty, 2006).

MATERIALS AND METHODS

Chemicals

p-tolylisocyanate (*p*TI), CAS-No. 622-58-2, *p*-toluidine (*p*TA), CAS-No.106-49-0, N-acetyl-L-cysteine (AcCys), CAS-No. 61-91-1, N-acetyl-L-cysteinemethyl ester (AcCysMe), CAS-No. 7652-46-2 and trifluoroacetic acid, CAS-No. 76-05-1, were obtained from Fluka; morpholine, CAS-No. 110-91-8, 2-methoxyethanol, CAS-No. 109-86-4, and formic acid, CAS-No. 64-18-6 from Merck; 4,4´-methylenediphenyl diisocyanate (4,4´-MDI), CAS-No. 101-68-8 and 4,4´-methylene dianiline (4,4´-MDA), CAS-No. 101-77-9 from Bayer AG.

Solvents: Dimethylacetamide (DMAc), CAS-No. 127-19-5, was distilled first over phosphorus pentoxide, then over MDI. Acetonitrile (ACN), from Promochem, HPLC gradient grade, CAS-No. 75-05-8, was used as received; toluene was distilled from sodium/potassium alloy, water was double distilled; *p*TI and 4,4´-MDI were distilled in vacuo prior to use.

Buffer solution was prepared as described previously (Mormann et al., 2006)

Synthesis of model compounds

Reactions involving isocyanates in organic media were carried out in pre-dried glass ware in an atmosphere of dry argon.

*p*TI-*AcCysMe adduct; N-acetyl-S-(p-tolyl-carbamoyl)-L-cysteine methyl ester*
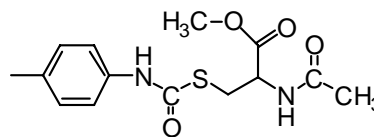
In a Schlenk flask equipped with a magnetic stirring bar 1.45 g (8.19 mmol) AcCysMe were dissolved in 25 mL dry toluene under gentle heating. 1.05 g (7.89 mmol) of *p*TI were added dropwise within 30 seconds. The solution was stirred for 30 minutes at 60 °C. The white precipitate formed was separated by filtration, washed with toluene and pentane and dried at 60 °C, 15 hPa.

Yield: 1.73 g, 71 %
M.p.: 151 °C, 247 °C$^{dec.}$ (DSC).

IR-spectrum $_{KBr}$: 3303 (N-H); 1735 (C=O, urethane/ester); 1651 cm^{-1} (amide).

^{1}H-NMR$_{CDCl3}$: 2.02 (s, **H$_3$C**-Ar); 2.31 (s, -NH-CO-**CH$_3$**); 3.44 (m, -S-**CH$_2$**); 3.74 (s, COO-**CH$_3$**); 4.84 (m, -OOC-**CH**-NH-); 6.81 (d, -CH-**NH**-CO-); 7.13 (d, H-Ar); 7.28(d, H-Ar); 7.91 ppm (s, Ar-**NH**-CO-S-).

^{13}C-NMR$_{CDCl3}$: 21.24 (**CH$_3$**-Ph): 22.64 (**CH$_3$**-CO); 31.93 (-**CH$_2$**-S); 53.20 (-**CH**-CH$_2$);53.61 (**C**O-); 119.33,129.55, 132.71, 136.71 (arom.); 164.03 (-**C**O-S); 171.08 (-**C**OCH$_3$); 172.26 (-**C**OOCH$_3$).

C$_{16}$H$_{18}$N$_2$O$_4$S calc.: C 54.18, H 5.85, N 9.03, S 10.33; found: C 53.85, H 5.85, N 8.79, S 10.33.

*p*TI-*morpholine adduct; N-(4-Methyl-phenyl)-morpholine-4-carbamide*
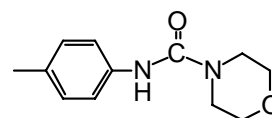
In the above setup 0.50 g (5.75 mmol) morpholine were dissolved in 20 mL dry toluene and 0.73 g (5.49 mmol) *p*TI were added at room temperature within 30 seconds. A white precipitate was formed immediately. After 30 minutes the solid was collected by filtration, washed with

toluene and with pentane and dried at 60 °C, 15 hPa.

Yield: 1.13 g, 90 %.
M.p.: 156 °C, 279 °C[dec.] (DSC).

IR-spectrum [KBr]: 3294 (N-H); 1634 cm^{-1} (C=O, urea).

^{1}H-NMR[DMSO]: 2.20 (s, **H**₃C-Ar); 3.39 (d, -N-CH₂-**CH**₂-O- + water from DMSO); 3.58 (d,- N-**CH**₂-CH₂-O-); 7.02 (d, **H**-Ar); 7.32 (d, **H**-Ar); 8.41 ppm (s, **H**-Amin).

^{13}C-NMR[DMSO]: 21.28 (**C**H₃-Ph): 45.08 (2**C**H-N-): 66.78 (2**C**H-O-); 129.01,129.55, 131.50, 138.53 (arom.); 156.43 (-**C**O-N).

$C_{12}H_{16}N_2O_2$ calc.: C 65.43, H 7.32, N 12.62; found: C 65.45, H 7.39, N 12.62.

*p*TI-*methoxyethanol adduct; 2-methoxyethyl N-(4-methylphenyl)carbamate*
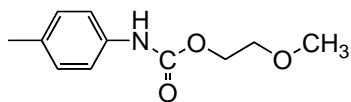
The above setup was charged with 5.58 g (73.4 mmol) methoxyethanol. 2.12 g (15.9 mmol) *p*TI were added dropwise. The mixture was stirred for 2 h at 50 °C. Excess methoxyethanol was removed at 50 °C and 30 hPa. The remaining liquid was filtered and purity was checked with HPLC.

Yield: 2.25 g, 68 %.

IR-spectrum [NaCl-Film]: 3314 (N-H); 1738, 1718, 1707 cm^{-1} (C=O).

^{1}H-NMR[CDCl3]: 2.28 (s, **H**₃C-Ar.); 3.39 (s, -O-**CH**₃); 3.62 (t, -CH₂-**CH**₂-O); 4.30 (t, -**CH**₂-CH₂-O); 6.96 (s, **H**-Amin); 7.08 (d, **H**-Ar); 7.25 (d, **H**-Ar).

^{13}C-NMR[CDCl3]: 21.24 (**C**H₃-Ph): 59.29 (**C**H₃-O); 64.37, 71.17 (-**C**H₂-O-); 119.21, 129.55, 133.35, 135.73 (arom.); 153.97 (-**C**O-O).

$C_{16}H_{18}N_2O_4S$ calc.: C 63.14, H 7.23, N 6.69; found: C 63.17, H 7.30, N 6.68.

p-tolylisocyanate-N-acetyl-L-cysteine ad-duct was prepared as described in a previous paper (Mormann et al., 2006).

MDI(AcCysMe)₂ adduct; N,N'-diacetyl-S,S'-[methylenebis(4,1-phenyleneiminocar-bonyl)]dicysteine dimethyl ester
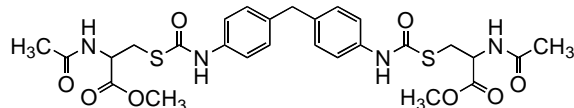
In the above setup 2.59 g (10.3 mmol) 4,4´-MDI were dissolved in 10 mL (14.2 g) DMAc. 3.61 g (20.8 mmol) AcCysMe were added and the mixture stirred at 60 °C for 24 h. Half of the solvent was removed in vacuo, then toluene was added. From this solution the adduct precipitated within 2 h at room temperature. The colorless solid was collected by filtration, washed with toluene and dried at 60 °C, 15 hPa.

Yield: 3.4 g, 55 %
M.p.: 180 °C, 200 °C[dec.] (DSC), 170 °C (microscope).

IR-spectrum [KBr]: 3299 (N-H); 1739 (C=O, urethane/ester), 1656 cm^{-1} (C=O, amide).

^{1}H-NMR[DMSO]: 1.84 (s, -CO-**CH**₃); 3.05 (m, -S-**CH**₂-); 3.62 (s, -O-**CH**₃); 3.80 (s, Ar-**CH**₂-Ar); 4.39 (m, -NH-**CH**(COOCH₃)-S-); 7.10 (d, **H**-Ar); 7.36 (d, **H**-Ar); 8.43 (d, -CO-N**H**-CH(COOCH₃)-); 10.31 (s, -CO-N**H**-Ar).

^{13}C-NMR[DMSO]:23.12 (**C**H₃-CO); 30.93 (-**C**H₂-S); 53.05 (-**C**H-NH); 119.33, 129.55, 130.25, 137.71 (arom.); 164.47 (-**C**O-S); 170.34 (-**C**O-CH₃); 171.81 (-**C**OOCH₃).

$C_{27}H_{32}N_4O_8S_2$ calc.: C 53.64, H 5.30, N 9.27, S 10.60; found C 53.73, H 5.28, N 9.35, S 10.37.

MDI-bis-morpholine adduct; N,N'-[methylenebis(4,1-phenylene)] di(morpholine-4-carbamide)
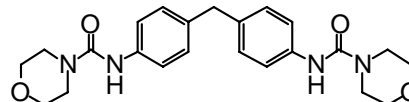
In a Schlenk flask 2.93 g (11.72 mmol) 4,4´-MDI were dissolved in 35 mL dry toluene and 2.14 g (24.60 mmol) morpholine were added dropwise at room temperature. A white precipitate was formed at once. It was filtered off, washed with toluene and pentane and dried at 60 °C, 15 hPa.

Yield: 4.84 g, 97 %.
M.p.: 293 °C, 336 °C$^{dec.}$ (DSC).

IR-spectrum $_{KBr}$: 3336 (N-H); 1643 cm^{-1} (C=O, urea.).

^{1}H-NMR$_{DMSO}$: 3.39 (d, -N-CH$_2$-**CH$_2$**-O-); 3.58 (t, -N-**CH$_2$**-CH$_2$-O-); 3.76 (s, Ar-**CH$_2$**-Ar); 7.04 (d, **H**-Ar); 7.32 (d, **H**-Ar); 8.44 ppm (s, **H**-Amin).

^{13}C-NMR$_{DMSO}$: 45.01 (2**CH**-N); 66.86 (2**CH**-O); 120.69, 129.75, 135.92, 139.07 (arom.); 156.12 (-**CO**-NH).

C$_{32}$H$_{28}$N$_4$O$_4$ calc.: C 65.08, H 6.65, N 13.02; found: C 65.08, H 6.72, N 12.97.

MDI-bis-methoxyethanol adduct
bis(2-methoxyethyl) N,N'-[methylenebis (4,1-phenylene)]dicarbamate
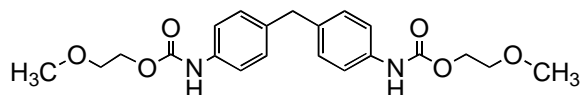
In a Schlenk flask 4.68 g (18.72 mmol) 4,4´-MDI were melted and 8.62 g (113.42 mmol) methoxyethanol added. The mixture was stirred for 18 h at 60 °C. After cooling down to roomtemperature a gel-like product was obtained. It was triturated with dry diethylether and a colorless powder was obtained. It was filtered off, washed with ether and dried at 35 °C, 15 hPa.

Yield: 6.61 g, 61 %.
M.p.: 94.1 °C (DSC).

IR-spectrum$_{KBr}$: 3316 (N-H); 1701 (C=O, urethane); 1604 cm^{-1} (arom.).

^{1}H-NMR$_{DMSO}$: 3.25 (s, **H$_3$**C-O); 3.54 (m, O-**CH$_2$**-CH$_2$-O-CO); 3.77 (s, Ar-**CH$_2$**-Ar); 4.16 (m, O-CH$_2$-**CH$_2$**-O-CO); 7.10 (d, **H**-Ar); 7.33 (d, **H**-Ar); 9.63 (s, N**H**).

^{13}C-NMR$_{DMSO}$: 58.76 (**CH$_3$**-O); 64.02, 70.97 (-**CH$_2$**-O); 119.15, 129.75, 136.34,

137.92 (arom.); 154.32 (-**CO**-O).

C$_{22}$H$_{30}$N$_2$O$_6$ calc.: C 63.14, H 7.23, N 6.69; found: C 62.84, H 6.90, N 7.01.

4,4'-MDI(AcCys)$_2$ adduct

The 4,4'-MDI-bis-N-acetyl-L-cysteine adduct was prepared as previously described (Mormann et al., 2006).

Mixed adducts of 4,4'-MDI

In a Schlenk flask equipped with a magnetic stirring bar 0.19 g (1.19 mmol) AcCys and 0.22 g (1.23 mmol) AcCysMe were dissolved in 4 mL DMAc. 0.2519 g (1.19 mmol) 4,4'-MDI dissolved in 5 mL DMAc were added. After all isocyanate had reacted (IR-control) a 0.13 % solution in acetonitrile / water (98:2) was investigated by HPLC (gradient 2, Table 1) to obtain a calibration curve. For a calibration curve concentration of the mixed adduct was calculated from injected mass minus mass of the symmetric adducts as determined from the calibration curves for these compounds.

Mixed adducts of 4,4'-MDI with morpholine or methoxyethanol and AcCys were obtained in a similar way. To 1.6 g (6.4 mmol) MDI dissolved in 5 mL DMAc 0.54 g (6.2 mmol) morpholine or 0.48 g (6.4 mmol) methoxyethanol were added. After 1 h 1.12 g (6.8 mmol) AcCys dissolved in 5 mL DMAc were added and the mixture was stirred for an additional hour (IR-control) and investigated by HPLC (gradient 2 for methoxyethanol mixed adduct, gradient 3 for the morpholine mixed adduct) as described above.

Solvolysis of pTI-AcCys and MDI(AcCys)$_2$ in aqueous buffer solutions of pH 7.4

In a Schlenk flask equipped with a magnetic stirring bar approximately 0.20 mmol *p*TI-AcCys (0.10 mmol for MDI(AcCys)$_2$) and AcCysMe were weighed in and dissolved in 20 mL buffer solution of pH 7.4. For alcoholysis and aminolysis 15 mL of buffer solutions were used. Methoxyethanol or morpholine were added from a buffer solution containing the equivalent amount in 5 mL solution. The pH was adjusted to 7.4 with 0.2 M sodium

hydroxide solution and the initial concentration of reactants was calculated.

After the times given in the corresponding tables approximately 0.5 g of the reaction mixture were weighed into a 10 mL volumetric flask and filled with buffer solution (density assumed to be 1.0 g/mL) to the mark. Volumes injected were between 3 and 10 µL.

Solvolysis of pTI-AcCys and MDI(AcCys)₂ in DMAc

In a 50 mL Schlenk flask equipped with magnetic stirring bar 1 mmol (296 mg) *p*TI-AcCys or 0.5 mmol (288 mg) MDI(AcCys)₂ were dissolved in 10 mL freshly distilled DMAc. The temperature was raised to 25, 37, 50 or 65 °C and a sample was taken for HPLC. Then 1 mmol of the nucleophile (morpholine or Ac-CysMe) was added. For HPLC samples of 150 to 200 mg were taken, diluted with 10 mL of ACN and analysed with gradient 2 or 3 as indicated in the corresponding tables. Injected volumes were between 3 and 5 µL.

Instrumental Analysis

^{1}H- and ^{13}C-nmr-spectra were recorded with a Bruker AC-200FT-spectrometer (200 MHz proton and 50.3 MHz ^{13}C-spectra) in DMSO-d6. The signal of non-deuterated impurities (δ = 2,49 ppm) was used as internal standard for proton nmr, the signal of the methyl-carbon (δ = 39.7 ppm) as reference for ^{13}C-spectra.

Thermal properties were investigated on a Mettler TC 11/15 system with a DSC 30 unit and a TG 50 unit, nitrogen atmosphere, heating rate 10 K/min.

HPLC analyses were performed on an Agilent 1100 Series liquid chromatographic system consisting of a model G1312A binary pump, G1379A vacuum degasser, G1313A autosampler, G1315B diode array detector (UV), and Agilent ChemStation data handling program (Agilent Technologies). The reaction mixtures were chromatographed on a Zorbax 300SB-C18, 150 mm, 4.6 mm i. d reversed phase C18 analytical column. Injection volumes varied

from 0.1 to 100 µL. For identification of unknown peaks a mass spectrometer was used as detector. Solvents for elution were acetonitrile (HPLC gradient grade) and bidistilled water. Solvent A was water containing either 0.1 % (v/v) of formic acid or 0.05 % of trifluoroacetic acid; solvent B was acetonitrile containing the same amount of the corresponding acid. Three different gradients used for separation of reaction products; details are given in Table 1. Flow rate was 1 mL per minute.

Table 1: Gradients used for HPLC-chromatography

Gradient 1 (0.05 % trifluoroacetic acid)		Gradient 2 (0.1 % formic acid)		Gradient 3 (0.1 % formic acid)	
t /min	% B	t /min	% B	t /min	% B
0	2	0	0	0	0
7	2	5	0	5	0
15	100	9	5	9	5
17	100	23	100	40	26
		25	100	49	100
				51	100

Retention times for model compounds are summarised in Table 2. Calibration curves were established for these components. Calibration curves of mixed adducts were obtained after determination of the amounts of the symmetrical adducts in the mixture from already established calibration curves. Concentration of the mixed adduct was determined as the remaining of the weighed in concentration minus the amounts of symmetrical adducts. Several peaks that could not be assigned from model compounds were assigned by HPLC-MS. These compounds are also listed in Table 2 and marked with an asterix. Quantitative analysis, however, was not possible because the compounds could not be isolated in quantities sufficient for calibration curves.

Table 2: Retention times (HPLC) of model compounds from *p*TI and MDI

Compound	Grad 1 Rt/min.	Grad 2 Rt/min.	Grad 3 Rt/min.
AcCys	3.37	4.31	4.31
(AcCys)$_2$*	7.75	11.91	11.91
AcCysMe	7.20	10.87	
(AcCysMe)$_2$*		13.32	
*p*TA	7.25	5.29	
*p*TI-AcCys	10.75	16.28	
*p*TI-AcCysMe	11.43	17.13	
*p*TI-morpholine	10.51	15.91	
*p*TI-methoxyethanol	11.64	17.33	
*p*T-urea	13.06	19.23	
MDI(AcCys)$_2$		16.5	43.8
MDI(AcCysMe)$_2$		17.7	
MDI(methoxyethanol)$_2$		18.3	
MDI-AcCys-AcCysMe		17.2	
MDI-AcCys-methoxy.		17.3	
MDA		7.2	7.2
MDI(morpholine)$_2$			42.1
MDI-AcCys-Morpholine			43.3
MDI-AcCys-Amine*		14.6	28.3
MDI-Urea-AcCys$_2$*		18.3	45.7

* (no calibration curves available, identified with HPLC-MS)

RESULTS

Synthesis of model compounds

Model compounds of p-TI

The chemistry of solvolysis reactions of the *p*TI-AcCys adduct is straightforward. Reactions of the adduct with AcCysMe, 2-methoxyethanol, morpholine and water lead to products that are identical to those obtained from reaction of *p*TI with these compounds. No mixed products are to be expected. Final products of the *p*TI reaction, shown in Scheme 1, were synthesised and their analytical and spectroscopic properties were determined.

Solvents for the reactions were chosen according to the solubility of the nucleophilic compounds. *p*TI-AcCys was synthesised as previously described (Mormann et al., 2006). Reaction of *p*-tolylisocyanate with AcCysMe was performed in toluene from which the adduct precipitated as analytically pure material. *p*-Tolyl(2-methoxyethyl)urethane was obtained using excess methoxyethanol as reagent and solvent. Residual methoxyethanol was removed, the remaining liquid was dissolved and freeze dried from benzene. *p*TI-morpholine urea was prepared in toluene from which the product precipitated as analytically pure material.
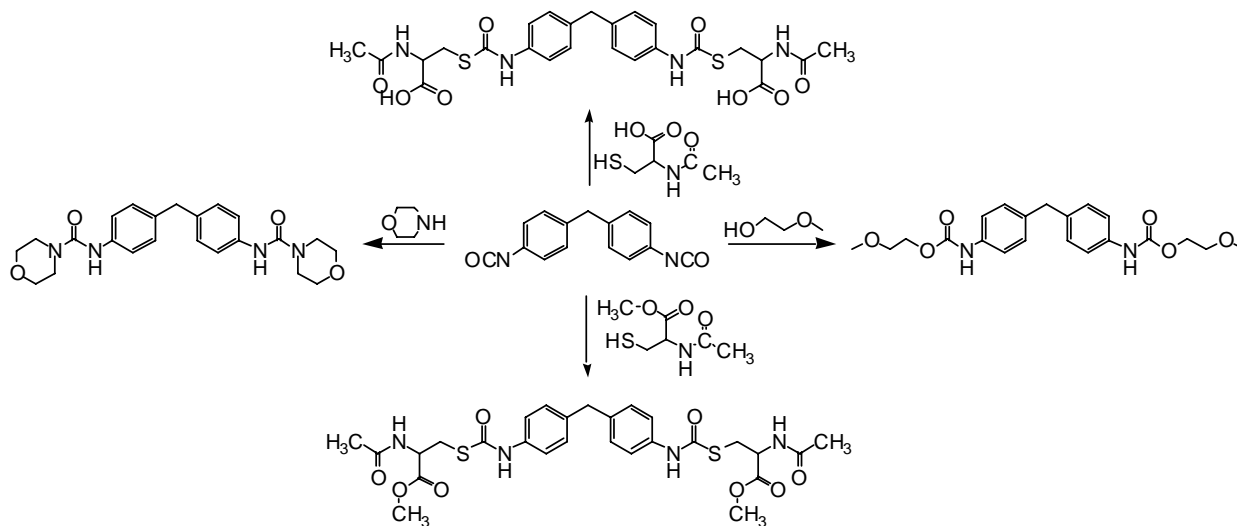
Model compounds of 4,4´-MDI

As in the *p*TI series products of complete transcarbamoylation reactions can be synthesised from MDI and the nucleophiles as shown in Scheme 2. However, apart from the bis-adducts mixed adducts are likely to be formed. These have AcCys added to one of the isocyanate groups of MDI and the nucleophile added to the other. These products were synthesised from MDI by reaction with half a mole of AcCys and half a mole of the nucleophiles used in the solvolysis reactions which is shown in Scheme 3.

Synthesis of the AcCys adduct of MDI in DMAc has been previously reported (Mormann et al., 2006). The reaction of 4,4´-MDI with AcCysMe was performed in the same solvent and the reaction product precipitated in toluene. The adduct of MDI and 2-methoxyethanol was synthesised as reported for *p*TI using excess alcohol as reagent and solvent. The morpholine diurea of MDI was made in toluene from which the product precipitated analytically pure.

Mixed adducts of MDI having one AcCys moiety and one of the solvolysis nucleophiles were not synthesised in pure state. MDI was dissolved in DMAc and reacted first with half the stoichiometric amount of AcCys. Then the appropriate amount of AcCysMe, methoxyethanol or morpholine dissolved in DMAc was added (cf. Scheme 3) and the reaction was monitored by infrared spectroscopy (disappearance of the isocyanate absorption at 2267 cm^{-1}). These mixtures were used for HPLC analysis without further work up.
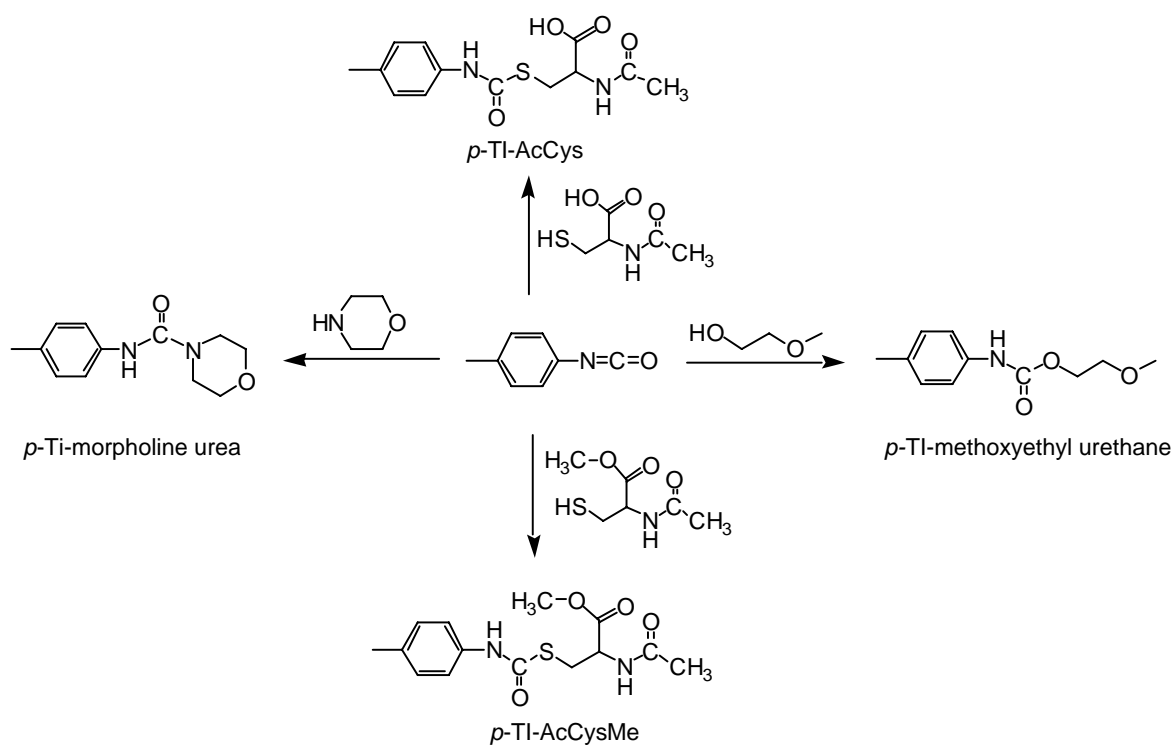
Scheme 1: Model compounds of *p*TI and hydroxy-, amino-, and mercapto nucleophiles

Scheme 2: Model compounds of 4,4′-MDI and hydroxy, amino and mercapto nucleophiles
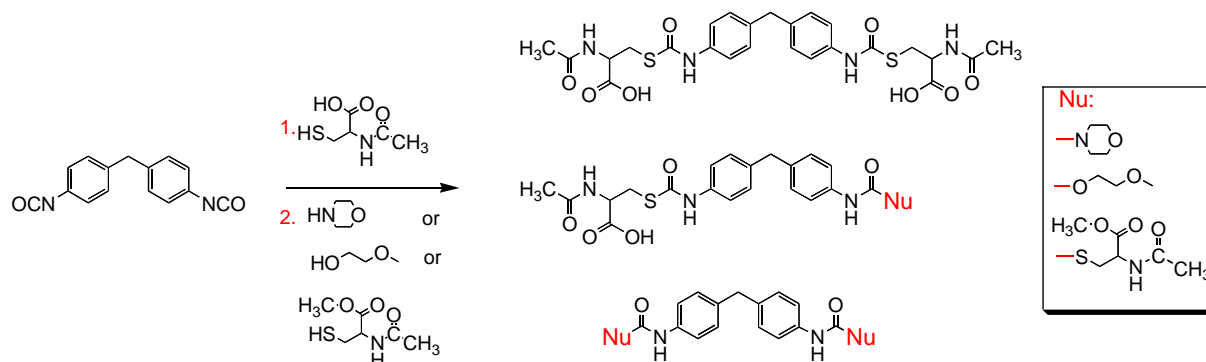
Scheme 3: Mixed adducts of MDI

Transcarbamoylation of *p*TI-AcCys

Solvolysis reactions of *p*TI-AcCys with morpholine, AcCysMe, 2-methoxyethanol and water were investigated in aqueous buffer solution of pH 7.4 to mirror physiological conditions. Three temperatures were chosen for aminolysis and for thiolysis to determine the activation energy of these reactions. Some of the reactions were also studied under anhydrous conditions in DMAc as solvent. Another advantage of this solvent is that reactions can be run under homogeneous conditions as all products were soluble. All experiments were performed in a similar way. Samples were taken from the reaction mixture, filtered to remove insoluble reaction products and analysed by HPLC. From the HPLC-results conversion-time-data for kinetic analysis were available.

Solvolysis reactions of pTI-AcCys in aqueous buffer of pH 7,4

Aminolysis of *p*TI-AcCys was investigated at 298, 310 and 323 K. Samples were taken at intervals shown in Tables 3-5. A precipitate formed after the first or second sample was taken. The solid was identified to be *p*T-urea containing approximately 1 % *p*TI-morpholine. Concentrations of *p*TI-AcCys, *p*TI-morpholine and the sum of the two were plotted vs. time as shown for aminolysis at 323 K in Figure 1. The amount of morpholine adduct detected in the liquid phase did not correspond to the amount of *p*TI-AcCys that disappeared. The difference is due to formation of insoluble

*p*T-urea which clearly indicates that hydrolysis competes with aminolysis under the conditions of these experiments. After approximately 24 h conversion of *p*TI-AcCys increased from 14 % at 298 K to 50 % at 310 K and more than 80 % at 323 K.

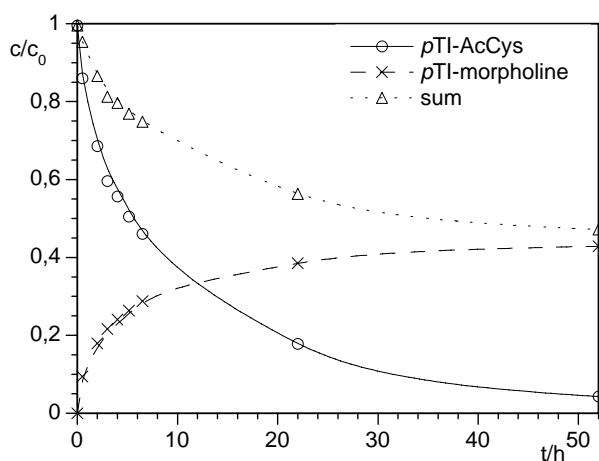**Table 3:** Reaction of *p*TI-AcCys and morpholine (298 K)

t/h	c/mmol L^{-1} (c_0: 12.85 mmol L^{-1})	
	*p*TI-AcCys	*p*TI-morpholine
0	12.80	0.000
0.5	12.72	0.035
3	12.42	0.190
5.5	12.22	0.319
25	11.05	0.997

Table 4: Reaction of *p*TI-AcCys and morpholine (310 K)

t/h	c/mmol L^{-1} (c_0: 12.24 mmol L^{-1})		
	*p*TI-AcCys	*p*TI-morpholine	*p*TA
0	11.82	0.00	0.000
0.5	10.88	0.35	0.139
4	9.19	1.14	0.456
6	8.75	1.50	0.616
26	6.15	2.82	0.593

Table 5: Reaction of *p*TI-AcCys and morpholine (323 K)

t/h	c / mmol L^{-1} (c_0: 11.05 mmol L^{-1})	
	*p*TI-AcCys	*p*TI-morpholine
0.0	11.00	0.00
0.5	9.50	1.04
2.0	7.58	1.99
3.0	6.59	2.39
4.0	6.15	2.66
5.0	5.58	2.91

Figure 2: Second order kinetics (298, 310, 323 K) of aminolysis of *p*TI-AcCys at pH 7.4

The plot of relative concentrations of *p*TI-AcCys and *p*TI-AcCysMe together with their sum in Figure 3 shows a smooth decrease *p*TI-AcCys and a maximum for the thiolysis product; the decrease in concentration of the ester adduct coincides with formation of precipitate. This also explains why the sum of solubles from that point on is less than 100 %. At 310 K the solid after 48 h consisted of 4.5 % *p*TI-AcCys, 4.9 % *p*T-urea and 90.6 % *p*TI-AcCysMe. The final reaction product will be *p*T-urea, minor amounts of *p*TA and the two AcCys-compounds. Second order kinetics gave best fits of data (cf. Figure 4). Rate constants and activation energy are included in Table 9.
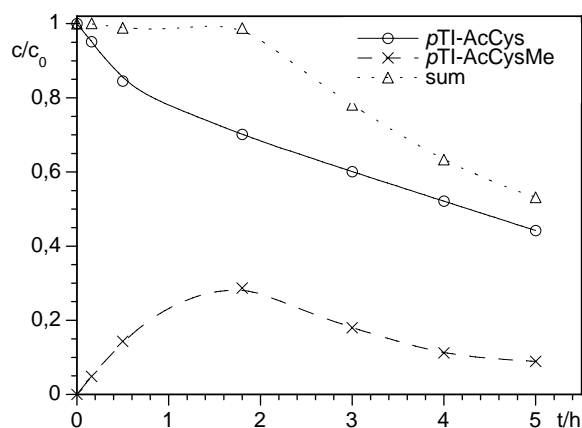

Figure 1: Relative concentrations of *p*TI-AcCys and *p*TI-morpholine (323 K)

Kinetics of aminolysis was studied assuming a second order reaction, which gave best fits of data (cf. Figure 2). As aminolysis to a significant extent is accompanied by hydrolysis, a more detailed interpretation of data was not attempted as this would involve speculation rather than results. An "incubation" period was also observed by Chipinda (Chipinda et al., 2006). Rate constants obtained from aminolysis of *p*TIAcCys and from all other lysis reactioins are included in Table 9. For control of reliability the experiment at 323 K was repeated showing almost no difference of rate constants.

Thiolysis of *p*TI-AcCys with AcCysMe was studied under the same conditions as aminolysis. Again a precipitate formed which initially consisted of the thiolysis product *p*TI-AcCysMe and less than 1 % of *p*T-urea and *p*TI-AcCys. The amount of *p*T-urea in the precipitate increased with time and accounted for 30 % in the experiment at 323 K after 26 h.

Figure 3: Relative concentrations of *p*TI-AcCys and *p*TI-AcCysMe (323 K, 5 h)
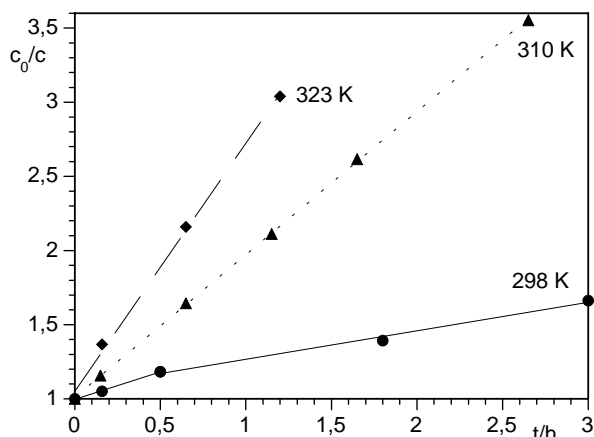
Figure 4: Second order kinetics (298, 310, 323 K) of thiolysis of *p*TI-AcCys at pH 7.4

Hydrolysis and alcoholysis were studied only at 310 K. Due to the poor solubility of *p*T-urea only the decrease of *p*TI-AcCys and formation of *p*TA were recorded by HPLC. The precipitate formed was *p*T-urea. Due to the high excess of water (factor of approx. 5500) first order kinetics were assumed in this reaction (cf. Figure 5).
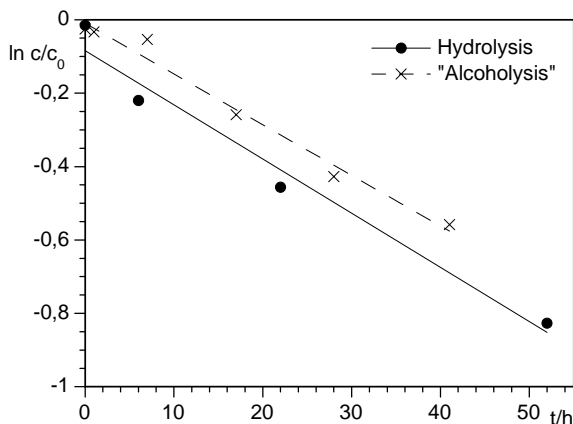

Figure 5: Pseudo first order kinetics of *p*TI-AcCys hydrolysis pH 7.4, 310 K

Alcoholysis also gave a precipitate of *p*T-urea. No *p*TI-methoxyethylurethane could be detected. As consequence of the great excess of water (factor of 5500) compared to alcohol again only hydrolysis took place and no rate of alcoholysis in aqueous buffer could be determined.

*Solvolysis reactions of p*TI-*AcCys in DMAC*

Solvolysis reactions proceed under homogeneous conditions in DMAc; they have

the advantage that reagents as well as reaction products are soluble. Thus the balance of compounds detected by HPLC should add up to 100 %. Aminolysis and thiolysis again were investigated at three temperatures to determine activation energies of these reactions.

Aminolysis of *p*TI-AcCys with morpholine was made in the usual way except that no filtration was required. As to be expected decrease of *p*TI-AcCys and formation of *p*T-morpholine urea corresponded well and added up to 100 % within experimental error as shown in Figure 6. The second order plots are shown in Figure 7. Rate constants and activation energy are contained in Table 9. Repetition of the reaction at 310 K showed reproducibility within ± 5 %.
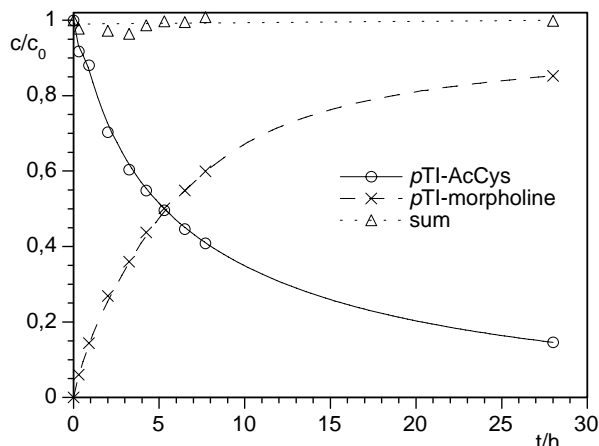

Figure 6: Relative concentrations of *p*TI-AcCys and *p*TI-morpholine (DMAc, 298 K)
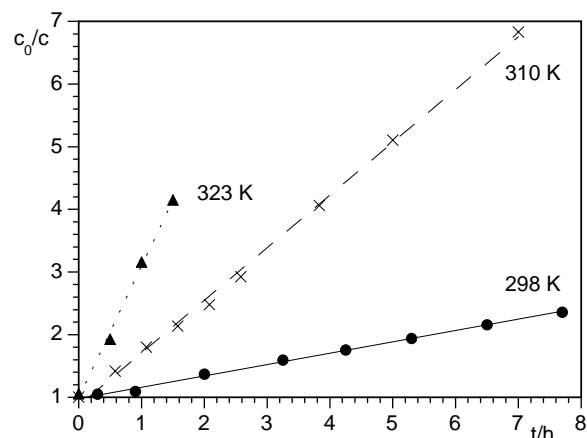

Figure 7: Second order kinetics (298, 310, 323 K) of aminolysis of *p*TI-AcCys in DMAc

Thiolysis of *p*TI-AcCys with AcCysMe was performed at 310, 323 and 338 K as the reaction proved to be quite slow in the

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aprotic DMAc. A time conversion plot for thiolysis at 323 K is shown in Figure 8, second order plots for the three temperatures in Figure 9, rate constants and activation energy in Table 9. No products other then the starting material and the solvolysis product were found.
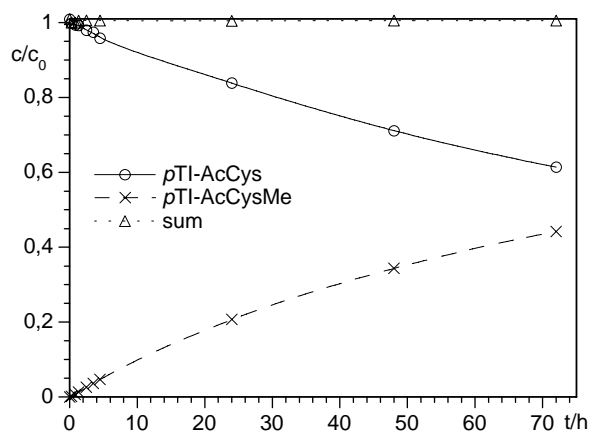

Figure 8: Relative concentrations of *p*TI-AcCys and *p*TI-AcCysMe (DMAc, 323 K)
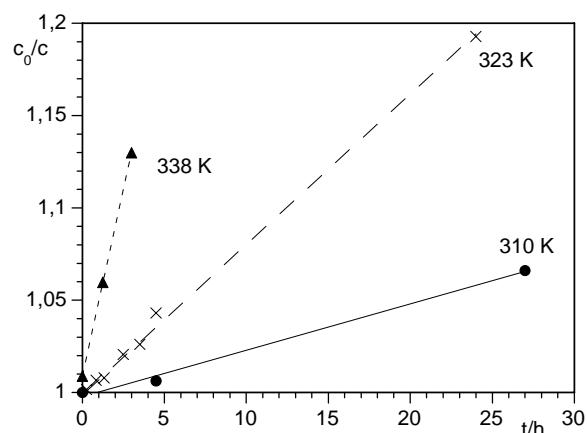

Figure 9: Second order kinetics of thiolyis of *p*TI-AcCys (310, 323, 338 K) in DMAc

No alcoholysis products of *p*TI-AcCys and methoxyethanol could be observed in DMAc at 310 K within 6 days. Aminolysis of the AcCysMe adducts of *p*TI and MDI at 310 K was included in the investigation to study the influence of the carboxyl group on the reaction rate (cf. Figure 10).
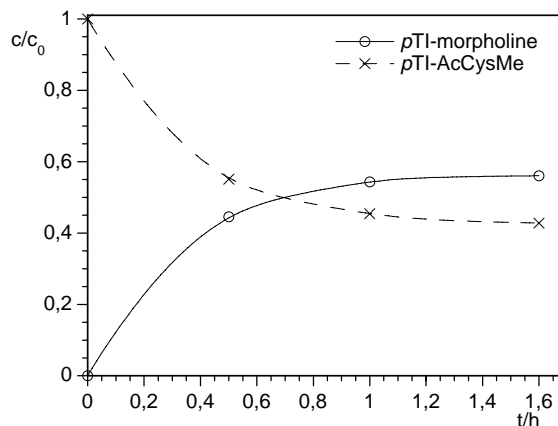
Figure 10: Relative concentrations of *p*TI-AcCysMe and *p*TI-morpholine in DMAc (310 K)

Transcarbamoylation of MDI(AcCys)$_2$ in aqueous buffer solution of pH 7.4

Transcarbamoylation of MDI(AcCys)$_2$ was studied in analogy to that of *p*TI-AcCys. Because of the bifunctional character of the MDI adducts oligomeric ureas with varying end groups were formed which could neither be identified nor completely dissolved for HPLC-analysis. Due to the more complicated nature of the solvolysis of MDI adducts in comparison to those of monofunctional isocyanates and due to the similarity of both isocyanates, reactions were carried out at 310 K only. With respect to electronic properties *p*TI has all the features of MDI since the two aromatic rings of MDI are decoupled by the methylene group. Therefore, activation energies should be rather similar.

Aminolysis of MDI(AcCys)$_2$ was studied in the usual way. The results are included in Table 6. Conversion time diagrams and kinetic curves are shown in Figures 11 and 12.

Table 6: Concentrations of MDI adducts in aminolysis (310 K)

t /h	c/mmol L^{-1} (c_0: 5.37 mmol L^{-1})				Area / %	
	MDI (AcCys)$_2$	MDI-mix	MDI (morph)$_2$	AcCys	AcCys-MDI-NH$_2$	AcCysMD-urea
0	4.89	0.26	0.000	0.00	0.0	2.6
1	3.68	0.96	0.058	1.91	2.9	1.5
2	3.22	1.19	0.107	2.65	3.5	3.4
3	2.82	1.30	0.146	2.84	4.1	4.5
4	2.62	1.43	0.189	3.55	4.4	5.4
5	2.39	1.48	0.210	4.10	4.9	6.4
6	2.23	1.54	0.273	4.13	5.0	8.0
7	1.95	1.50	0.310	4.52	5.4	9.2
24	0.86	1.03	0.104	6.81	8.1	4.4

(gradient 3, samples filtered after 7 h)

After 24 h 80 % of the MDI(AcCys)$_2$ has reacted. Relative concentration of the mixed adduct reaches a maximum of 30 % after 6 h; that of morpholine urea goes through a maximum of 6 % after 7 h and then adopts a practically constant value. This maximum is most likely due to super-saturation before precipitation of the urea. Another product that remains soluble is the bis-MDI-urea with AcCys end groups (Ac-CysMD-urea). The concentration of Ac-CysMD-urea has a maximum after 24 h, subsequent decrease is caused by further reaction to insoluble higher homologues. Free amine (MDA) could not be detected. Hydrolysis, however, also takes place as indicated by the formation of AcCys-MDI-NH$_2$. The maximum concentration of this asymmetric adduct is 8 % after 24 h. It is worth mentioning that after this time only 50 % of the products are soluble. 80 % of MDI(AcCys)$_2$ have reacted after 24 h but only 60 % of the theoretical amount of Ac-Cys have been formed (cf. Figure 11).

The precipitate formed was not completely soluble in acetonitrile/water (98:2 vol/vol). The soluble part contains traces of the mixed adduct and 60 % of morpholine urea. The remainder are oligomeric products, some of which are formed through hydrolysis. Aminolysis of MDI(AcCys)$_2$ follows second order kinetics (cf. Figure 12).
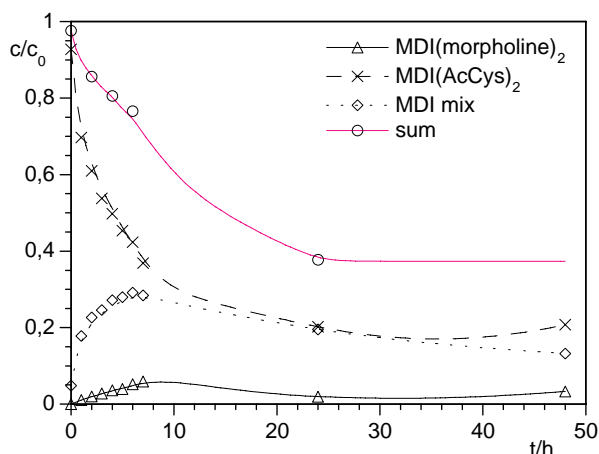

Figure 11: Relative concentrations of MDI adducts of aminolysis (310 K, pH 7.4)
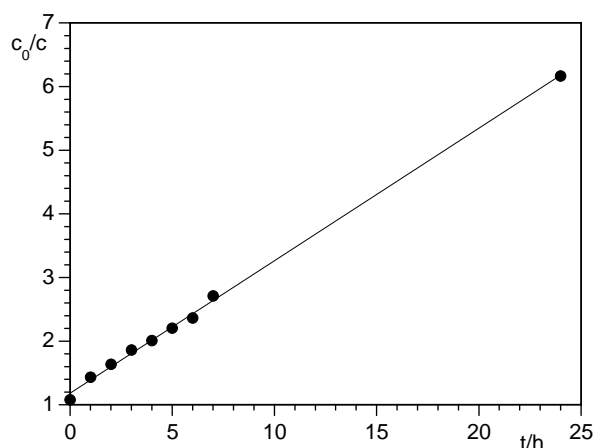

Figure 12: Second order kinetics (310 K) of aminolysis of MDI(AcCys)$_2$ at pH 7.4

Thiolysis of MDI(AcCys)$_2$ was very fast; 80 % had undergone solvolysis to Ac-Cys-MDI-AcCysMe and MDI(AcCysMe)$_2$ within one hour. Concentration of AcCys-MDI-AcCysMe went through a maximum,

$MDI(AcCysMe)_2$ is practically insoluble in the buffer solution.

A precipitate formed from the very beginning, hence all samples were filtered. Precipitates after 2, 7.5 and 24 h were analyzed. They consisted of traces of $MDI(AcCys)_2$, AcCys-MDI-AcCysMe and $MDI(AcCysMe)_2$ in a ratio between 1:4.5 and 1:5. Further compounds with retention times of 15.3, 18.8 and 19.2 min were detected in HPLC. The area percentage of individual peaks was between 4 and 5 %, that of all unknown peaks between 7 and 12 % (cf. Table 7, Figures 13-15).
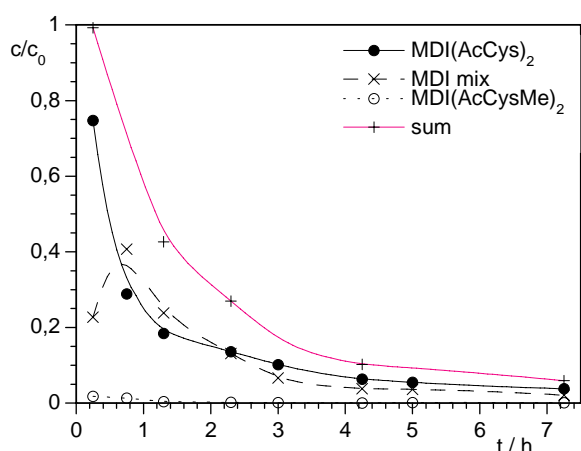
Figure 13: Relative concentrations of MDI adducts of thiolysis (310 K, pH 7.4)
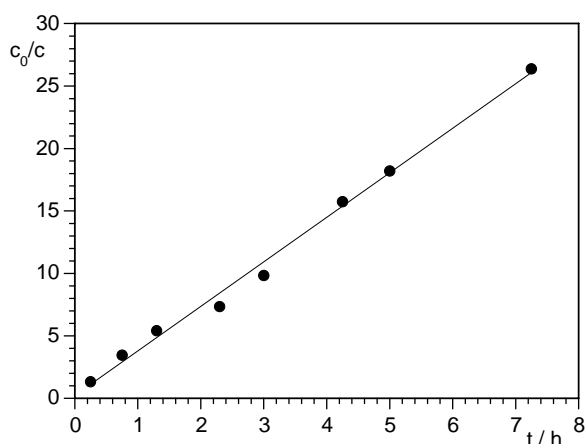
Figure 14: Second order kinetics of thiolysis of $MDI(AcCys)_2$
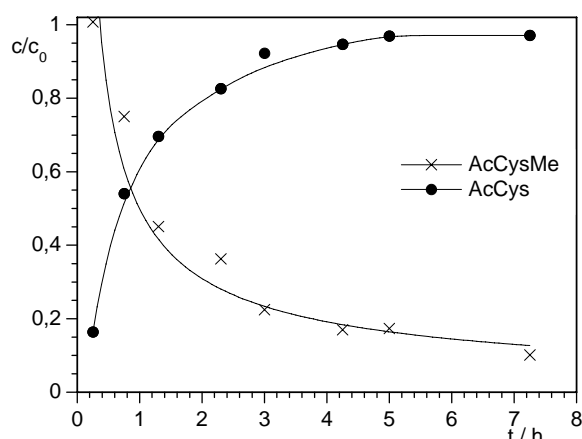
Figure 15: Relative concentrations of AcCys and AcCysMe (310 K, pH 7.4)

Hydrolysis of MDI(AcCys)₂

Hydrolysis at 310 K was by a factor of approximately ten slower than thiolysis. Soluble compounds that could be detected were $AcCys-MDI-NH_2$, $MDI-urea-AcCys_2$ and $AcCys-MDI-Urea-NH_2$, besides the initial adduct. Peak areas and concentrations of known compounds are given in Table 8, time conversion and kinetic curves are shown in Figures 16-17. $MDI(AcCys)_2$ had reacted to approximately 90 % after three days. Concentrations of $AcCys-MDI-NH_2$ und $MDI-Urea-AcCys_2$ reached a maximum after 7 h which coincides with the formation of solids.
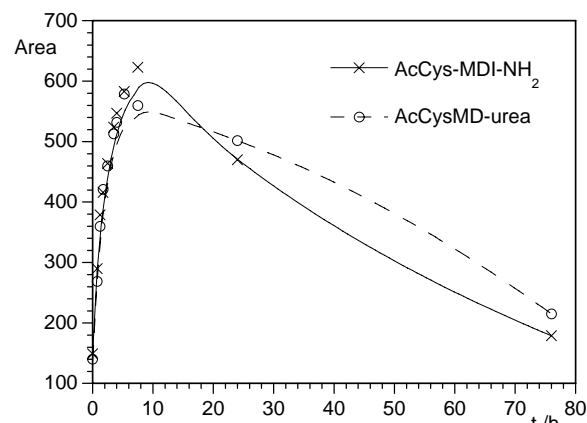
Figure 16: $AcCys-MDI-NH_2$ (14.6 min) and MDI-Urea$(AcCys)_2$ (18.3 min); (arbitrary units)

Table 7: Concentrations of MDI adducts and cysteine derivatives in thiolysis (310 K)

t / h	c / mmol L^{-1} (c_0: 5.085 mmol L^{-1})				
	MDI (AcCys)$_2$	MDI mix	MDI (AcCysMe)$_2$	AcCys	AcCysMe
0.3	3.773	1.147	0.089	1.67	11.43
0.7	1.457	2.054	0.065	5.49	9.43
1.3	0.932	1.203	0.198	7.08	7.10
2.3	0.687	0.662	0.010	8.20	6.72
3.0	0.512	0.334	0.006	9.38	5.34
4.3	0.320	0.193	0.003	9.22	4.91
5.0	0.277	0.184	0.007	9.85	4.94
7.3	0.191	0.105	0.003	8.86	4.37
24.0	0.118	0.045	0.001	9.86	3.38
120.0	0.162	0.034	0.000	9.22	1.51
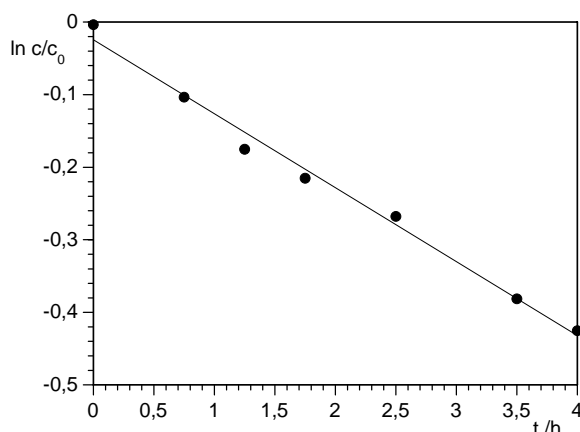
Figure 17: Pseudo first order kinetics (310 K) of hydrolysis of MDI(AcCys)$_2$

Table 8: Concentrations of MDI(AcCys)$_2$ and AcCys in hydrolysis (310 K)

t/h	c/mmol L^{-1} (c_0: 5.283 mmol L^{-1})	
	MDI(AcCys)$_2$	AcCys
0.0	5.26	0
0.7	5.05	0
1.3	4.43	0.0078
1.7	4.26	0.0048
2.5	4.04	0.0056
3.5	3.61	0.0089
4.0	3.45	0.0112
5.3	3.32	0.0099
7.5	3.12	0.0130
24.0	1.83	0.0220
76.0	0.55	0.0067

(gradient 2, samples filtered after 5.25 h)

Solvolysis reactions of MDI(AcCys)$_2$ in DMAc at 310 K

Reactions of MDI(AcCys)$_2$ proceeded without formation of oligomeric compounds under these homogeneous anhydrous conditions.

Aminolysis of MDI(AcCys)$_2$ with morpholine was the fastest reaction in DMAc. Time conversion plots and kinetic curves are given in Figure 18. After 10 h MDI(AcCys)$_2$ had reacted to 99 % and 80 % bis morpholine urea formed which increased to 90 % after 24 h. The mixed adduct reaches a maximum of 46 % after 2 h and decreases to 6 % after 24 h.
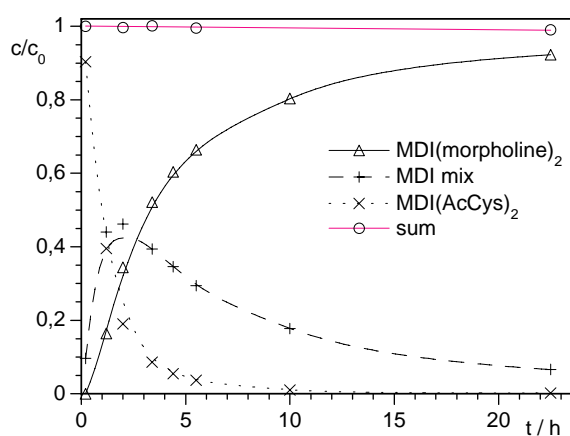
Figure 18: Concentrations of MDI adducts in aminolysis of MDI(AcCys)$_2$ (DMAC, 310 K)

Thiolysis, alcoholysis of MDI(AcCys)$_2$, and aminolysis of MDI(AcCysMe)$_2$ were investigated in the same way. Rate constants are included in Table 9. Thiolysis is slower than aminolysis and approaches an equilibrium. After 36 days MDI(AcCys)$_2$ had undergone 65 % exchange reaction with AcCysMe. The reaction mixture contained 35 % MDI(AcCys)$_2$, 45 % mixed adduct and 20 % MDI(AcCysMe)$_2$.

Alcoholysis of MDI(AcCys)$_2$ in DMAc was very slow. Small amounts of the mixed adduct MDI-AcCys-methoxyethanol were formed and the reaction almost came to a halt after two days. MDI(methoxyethanol)$_2$ was not found in HPLC. Therefore, the rate constant was calculated on the basis of the first three data points. Aminolysis of the adduct MDI(AcCysMe)$_2$ was included in this investigation in order to study the influence of the carboxyl group on the reaction rate (cf. Table 9).

DISCUSSION

General aspects of kinetic analysis

Reactions of *p*TI-AcCys and MDI (AcCys)$_2$ with nucleophilic compounds susceptible to transcarbamoylation (morpholine, AcCysMe, and 2-methoxyethanol) are expected to follow second order kinetics in case substrate and reagent are present in equimolar amounts. Hydrolysis should follow pseudo-first order kinetics because of the high excess of water with respect to the isocyanate adducts.

The general reaction scheme is represented by the following three equations which stand for the second order lysis reaction, the pseudo-first order hydrolysis reaction and the subsequent aminolysis reaction that leads to the formation of a urea (cf. Scheme 4). In thiolysis the primary lysis product *p*TIAcCysMe also can undergo hydrolysis and aminolysis; these reactions are also included in Scheme 4.

In thiolysis an equilibrium between the AcCys and the AcCysMe conjugates (two symmetric and one mixed thiocarbamate for MDI(AcCys)$_2$) should be reached under homogeneous conditions in the absence of hydrolysis. Both assumptions are not valid in aqueous buffer. AcCysMe and its adducts are less soluble than AcCys and its conjugate, hence the equilibrium will be shifted to the AcCysMe-conjugate and finally to a urea. Supersaturation with *p*TI-AcCysMe and delayed precipitation (Figure 3) cause further problems with the analysis of lysis reactions.

Clean reaction kinetics can be expected only, if the rate constant for reaction of the nucleophile is by several orders of magnitude larger than that of hydrolysis, since concentration of water is by a factor of approximately 5500 larger than that of the other nucleophiles. If one assumes HO$^-$ as the true nucleophile, concentration will be lower but the rate constant will be much higher. A further complication arises from the formation of insoluble reaction products. All these complications do not allow to exactly solve the kinetic expressions that describe the reaction schemes.

Products formed in lysis reactions

Reactions of *p*TI-AcCys and MDI(AcCys)$_2$ with morpholine, AcCysMe, water and 2-methoxyethanol have been monitored and analysed to provide rate constants and in some cases also activation energies. Rate constants obtained are for consumption of conjugates, which means that for MDI(Ac-Cys)$_2$ reaction of the first AcCys has been used for determination of rate constants. All possible reaction products that did not result from oligomerization have been identified, calibration curves were established except for MDI-AcCys-Amine and AcCysMD-urea.

Insoluble products, mostly oligomers, ureas and AcCysMe terminated compounds have been observed in aqueous buffer solution of pH 7.4. Oligomerisation which requires hydrolytic cleavage and reaction of the resulting amino group containing moieties with an isocyanate-AcCys conjugate is an important side reaction under aqueous conditions. Insoluble products in lysis reactions of *p*TI-AcCys were *p*T-urea and *p*TI-AcCysMe.

$$p\text{TI-AcCys} + \text{Nu} \xrightarrow{\text{k}_{\text{Nu}}} p\text{TI-Nu} + \text{AcCys}$$

$$p\text{TI-AcCys} + \text{H}_2\text{O} \xrightarrow{\text{k}_{\text{H2O}}} p\text{TA} + \text{AcCys} + \text{CO}_2$$

$$p\text{TI-AcCys} + \text{pTA} \xrightarrow{\text{k}_{p\text{TA}}} p\text{T-urea} + \text{AcCys}$$

additional reactions in thiolysis

$$p\text{TI-AcCysMe} + \text{H}_2\text{O} \xrightarrow{\text{k}'_{\text{H2O}}} p\text{TA} + \text{AcCysMe} + \text{CO}_2$$

$$p\text{TI-AcCysMe} + \text{pTA} \xrightarrow{\text{k}'_{p\text{TA}}} p\text{T-urea} + \text{AcCysMe}$$

Scheme 4: Reactions occurring during solvolysis of AcCys conjugates in aqueous buffer

$$-\frac{d[p\text{TI–AcCys}]}{d[t]} = k_{\text{Nu}}[p\text{TI–AcCys}][\text{Nu}] + k_{\text{H}_2\text{O}}[p\text{TI–AcCys}][\text{H}_2\text{O}] + k_{p\text{TA}}[p\text{TI–AcCys}][p\text{TA}]$$

Equation 1: Rate law for the decay of pTI-AcCys

For MDI(AcCys)$_2$ insoluble urea group containing oligomers were formed, which involve at least one hydrolysis-step and can have two identical or two different end groups X and Y as shown in Scheme 5.

X, Y:

Scheme 5: Possible oligomeric reaction products from transcarbamoylation of MDI(AcCys)$_2$ combined with hydrolysis

AcCysMD-urea has been detected as soluble material, other oligomers could not be dissolved for HPLC/MS. This is why homogeneous lysis reactions in dry DMAc were easier to analyse.

Reactivity of conjugates in lysis reactions

All rate constants from lysis reations that could be determined, are collected in Table 9. Activation energies for reactions of pTI-AcCys are also included and for those reactions that have been studied with both isocyanates the ratio of the rate constants is also given. In case there is more than one rate constant for a given temperature, the second value was obtained from a repetition of the corresponding experiment.

Time conversion curves and second order plots show that there is fast reaction taking place in the beginning followed by retardation (as can be seen in Figure 2 for aminolysis and in Figure 4 for thiolysis). For the MDI-conjugate this effect is much smaller (cf. Figures 12 and 14). In thiolysis an equilibrium between AcCys- and AcCysMe conjugates could be the reason, this, however, is absent in aminolysis. Some kind of complexation or preferential solvation can be responsible of this behaviour. In those cases where retardation occurred data points of the first state of the reaction have been chosen for determination of rate constants in order to have reliable and comparable kinetic information.

Table 9: Kinetic data of solvolysis reactions of *p*TI-AcCys and MDI(AcCys)₂

Reaction	*p*TI-AcCys				MDI(AcCys)₂	k$_{MDI}$/k$_{pTI}$
	T /K	10^3 k /l mol^{-1} s^{-1}	Corr. coeff.	E$_A$ /kJ mol^{-1}	10^3 k /l mol^{-1} s^{-1}	
Aminolysis aq. buffer pH 7.4	298	1.33	0.995	119 (0.996)		
	310	7.78	0.986		53.8 (0.999)	6.9
	323	55.5	0.998			
	323	54.7	0.997			
Thiolysis aq. buffer pH 7.4	298	100.4 (0.5 h) 52.2 (0.5-3 h)	0.999	44 (0.988) 68 (0.96)		
	310	228.2	0.999			
	310	247.8	0.999		974 (0.997)	3.9
	323	401.2	0.998			
	323	433.6	0.999			
Hydrolysis aq. buffer pH 7.4	310	(4.25) 0.0407 s^{-1}	0.998		(33.8) 0.2828 s^{-1} (0.995)	7.9 6.9
”Alcoholysis“	310	(4.60) 0.039 s^{-1}	0.991			
Aminolysis in DMAc	298	5.20 4.70	0.999	77 (0.999) 80 (0.989)		
	310	17.52 22.48	0.999		77.3 (0.993)	4.4 (3.4)
	310	16.52	0.999			
	323	57.42	0.995			
Thiolysis in DMAc	310	0.075 0.078	0.989	88.5 (0.997) 85.5 (0.994)	0.13 (0.999)	1.7
	323	0.241 0.227	0.999			
	338	1.198 1.202	0.999			
Alcoholysis in DMAc	310	-	-		0.028 (0.997)	
	*p*TI-AcCysMe				MDI(AcCysMe)₂	
Aminolysis in DMAc	310	37.3	0.998		557 (0.996)	15.5

The order of reactivity of nucleophiles found in aqueous buffer solution of pH 7.4 and in DMF for both *p*TI-AcCys and MDI(AcCys)₂ is:

aqueous buffer pH 7.4
thiolysis > aminolysis > hydrolysis
DMAc
aminolysis > thiolysis > hydrolysis

In aqueous buffer solution of pH 7.4 alcoholysis with 2-methoxyethanol could not be observed. Rates constants of both reactions seem to be comparable and the very large excess of water suppresses alcoholysis under these conditions. Hence, under physiological conditions the "isocyanate" group is not transferred to aliphatic hydroxy-groups of bio-molecules, e. g., serin.

At 37 °C thiolysis is by a factor 30 faster than aminolysis. In addition to first order rate constants, second order rate constants have been calculated for hydrolysis since the latter are better suited for comparison. On that basis aminolysis proceeds only by a factor of 2 faster than hydrolysis. This also suggests that hydrolytic cleavage of thiocarbamates should be an important reaction under physiologcal conditions.

In DMAc aminolysis is faster than under physiological conditions by a factor of 2 at 37 °C. More striking is that in DMAc aminolysis is by a factor of more than 200 faster than thiolysis.

It also is remarkable, that reactions of MDI(AcCys)$_2$ are faster by a factor of 4 to 8 than those of pTI-AcCys. The only case where rates are close together is thiolysis in DMAc with a factor of only 1.7. A factor of 2 arises from probability arguments, as MDI(AcCys)$_2$ provides two reactive groups per molecule versus one group per moleculs in pTI-AcCys.

From the electronic point of view pTI is a perfect model for MDI because the methylene group interrupts any mesomeric influence of substituents. Hence higher reactivity must be due to some favourable spatial conformation of MDI(AcCys)$_2$. One possible conformation is shown in Figure 19. This may facilitate optimal orientation of the active form of the nucleophile that cleaves the thiocarbamate.
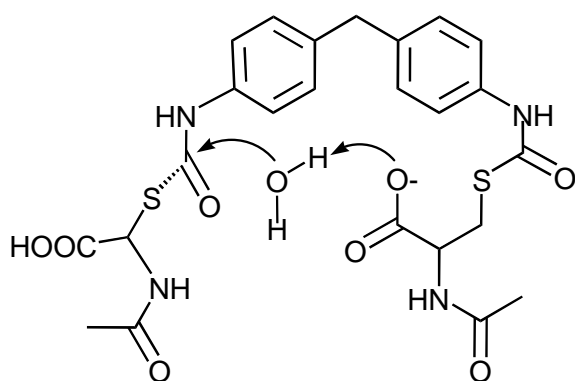

Figure 19: Tentative mechanism of activation of water in hydrolysis of MDI(AcCys)$_2$

The deprotonated carboxyl group can act as an activator, e. g., for water, as shown in Figure 19. Activation by carboxyl groups is well known from the carboxylesterases class of enzymes (Satoh and Hosokawa, 2006).

Activation energies have been determined for aminolysis and thiolysis of pTI-AcCys both in DMAc and under physiological conditions. First of all it is noteworthy that the activation energy of aminolysis is roughly 50 % higher in buffer of pH 7.4 (119 kJ/mol) than in DMAc (77 kJ/mol). Activation energy of thiolysis is rather small in buffer (44 kJ/mol) and has twice that value in DMAc (88 kJ/mol). This different behaviour of conjugates in DMAc and under physiological conditions can be understood if acid-base reactions are taken into account. In aqueous buffer of pH 7.4 the amine is protonated to some extent. This reaction is reversed by thermal dissociation at higher temperature. It should be noted that the activation energy for the hydrolysis of MDI(AcCysMe)$_2$ was reported to be 44.5 kJ/mol (Chipinda et al., 2006).

The rather low activation energy for thiolysis in aqueous buffer can also be understood from the acid-base behaviour of the sulfhydryl group. At 25 °C, the pK$_a$ of the sulfhydryl group of cysteine is 8.33 (Burner, 1999). At pH = 7.4 approximately 10 % of the AcCysMe is present as highly nucleophilic mercaptide, which explains the higher reactivity of the sulfhydryl group compared to the amino group of morpholine. It also explains the high activation energy of aminolysis and the low activation energy of thiolysis in aqueous buffer.

In aprotic DMAc acid-base reactions do not play any important role for the AcCysMe; morpholine will at least partly be protonated by acid-base reaction with pTI-AcCys or MDI(AcCys)$_2$, respectively. The lack of thiolate-anion in DMAc results in higher reaction rates of morpholine in comparison to AcCysMe; activation energies of both reactions are comparable.

The inhibiting influence of the carboxy group of N-acetyl-L-cysteine on aminolysis in DMAc is demonstrated in experiments with pTI-AcCysMe and MDI(AcCysMe)$_2$ (cf. Table 9). The esters react faster: more than two times with pTI and more than

three times with MDI-adducts. Carboxy acid groups of N-acetyl-L-cysteine either partially protonate the amino group of morpholine or donate the proton for a hydrogen bond; both decrease nucleophilicity. This deactivation of the amine is not possible for *p*TI-AcCysMe and MDI(AcCysMe)$_2$.

Formation of aromatic amines

Formation of amines as intermediates or final products is one reaction of isocyanates in aqueous media. This has been dealt with in previous reports (Mormann et al., 2003, 2006). In this work, no MDA has been observed while *p*TA was formed to some extent.

From Table 10 can be seen that up to 8.7 % of *p*TA are present in the reaction mixture in the course of reaction. The *p*TA concentration is highest in thiolysis because hydrolysis is the only reaction by which *p*TI is irreversibly consumed. Cysteine derivatives compete with the amine in solvolysis reactions suppressing formation of ureas. Again no MDA has been found although significant amounts of the monohydrolysis product AcCys-MDI-NH$_2$ could be detected. The detection limit of MDA is in the order of 10 nano grams or 10^{-4} mmol /L.

Table 10: *p*TA formed during lysis reactions of *p*TI-AcCys

Lysis reaction	T/K	amount /mmol L^{-1}	amount / %
Aminolysis	298	0.342	3.2
	310	0.615	6.1
	323	0.594	5.9
Thiolysis	298	0	0
	310	0.866	8.7
	323	0.861	8.5
Hydrolysis	310	0.662	6.6

Solvolysis of thiocarbamates: E1cB with free isocyanate as intermediate or BAc2

Solvolysis reactions of carboxylic acid and carbonic acid derivatives involve nucleophilic compounds which can also behave as bases. There are two possible mechanism according to which solvolysis reactions of carbamic acid derivatives can proceed. These are shown in Scheme 6 for aminolysis. In the addition/elimination mechanism (base catalysed bimolecular substitution: BAc2) nucleophilicity is dominating and attack of the electrophilic carbonyl carbon is the first step followed by elimination of the best leaving group. The elimination/addition mechanism (E1cB) involves base catalysed deprotonation of the nitrogen and elimination of a nucleophile to yield an isocyanate as intermediate followed by addition of another nucleophile to give the reaction product. An E1cB mechanism has first been claimed for hydrolysis of S-aryl thiocarbamates and N,S-diaryl thiocarbamates (Bourne et al., 1984). The main argument for this mechanism was the high ρ-value in the Hammett equation of this reaction which is consistent with a substantial negative charge of the sulfur in the transition state of the rate determining step.

This mechanism seems to have been generally adopted also for S-alkyl thiocarbamate (glutathione or cysteine conjugates) reactions (Day et al., 1997; Reisser et al., 2002; Chipinda et al. 2006). The mechanism, however, has no influence on the physiological activity of isocyanates as transcarbamoylation is the result of both reactions in Scheme 6. Both reaction mechanisms may act in parallel. In the E1cB mechanism, the strong nucleophile RS$^-$ is generated.

It is well known that isocyanate addition products undergo reverse reaction at elevated temperature. The cleavage temperature depends on the type of isocyanate and on the leaving group quality of the nucleophile that has been added. Thiocarbamates and also biurets are supposed to be cleaved to a significant extent at temperatures above 100 °C (Petersen, 1949; Oertel, 1993). Therefore some comparative experiments have been made to support one of the mechanisms.

The only piece of evidence for isocyanate formation is a peak observed in a HPLC/ESI-mass spectrometry experiment which has the mass of the TDI-glutathione mono-adduct (Day et al., 1997). Free TDI

has not been observed and formation of a cyclic adduct of glutathione/TDI, which has the same molar mass as a TDI-glutathion mono-adduct with a free NCO-group, has not been discussed. The structure of this cyclic adduct is shown in Figure 20.

No isocyanate could be detected in the reaction of isocyanates with AcCys neither in aqueous buffer nor in anhydrous DMAc, only the amine was found with *p*TIAcCys. Temperature dependent IR-spectroscopy of *p*TI-AcCysMe was used as a sensitive probe to detect free isocyanate in case it was formed. The methyl ester has the advantage that no carboxy group can interfere by trapping any isocyanate groups formed. Samples prepared under different conditions were studied in a heatable cell by IR-spectroscopy. No isocyanate could be detected. Repetition of this procedure with *p*TI-biuret clearly showed elimination at temperatures well below 120 °C forming *p*TI and *p*T-urea as shown in Equation 3 and Figure 21.
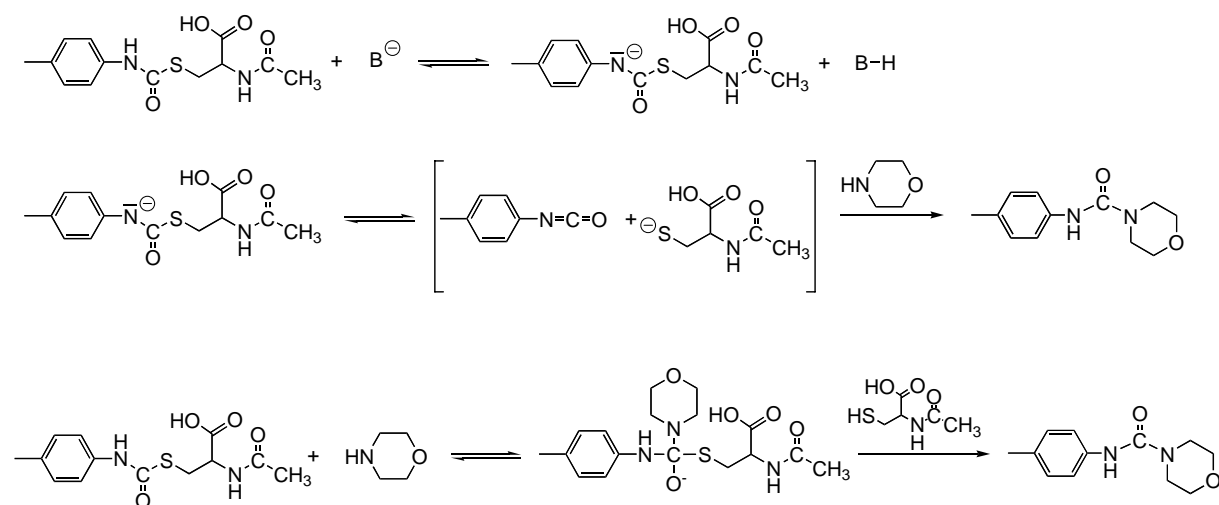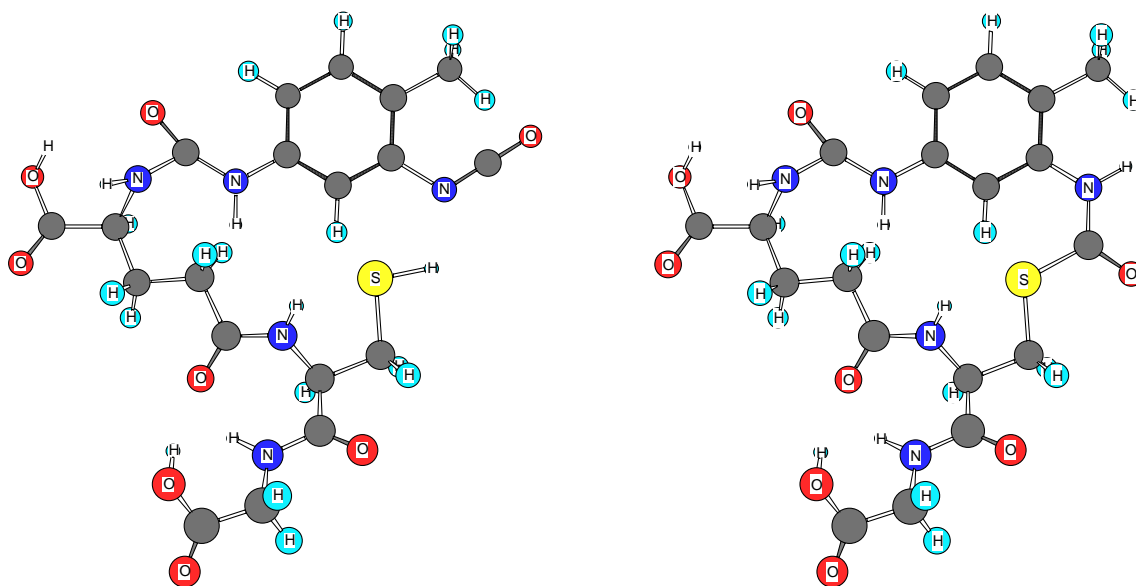

Scheme 6: E1cB and BAc2 reaction pathways

Figure 20: Cylindrical bond model of open (isocyanate containing) and cyclic (isocyanate free) TDI–glutathione mono-adduct
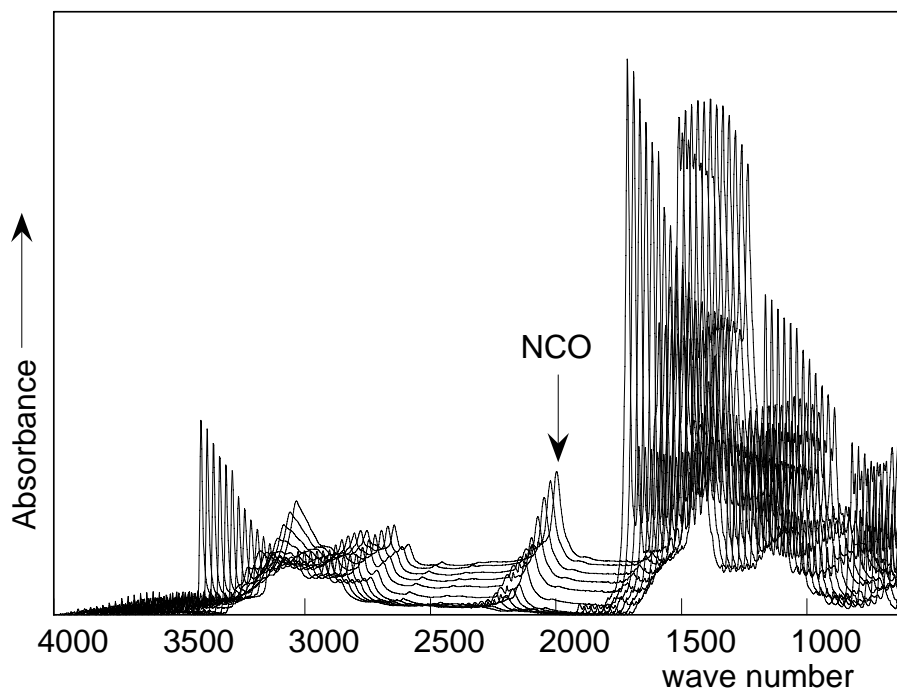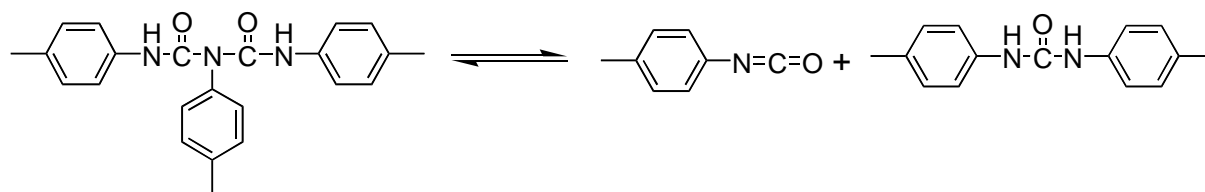
Figure 21: IR-spectra of *p*-TI-biuret from 20 to 120 °C

Equation 3: Thermal dissociation of *p*TI-biuret

Another argument in favour of an addition/elimination mechanism comes from the activation energies. Results on ammonolysis of several carbamic acid derivaties shown in Table 11 reveal two sets of activation energies: A high value above 120 kJ/mol and a lower value in the order of 70 kJ/mol. The high value is in the range of the elimination/addition reactions, whereas the lower value is typical for reactions that must proceed according to an addition/elimination mechanism to give the desired product (cf. Scheme 7). Comparison with activation energies from Table 9 strongly suggests that lysis reactions also proceed by the latter mechanism. The high activation energy for aminolysis in aqueous buffer has been explained by an acid base reaction.

Table 11: Activation energies of ammonolysis of carbamates

Model compound	E_A kJ/mol
	117.5
	69.2
	74.7
	136.4

Finally, it is worth mentioning that Bourne et al. (1984) used thiocarbamates based on thiophenol ($pK_a = 6.5$), while cysteine has an aliphatic SH-group with pK_a

8.3, whereas N-acetylcysteine has pK_a of 9.5 and Cysteine methyl ester pK_a 6.6 (Burner et al., 1999) with respect to the SH-group. As glutathione has a pK_a (SH) of 8.8 (Burner et al., 1999), N-acetylcysteine seems to represent to more reliable model for this tri-peptide. Further, due to the similar pK_a values, it is not too surprising that cysteine methyl ester behaves similar to thiophenol. S-glutathionyl and S-(N-acetyl-cysteinyl) residues of thiocarbamates are – due to the lower acidity - by far not as good a leaving group as the thiophenolate. In addition, the acidifying effect of the aromatic SH is stronger than that of the aliphatic SH; therefore the pKa of the NH in aromatic thiocarbamates from cysteine conjugates with isocyanates is higher than the corresponding thio-phenol products; in an S-alkyl thiocarbamate the NH is less likely to be deprotonated under the conditions of the aqueous buffer of pH 7.4 than in an S-aryl thiocarbamate. These general considerations are further supported by a paper of Baudet (Baudet et al., 1965). These authors reacted S-arylthiocarbamates of aminoacid esters and anilin with diazomethane. Free isocyanate was observed with N-alkyl,S-arylthiocarbamates, while no deprotonation occurred with N-phenyl-S-arylthiocarbamate. Furthermore no reaction took place with N-alkyl,O-phenyl carbamates. This demonstrates once more that the results of Bourne et al. cannot be generalized for all lysis reactions.

All the evidence from our own experiments as well as from the literature suggests that no free isocyanate is released from N-aryl-S-alkylthiocarbamates in aqueous solutions. An elimination/addition mechansim for reactions of cysteine-isocyanate conjugates is not backed by experimental evidence. Finally it should be emphasized once more that the difference between the two mechanisms is the difference of transition state not of reaction products. E1cB means that in the transition state of this mechanism an elimination step is involved not that isocyanate is a real intermediate.

Based on the experimental evidence given in this work, formation of free isocyanate groups in analytical concentration during lysis reactions of cysteine-based thiocarbamates is highly unlikely.

CONCLUSIONS

Transcarbamoylation reactions of *p*TI and MDI cysteine conjugates with SH-, NH-, alcoholic OH and water in aqueous buffer solution of pH 7.4 revealed that all reactions are accompanied by hydrolysis, which leads to side-products, most of which are insoluble. Complete kinetic analysis was not possible under these conditions. Transcarbamoylation with 2-methoxy ethanol was not detectable. It can be assumed therefore that transfer of the isocyanate-moiety from thiocarbamates to aliphatic OH-groups in biomolecules is negligible.

Reactions of MDI(AcCys)$_2$ are generally by a factor of 4–8 faster than the corresponding reactions of *p*TI-AcCys, with the exception of thiolysis in DMAc where rates are very similar with a factor of only 1.7.

Elimination/addition mechanism: high activation energy > 120 kJ/mol
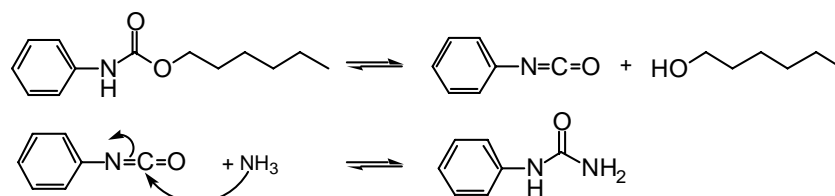
Addition/elimination mechanism: lower activation energy approx. 70 kJ/mol
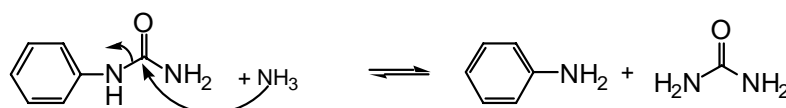
Scheme 7

No free isocyanate could be detected neither in aqueous buffer nor in an organic solvent. A number of arguments and experimental observations have been put forward which suggest that transcarbamoylation reactions of AcCys adducts proceed via an addition/elimination mechanism and not via free isocyanate groups in analytical concentrations as suggested by other research groups.

Small amounts of *p*-toluidine were found in solvolysis reactions of *p*TI-AcCys while no MDA was detected in corresponding reactions of MDI(AcCys)$_2$. This confirms the results of earlier findings (Mormann et al., 2006).

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