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Laboratory note

Synthesis of isothiocyanate-derived mercapturic acids

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Abstract

Twelve mercapturic acids derived from saturated and unsaturated aliphatic and aromatic isothiocyanates were synthesised, by adding isothiocyanate to a solution of *N*-acetyl-L-cysteine and sodium bicarbonate, in a typical yield of 77%. Isothiocyanates were synthesised first by adding the corresponding alkyl bromide to phthalimide potassium salt. The obtained *N*-alkyl-phthalimide was hydrazinolysed yielding the alkyl amine, which subsequently was reacted with thiophosgene yielding the isothiocyanate with an overall yield of 16%. Mercapturic acids in urine can serve as a biomarker of intake to determine the health promoting potential of isothiocyanates present in cruciferous vegetables.

Keywords: Nucleophilic addition; Glucosinolates; Isothiocyanates; Mercapturic acids; Biomarker

1. Introduction

Consumption of fruits and vegetables is associated with a reduced risk on degenerative diseases such as cancer and cardiovascular diseases, as indicated by epidemiological studies [1,2]. A reasonable estimate of the overall extent to which dietary modification may be expected to reduce cancer risk is 30–40% [3]. In particular, cruciferous vegetables appear to have beneficial health potential [4,5]. Since cruciferous vegetables differ from other vegetables by the presence of glucosinolates, these health promoting effects seem to be attributable to these phytochemicals or breakdown products thereof [6]. Glucosinolates are broken down into indoles, nitriles and isothiocyanates (Fig. 1) by a thioglucosidase (myrosinase, EC 3.2.3.1) present in cruciferous vegetables [7,8] and to a lesser degree by microbes present in the human gut [9,10]. Isothiocyanates are strong inhibitors of phase I enzymes and inducers of phase II enzymes, and therefore are thought to be strong cancer chemopreventors [11–13]. Many different isothiocyanates (more than 25) block the carcinogenic effects of more than 12 chemically different types of carcinogens in at least 10 different target sites in three species of rodents [14]. Phenethyl isothiocyanate is a particularly effective inhibitor of lung tumor induction by the tobacco-specific nitrosamine-4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and, therefore, is currently being developed as a chemopreventive agent against lung cancer [14]. Human intervention trials with large quantities of cruciferous vegetables gave similar effects on phase I and phase II enzymes [15,16].

Isothiocyanates are conjugated to glutathione in the body and excreted into the urine as their corresponding mercapturic acids (Fig. 1) as was demonstrated in rats [17], guinea pigs and rabbits [18] and in humans [19,20]. Mercapturic acids reflect the intake of glucosinolates present in cruciferous vegetables [21–23] and could be used as a selective biomarker for cruciferous vegetable intake. Different cruciferous vegetables can botanically be differentiated by the variety and amount of glucosinolates [24]. Broccoli, for instance, is rich in 4-methylsulfinylbutyl glucosinolate, whereas Brussels sprouts are rich in 2-propenyl and 2-hydroxy-3-butenyl glucosino-

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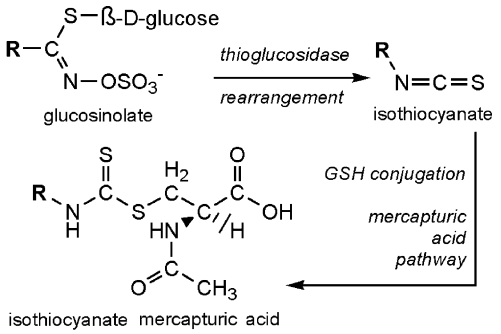


Fig. 1. Isothiocyanates are enzymatically hydrolysed from glucosinolates, in the body conjugated to glutathione and excreted as mercapturic acids in the urine.

late (progoitrin), and cress is rich in aromatic glucosinolates [7].

Several reports describing the synthesis of isothiocyanates and the corresponding mercapturic acids are available (Table 1, comments in Section 3). We here describe the synthesis of 12 mercapturic acids derived from saturated and unsaturated aliphatic and aromatic isothiocyanates, namely methyl, butyl, isopropyl, 1-methylpropyl, 2-propenyl, 3-butenyl, 4-pentenyl, phenyl, benzyl, phenylethyl, 4-methylthiobutyl and 4-methylsulfinylbutyl isothiocyanate (Fig. 2). The HPLC–MS/MS analysis of isothiocyanate-derived mercapturic acids in urine is described in Ref. [41].

2. Chemistry

In cruciferous plants, glucosinolates are formed from amino acids [25]. Tissue disruption by, e.g. chewing starts the breakdown of glucosinolates into isothiocyanates and this provides protection from plants towards insects as isothiocyanates are pungent metabolites. In humans, chewing of Brussels sprouts releases 39% of the glucosinolates as isothiocyantes, measured in the urine, whereas no chewing results in the excretion of 26% of isothiocyanates [26]. Ingestion of pure benzyl isothiocyanate, introduced as a drug for the treatment of infections of the respiratory and urinary tract under the trademark Tromacaps®, resulted in 43–60% excretion of the compound in the urine as its mercapturic acid [20]. Sulforaphane is the bioactive compound found in broccoli [27]. This synthesis is described in Scheme 1.

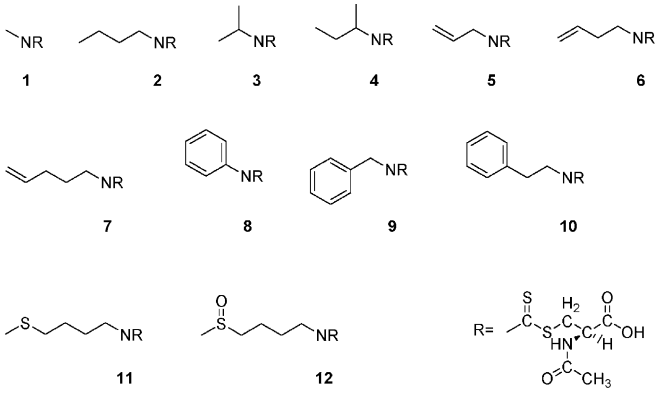


Fig. 2. Structures of **12** synthesised mercapturic acids derived from isothiocyanate.

3. Results and discussion

Twelve isothiocyanate mercapturic acids have been synthesised (Fig. 2, Table 1). The synthesis of isothiocyanates from phthalimide is a multistep reaction with an overall yield of 16%. The synthesis from *N*-acetyl-L-cysteine (NAC) and isothiocyanate was a convenient one-step reaction which proceeds in a typical yield of 77%. Before adding isothiocyanate to NAC, we converted NAC to its sodium salt using sodium bicarbonate, thus enhancing the reaction rate. After the reaction was completed, the mercapturic salt was converted into the poorly water-soluble mercapturic acid using hydrochloric acid. Several solvents were tested for recrystallisation. The products dissolved best in boiling ethyl acetate which allowed crystallisation on cooling. The mercapturic acids derived from isopropyl and of 1-methylpropyl isothiocyanate were obtained in a yield of 50%. The solubility of these products in ethyl acetate is perhaps higher than, for instance, the mercapturic acid derived from benzyl isothiocyanate. A better solvent for recrystallisation could not be found.

The synthesis of 3-butenyl isothiocyanate has previously been described by Kjær et al. [28] starting from allyl cyanide. The boiling point observed by us and that reported [28] are the same (60 °C at 12 mmHg). Ettlinger and Hodgkins [29] started from allylcarbinol and reported the boiling point to be 77.5 °C at 28 mmHg. Leoni et al. [30] obtained 3-butenyl isothiocyanate by enzymatic hydrolysis of gluconapin. Our NMR data are identical with that reported. 4-Pentenyl isothiocyanate has been synthesised previously by Kjær and Jensen [31] and in the same way by Gilbert and Nursten [32]. Only the boiling point, density and elemental analysis were mentioned. 4-Methylthiobutyl isothiocyanate (trivial name erucin) has first been synthesised by Schmid and Karrer [33] and later by Kjær and Gmelin [34]. Our boiling point and NMR data are identical with those reported. 4-Methylsulfinylbutyl isothiocyanate (trivial name sulforaphane) has first been synthesised by Schmid and Karrer [33] who also described the synthesis of optically pure L- and D-sulforaphane. Sulforaphane was later synthesised by Zhang et al. [27] and Kuhnert et al. [35] (conversion of amine into the isothiocyanate unit precedes the oxidation of sulfur). The physical and spectral data are identical. All authors who published their results between 1948 and 1972 used pyridine and benzene. These toxic compounds can be replaced by

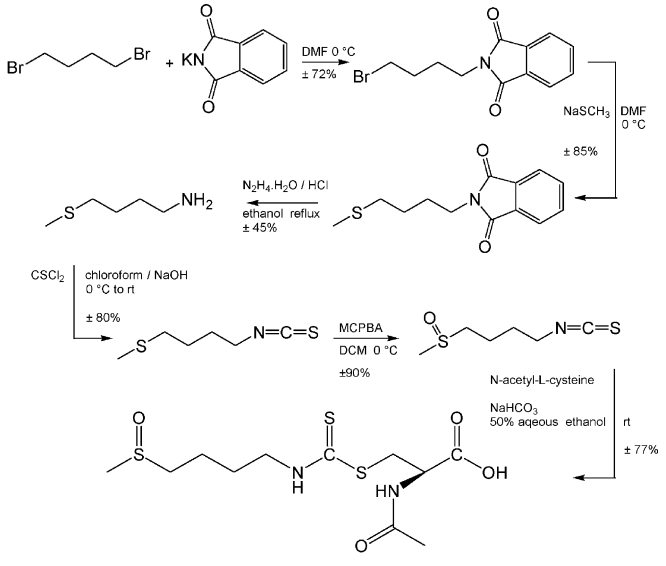
Table 1
Trivial names of isothiocyanate yielding glucosinolates, structures of the corresponding isothiocyanates, (main) dietary source and literature references to isothiocyanate and mercapturic acid synthesis

Glucosinolate	Isothiocyanate	Main dietary source	[a]	Previous isothiocyanate synthesis by others [b]	Previous isothiocyanate mercapturic acid synthesis by others [b]
Glucocapparin	H_3CNCS	cauliflower, capers	**1**	commercially available	(36)
Unknown	$H_3C(CH_2)_3NCS$	cabbage, horseradish	**2**	commercially available	(36)
Glucoputranjivin	$H_3CCH(CH_3)NCS$	Brussels sprouts, turnip	**3**	commercially available	not yet reported
Glucocochlearin	$H_3CCH_2CH(CH_3)NCS$	black mustard	**4**	commercially available [c]	not yet reported
Sinigrin	$H_2C{=}CHCH_2NCS$	cabbage, brown mustard	**5**	commercially available	(36, 37)
Gluconapin	$H_2C{=}CH(CH_2)_2NCS$	Chinese cabbage	**6**	(28, 29)	not yet reported
Glucobrassicanapin	$H_2C{=}CH(CH_2)_3NCS$	Chinese cabbage	**7**	(31, 32)	not yet reported
Unknown	C_6H_5NCS	ambiguous[d]	**8**	commercially available	not yet reported
Glucotropaeolin	$C_6H_5CH_2NCS$	garden cress	**9**	commercially available	(17)
Gluconasturtiin	$C_6H_5(CH_2)_2NCS$	water cress	**10**	commercially available	(38, 39)
Glucoerucin	$H_3CS(CH_2)_4NCS$	broccoli, salad rocket	**11**	(27, 33, 34)	(40)
Glucoraphanin	$H_3CS(=O)(CH_2)_4NCS$	cabbage	**12**	(27, 33, 35)	(40)

[a] Numbers refer to structures in Fig. 2.
[b] For comments, see Section 3.
[c] Not optically pure.
[d] Presence in nature is unlikely.

aqueous ethanol and heptane, respectively. The presence in nature and isolation from plants of isothiocyanates was summarised by Kjær [7].

Mennicke et al. [36] described the synthesis of **1**, **2** and **5**. Physical data were only given for the dicyclohexylamine salt. Ioannou et al. [37] (only brief description of the synthesis) described the isolation of **5** from rat urine and the synthesis of ^{14}C radio-labeled **5** using column chromatography to obtain the pure compound. All NMR data are comparable with ours. Brüsewitz et al. [17] described the synthesis of benzyl isothiocyanate



Scheme 1. Synthesis of *N*-acetyl-*S*-(*N*-4-methylsulfinylbutylthiocarbamoyl)-L-cysteine (sulforaphane mercapturic acid).

conjugates. They reported an m.p. of 58–62 °C for **9**, which is very different from ours (136.12 °C). Since optical rotation, elemental analysis and NMR data are in agreement with ours, the product obtained by Brüsewitz is probably contaminated by some solvent which lowers the m.p. Toxic solvents can be replaced by non-toxic. Eklind et al. [38] synthesised **10**, by adding the isothiocyanate to a solution of *N*-acetyl-L-cysteine in methanol in poor yield (39%). The NMR data are the same as ours. Jiao et al. [39] used the method of synthesis described by Brüsewitz, however, their NMR data for the obtained product (**10**) differ from those reported by Eklind and found by us. Kassahun et al. [40] prepared **11** and **12** by adding the isothiocyanate to an aqueous ethanol solution of *N*-acetyl-L-cysteine at basic pH. These products were purified by column chromatography, the NMR data (in $D_2O/CDCl_3$) are in agreement with ours.

In our synthesis of **11** and **12**, phthalimide potassium salt was added to three equivalents of 1,4-dibromobutane to minimise the formation of 1,4-diphthalimidylbutane. A top piece containing potassium hydroxide was used to avoid moisture and to prevent the reaction of sodium methylmercaptide with carbon dioxide to give methylmercaptan. We compared triethylamine, sodium hydroxide and sodium bicarbonate as the basic reagent in the formation of isothiocyanate from the amine and thiophosgene. Three equivalents of the base were used, after which chloroform and excess of thiophosgene were evaporated and the residue was pored in water and diethyl ether. This resulted in black solids when

triethylamine was used. Because of gas forming after adding sodium bicarbonate, we preferred the use of sodium hydroxide as a base. Distillation of the isothiocyanate formed was performed immediately after evaporating chloroform and thiophosgene in order to prevent residues of thiophosgene to react with the isothiocyanate. 4-Methylsulfinylbutyl isothiocyanate was obtained by oxidation of 4-methylthiobutyl isothiocyanate. 4-Methylsulfinylbutylamine could be obtained by oxidising *N*-(4-methylthiobutyl)-phthalimide with MCPBA, followed by hydrazinolysis. Subsequent reaction of the amine with thiophosgene led to complete reduction of the sulfinyl group yielding 4-methylthiobutylamine rendering this route of synthesis impossible. This deoxygenation in the absence of a reducing agent has been observed also by Kuhnert et al. [35] and cannot be explained. Thiophosgene used to convert amines to isothiocyanates can be replaced by di-2-pyridyl thionocarbonate which is not toxic and easier to handle. We were unable to derive optimal conditions for this conversion using 1,1′-thiocarbonyl diimidazole.

Product **7** has an brownish-orange color, while all other mercapturic acids are colored yellow to white. The reaction mixture containing 4-pentenyl isothiocyanate and NAC also contained some black residue which may be due to side products resulting from thiophosgene.

Isothiocyanate-derived mercapturic acids and its precursors can be separated using TLC. Isothiocyanates and their mercapturic acids are UV 254 nm active, whereas NAC is not UV active. Potassium dichromate followed by heat colors all products and precursors and has the advantage that it colors NAC orange before heat treatment. In ethyl acetate–water–formic acid (60:35:18, v/v/v), all components showed only minor retention, NAC $R_F = 0.78$, phenyl mercapturic acid $R_F = 0.90$ and phenyl isothiocyanate $R_F = 0.99$. Adding just enough acetic acid (8 mL) to mix 80 mL of ethyl acetate with 10 mL of water gave a satisfactory retention and separation of the components.

The melting of the mercapturic acid derived from phenyl isothiocyanate resulted in the explosion of a DSC sample cell. This is probably due to an exothermal reaction with formation of gas following the melt. Melting of NAC also resulted in the formation of gas but the reaction products could not be analysed.

4. Conclusions

In conclusion, we have synthesised 12 mercapturic acids derived from isothiocyanates in good yield. These 12 compounds were prepared because they probably represent the most important urinary excretion products after eating a cruciferous vegetable meal. Phenyl glucosinolate is not a natural compound so that the mercapturic acid derived from phenyl isothiocyanate can be used as internal standard for the analysis of mercapturic acids obtained from isothiocyanates. Future research will include the development of a liquid chromatographic method to analyse mercapturic acids in urine and validation of mercapturic acids as biomarkers to measure the effective dose of isothiocyanates absorbed.

5. Experimental protocols

5.1. Chemistry

Methyl, 2-propenyl, phenyl, benzyl and phenylethyl isothiocyanate as well as *N*-acetyl-L-cysteine (NAC), 1,4-dibromobutane, 4-bromo-1-butene and 5-bromo-1-pentene were purchased from Acros Organics. Butyl isothiocyanate, iso-propyl isothiocyanate and sodium methylmercaptide were purchased from Sigma-Aldrich Co., 1-methylpropyl isothiocyanate was obtained from Maybridge Chemical Company Ltd. and phthalimide potassium salt was purchased from Merck. Solvents were dried using the following methods. Dichloromethane was distilled from P_2O_5. Diethyl ether was distilled from NaH. Hexane and heptane were distilled from CaH_2. All other chemicals were of analytical grade. All chemical and physical data are mentioned in Table 2.

^{1}H-NMR (100 MHz) spectra were recorded on a Bruker AC 100 spectrometer, 300 MHz ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker AC 300 spectrometer, 400 MHz ^{1}H-NMR spectra were recorded on a Varian Unity-400 spectrometer, and 600 MHz ^{1}H-NMR spectra were recorded on a Bruker Avance spectrometer with DMSO-d_6 as internal standard unless stated otherwise (Aldrich, $\delta = 2.49$ and 39.7 ppm, respectively, J in Hz). Optical rotation (OR) was measured on a Perkin–Elmer 241 Polarimeter at 589.3 nm, products were dissolved in methanol (10 mg mL^{-1}), measured three times and the average was taken. Melting points (m.p.) were determined with a Reichert Thermopan microscope and figures between brackets on a differential scanning calorimeter (DSC-2920, TA Instruments), both are uncorrected. Elemental analyses (EA) were performed on a Carlo Erba instruments CHNS-O 1108 elemental analyser at the Department of Microanalysis of the University of Nijmegen. The formula weight (FW) is the average molecular weight. The exact molecular mass was calculated using the atomic masses of the most abundant isotopes. Mass spectra (MS) were collected on a Finigan MAT LCQ mass spectrometer coupled to a Waters 2690 liquid chromatograph (HPLC). All isothiocyanate-derived mercapturic acids were separated on a C18 column using a gradient of acetonitrile in water (both with 0.1% formic acid) and detected using an LCQ ion-trap MS with electrospray ionisation (positive mode). Mercaptu-

Table 2
Chemical and physical data of the synthesised isothiocyanate-derived mercapturic acids

No. [a]	Melting point (°C) [b]	Optical rotation (°)	^{1}H-NMR (δ in ppm, J in Hz)	Elemental analysis calculated/found	Mass	UV (absorbance maximum in nm)
1	144–146	−35.0	(300 MHz) 1.82 (s, 3H, C(O)CH_3), 3.00 (d, 3H, $J = 4.4$ Hz, NCH_3), 3.30 (dd, 1H, $J_{AB} = 13.6$ Hz, $J_{BX} = 9.2$ Hz, CH_B), 3.75 (dd, 1H, $J_{AX} = 5.0$ Hz, CH_A), 4.38 (m, 1H, CH_X), 8.30 (d, 1H, $J = 8.1$ Hz, OCNH), 9.96 (t, 1H, SCNH), 11.87 (s, 1H, OH)	$C_7H_{12}N_2O_3S_2$; C 35.58, H 5.12, N 11.85, S 27.14; C 35.82, H 5.01, N 11.70, S 27.29	FW 236.316; exact 236.0; MS 237.0	267 (1st max); 249 (2nd max)
2	rt	−19.2	(100 MHz) 0.86 (t, 3H, NCCCCH_3), 1.23 (m, 2H, NCCCH_2), 1.45 (m, 2H, NCCH_2), 1.82 (s, 3H, C(O)CH_3), 3.27 (dd, 1H, $J_{AB} = 13.6$ Hz, $J_{BX} = 9.2$ Hz, CH_B), 3.54 (t, 2H, NCH_2), 3.76 (dd, 1H, $J_{AX} = 5.1$ Hz, CH_A), 4.37 (m, 1H, CH_X), 8.32 (d, 1H, $J = 8.0$ Hz, OCNH), 10.00 (t, 1H, SCNH)	$C_{10}H_{18}N_2O_3S_2$; C 43.14, H 6.52, N 10.06, S 23.04; C 43.61, H 6.81, N 9.67, S 21.64	FW 278.397; exact 278.1; MS 279.0	251 (1st max); 270 (2nd max)
3	165–169	−17.6	(100 MHz) 1.13 (d, 6H, $J = 6.5$ Hz, C(CH_3)CH_3), 1.81 (s, 3H, C(O)CH_3), 3.26 (m, 1H, CH_B), 3.75 (dd, 1H, $J_{AB} = 13.6$ Hz, $J_{AX} = 5.0$ Hz, CH_A), 4.41 (m, 2H, CH_X and CH(C)C), 8.31 (d, 1H, $J = 7.9$ Hz, OCNH), 9.88 (d, 1H, $J = 7.3$ Hz, SCNH), 12.83 (s, 1H, OH)	$C_9H_{16}N_2O_3S_2$; C 40.89, H 6.10, N 10.60, S 24.26; C 40.85, H 6.12, N 10.40, S 24.01	FW 264.370; exact 264.1; MS 265.0	253 (1st max); 270 (2nd max)
4	140–144	−14.2	(100 MHz) 0.86 (t, 3H, C(C)CH_3), 1.15 (d, 3H, $J = 6.6$ Hz, C(CH_3)CC), 1.52 (dd, 2H, C(C)CH_2C), 1.87 (s, 3H, C(O)CH_3), 3.34 (dd, 1H, $J_{AB} = 13.3$ Hz, $J_{BX} = 9.1$ Hz, CH_B), 3.80 (dd, 1H, $J_{AX} = 5.1$ Hz, CH_A), 4.41 (m, 2H, CH_X and CH(C)CC), 8.36 (d, 1H, $J = 8.0$ Hz, OCNH), 9.89 (d, 1H, $J = 7.7$ Hz, SCNH), 12.87 (s, 1H, OH)	$C_{10}H_{18}N_2O_3S_2$; C 43.14, H 6.52, N 10.06, S 23.04; C 43.13, H 6.63, N 10.09, S 22.66	FW 278.397; exact 278.1; MS 279.0	253 (1st max); 270 (2nd max)
5	rt	−21.7	(100 MHz) 1.82 (s, 3H, C(O)CH_3), 3.29 (dd, 1H, $J_{AB} = 13.6$ Hz, $J_{BX} = 9.2$ Hz, CH_B), 3.77 (dd, 1H, $J_{AX} = 5.0$ Hz, CH_A), 4.20 (dd, 2H, NCH_2), 4.39 (m, 1H, CH_X), 5.14 (dd, 2H, CH_2=C), 5.84 (m, 1H, C=CH), 8.32 (d, 1H, $J = 8.1$ Hz, OCNH), 10.17 (t, 1H, SCNH), 12.85 (s, 1H, OH)	$C_9H_{14}N_2O_3S_2$; C 41.20, H 5.38, N 10.68, S 24.44; C 40.53, H 5.47, N 10.26, S 23.40	FW 262.354; exact 262.1; MS 263.0	251 (1st max); 269 (2nd max)
6	rt	−21.8	(100 MHz) 1.82 (s, 3H, C(O)CH_3), 2.31 (m, 2H, NCCH_2), 3.28 (dd, 1H, $J_{AB} = 13.6$ Hz, $J_{BX} = 9.2$ Hz, CH_B), 3.61 (m, 2H, NCH_2), 3.75 (dd, 1H, $J_{AX} = 5.1$ Hz, CH_A), 4.38 (m, 1H, CH_X), 5.05 (dd, 2H, CH_2=C), 5.78 (m, 1H, C=CH), 8.31 (d, 1H, $J = 8.0$ Hz, OCNH), 10.03 (t, 1H, SCNH), 12.84 (s, 1H, OH)	$C_{10}H_{16}N_2O_3S_2$; C 43.46, H 5.84, N 10.14, S 23.20; C 42.95, H 5.89, N 9.94, S 22.19	FW 276.381; exact 276.1; MS 277.0	251 (1st max); 269 (2nd max)
7	rt	missing	(100 MHz) 1.66 (m, 2H, NCCH_2), 1.82 (s, 3H, C(O)CH_3), 2.03 (m, 2H, NCCCH_2), 3.28 (dd, 1H, $J_{AB} = 13.6$ Hz, $J_{BX} = 9.2$ Hz, CH_B), 3.54 (m, 2H, NCH_2), 3.76 (dd, 1H, $J_{AX} = 5.0$ Hz, CH_A), 4.37 (m, 1H, CH_X), 5.01 (dd, 2H, CH_2=C), 5.77 (m, 1H, C=CH), 8.32 (d, 1H, $J = 8.1$ Hz, OCNH), 10.02 (t, 1H, SCNH), 12.85 (s, 1H, OH)	$C_{11}H_{18}N_2O_3S_2$; missing	FW 290.41; exact 290.1; MS 291.0	251 (1st max); 269 (2nd max)
8	176–178 (176.50)	−16.7	(400 MHz) 1.83 (s, 3H, CH_3), 3.28 (dd, 1H, $J_{AB} = 13.7$ Hz, $J_{BX} = 10.0$ Hz, CH_B), 3.81 (dd, 1H, $J_{AX} = 4.8$ Hz, CH_A), 4.46 (m, 1H, CH_X), 7.22 (m, 1H, aromatic), 7.40 (m, 2H, aromatic), 7.70 (m, 2H, aromatic), 8.39 (d, 1H, $J = 8.0$ Hz, OCNH), 11.70 (s, 1H, SCNH), 12.90 (s, 1H, OH)	$C_{12}H_{14}N_2O_3S_2$; C 48.30, H 4.73, N 9.39, S 21.49; C 48.36, H 4.72, N 9.29, S 20.77	FW 298.387; exact 298.1; MS 299.0	277
9	133–135 (136.12)	−15.8	(100 MHz) 1.82 (s, 3H, CH_3), 3.32 (dd, 1H, $J_{AB} = 13.7$ Hz, $J_{BX} = 10.0$ Hz, CH_B), 3.79 (dd, 1H, $J_{AX} = 4.8$ Hz, CH_A), 4.41 (m, 1H, CH_X), 4.82 (d, 2H, $J = 5.3$ Hz, NCH_2), 7.29 (m, 5H, aromatic), 8.33 (d, 1H, $J = 8.0$ Hz, OCNH), 10.50 (t, 1H, SCNH), 12.85 (s, 1H, OH)	$C_{13}H_{16}N_2O_3S_2$; C 49.98, H 5.16, N 8.97, S 20.53; C 50.10, H 5.37, N 9.06, S 20.50	FW 312.414; exact 312.1; MS 313.0	251 (1st max); 270 (2nd max)
10	50–58	−14.9	(300 MHz) 1.83 (s, 3H, CH_3), 2.88 (t, 2H, $J = 7.6$ Hz, ØCH_2), 3.30 (dd, 1H, $J_{AB} = 13.6$ Hz, $J_{BX} = 9.0$ Hz, CH_B), 3.75 (m, 1H, $J_{AX} = 5.0$ Hz, CH_A), 3.75 (m, 2H, NCH_2), 4.39 (m, 1H, CH_X), 7.25 (m, 5H, aromatic), 8.28 (d, 1H, $J = 8.0$ Hz, OCNH), 10.17 (t, 1H, SCNH), 12.86 (s, 1H, OH)	$C_{14}H_{18}N_2O_3S_2$; C 51.51, H 5.56, N 8.58, S 19.64; C 50.92, H 5.67, N 8.46, S 18.82	FW 326.441; exact 326.1; MS 327.0	253 (1st max); 270 (2nd max)

Table 2 (*Continued*)

No. [a]	Melting point (°C) [b]	Optical rotation (°)	^{1}H-NMR (δ in ppm, J in Hz)	Elemental analysis calculated/found	Mass	UV (absorbance maximum in nm)
11	rt	−17.6	(100 MHz) 1.57 (m, 4H, $NCCH_2$ and $NCCCH_2$), 1.82 (s, 3H, $C(O)CH_3$), 2.01 (s, 3H, SCH_3), 2.46 (t, 2H, $NCCCCH_2S$), 3.28 (dd, 1H, J_{AB} = 13.6 Hz, J_{BX} = 9.2 Hz, CH_B), 3.56 (t, 2H, NCH_2), 3.76 (dd, 1H, J_{AX} = 5.1 Hz, CH_A), 4.37 (m, 1H, CH_X), 8.31 (d, 1H, J = 8.1 Hz, OCNH), 10.02 (t, 1H, SCNH), 12.8 (s, 1H, OH)	$C_{11}H_{20}N_2O_3S_3$; C 40.72, H 6.21, N 8.63, S 29.65; C 40.04, H 6.07, N 8.34, S 28.34	FW 324.490; exact 324.1; MS 325.0	251 (1st max); 269 (2nd max)
12	60–62	missing	(600 MHz) 1.66 (m, 4H, $NCCH_2CH_2$), 1.82 (s, 3H, CH_3CO), 2.50 (s, 3H, CH_3SO), 2.70 (m, 2H, CCH_2SO), 3.28 (dd, 1H, J_{AB} = 13.2 Hz, J_{BX} = 8.1 Hz, CH_B), 3.58 (wide m, 2H, NCH_2), 3.68 (dd, 1H, J_{AX} = 4.8 Hz, CH_A), 4.30 (m, 1H, CH_X), 8.15 (d, 1H, J = 7.2 Hz, OCNH), 10.34 (wide s, 1H, SCNH), 12.86 (wide s, 1H, OH)	$C_{11}H_{20}N_2O_4S_3$; missing	FW 340.486; exact 340.1; MS 341.0	252 (1st max); 269 (2nd max)

[a] Numbers refer to compounds as depicted in Fig. 2, methods of analysis are described in Section 5.1.
[b] rt = room temperature, indicating a non-crystalline product.

ric acids were detected as their $[M+H]^+$ ion which is the MS data mentioned in the syntheses [41]. Ultraviolet (UV) spectra were collected on a Waters 996 Diode-array detector (DAD) after chromatographic separation as described for MS. Thin-layer chromatography (TLC) was carried out on Merck precoated silica gel 60 F254 plates (0.25 mm) using the eluents indicated. Spots were visualised with UV and using a potassium dichromate spray. Potassium dichromate colors NAC orange, but not its isothiocyanate mercapturic acid, the latter is made visible by heating the TLC plate with hot air.

5.1.1. General procedure for commercially available isothiocyanates

Compounds **1**, **2**, **3**, **4**, **5**, **8**, **9**, and **10** were synthesised by gradually adding the diluted isothiocianate to a solution of *N*-acetyl-L-cysteine and sodium bicarbonate. The reaction was monitored by TLC with ethyl acetate–water–acetic acid (80:10:8, v/v/v) as eluent.

*5.1.1.1. N-Acetyl-S-(N-methylthiocarbamoyl)-L-cysteine (**1**), mercapturic acid derived from methyl isothiocyanate.* Methyl isothiocyanate (16.5 mmol) was dissolved in 20 mL of ethanol and gradually added to a solution of *N*-acetyl-L-cysteine (NAC, 15.0 mmol) and sodium bicarbonate (15.8 mmol) in 20 mL of water. TLC: NAC $R_F = 0.48$, **1** $R_F = 0.43$. All NAC had reacted immediately after methyl isothiocyanate was added. Using a Dowex 50X8-200 cation exchange column, the sodium salt of the mercapturic acid was converted to the free carboxylic acid. The product was crystallised from ethyl acetate yielding 11.0 mmol (73.3%) of **1** as bright white crystals.

*5.1.1.2. N-Acetyl-S-(N-butylthiocarbamoyl)-L-cysteine (**2**), mercapturic acid derived from butyl isothiocyanate.* Butyl isothiocyanate (14.0 mmol) was dissolved in 15 mL of ethanol and gradually added to a solution of NAC (12.8 mmol) and sodium bicarbonate (13.4 mmol) in 15 mL of water. TLC: NAC $R_F = 0.47$, **2** $R_F = 0.57$. All NAC has reacted within a few hours after butyl isothiocyanate was added. The aqueous ethanol was evaporated and the residue was dissolved in 15 mL of brine. The excess of butyl isothiocyanate was extracted with heptane–diethyl ether (10:5, v/v) and the mixture was acidified. The mercapturic acid was extracted with 15 mL of ethyl acetate, concentrated until dryness and traces of ethyl acetate were removed by subsequent washing and evaporating with dichloromethane yielding 9.3 mmol (72.7%) of **2** as a yellow sticky product.

*5.1.1.3. N-Acetyl-S-(N-isopropylthiocarbamoyl)-L-cysteine (**3**), mercapturic acid derived from isopropyl isothiocyanate.* Isopropyl isothiocyanate (27.2 mmol) was dissolved in 20 mL of ethanol and gradually added to a solution of NAC (24.8 mmol) and sodium

bicarbonate (26.0 mmol) in 20 mL of water. TLC: NAC $R_F = 0.45$, **3** $R_F = 0.53$. All NAC has reacted immediately after isopropyl isothiocyanate was added. Ethanol was evaporated, the mixture was acidified with HCl and the product was crystallised from boiling ethyl acetate yielding 12.3 mmol (49.6%) of **3** as bright white crystals.

*5.1.1.4. N-Acetyl-S-(N-1-methylpropylthiocarbamoyl)-L-cysteine (**4**), mercapturic acid derived from 1-methylpropyl isothiocyanate.* 1-Methylpropyl isothiocyanate (105.1 mmol) was dissolved in 50 mL of ethanol and gradually added to a solution of NAC (100.0 mmol) and sodium bicarbonate (110.0 mmol) in 50 mL of water. TLC: NAC $R_F = 0.44$, **4** $R_F = 0.54$. All NAC has reacted within a few hours after 1-methylpropyl isothiocyanate was added. Ethanol was evaporated and the excess of 1-methylpropyl isothiocyanate was extracted with heptane. The mixture was acidified with HCl and the product was crystallised from boiling ethyl acetate, yielding 49.7 mmol (49.7%) of **4** as bright white crystals.

*5.1.1.5. N-Acetyl-S-(N-2-propenylthiocarbamoyl)-L-cysteine (**5**), mercapturic acid derived from 2-propenyl isothiocyanate.* 2-Propenyl isothiocyanate (57.3 mmol) was dissolved in 50 mL of ethanol and gradually added to a solution of NAC (51.2 mmol) and sodium bicarbonate (56.3 mmol) in 50 mL of water. TLC: NAC $R_F = 0.46$, **5** $R_F = 0.57$. All NAC has reacted within a few hours after 2-propenyl isothiocyanate was added. Ethanol was evaporated and the mixture was acidified with HCl which gave a yellow oily precipitate. The mercapturic acid was extracted with ethyl acetate and traces of organic solutes were evaporated using high vacuum, yielding 42.5 mmol (83.0%) of **5** as a yellow sticky product.

*5.1.1.6. N-Acetyl-S-(N-phenylthiocarbamoyl)-L-cysteine (**8**), mercapturic acid derived from phenyl isothiocyanate.* Phenyl isothiocyanate (13.3 mmol) was dissolved in 15 mL of ethanol–water (8:2, v/v) and gradually added to a solution of NAC (12.3 mmol) in 70 mL of tetrahydrofuran (THF). TLC: NAC $R_F = 0.39$, **8** $R_F = 0.46$. Sodium bicarbonate (14.0 mmol) and 30 mL of water were added to the mixture which started the reaction. All NAC had reacted within 24 h. THF was evaporated, the mixture was acidified with HCl and the product was crystallised from boiling ethyl acetate–methanol, yielding 8.9 mmol (72.4%) of **8** as white crystals.

*5.1.1.7. N-Acetyl-S-(N-benzylthiocarbamoyl)-L-cysteine (**9**), mercapturic acid derived from benzyl isothiocyanate.* Benzyl isothiocyanate (14.9 mmol) was gradually added to a solution of NAC (13.6 mmol) and sodium bicarbonate (13.6 mmol) in 33 mL of aqueous 82% (v/v) ethanol. TLC: NAC $R_F = 0.42$, **9** $R_F = 0.55$. All NAC had reacted within a few hours after benzyl isothiocyanate was added. Ethanol was evaporated and the mixture was acidified with HCl. After crystallisation, the product was washed on filter with cold water and dried on filter with gentle suction, yielding 11.5 mmol (84.6%) of **9** as yellowish-white crystals.

*5.1.1.8. N-Acetyl-S-(N-phenylethylthiocarbamoyl)-L-cysteine (**10**), mercapturic acid derived from phenylethyl isothiocyanate.* Phenylethyl isothiocyanate (13.8 mmol) was gradually added to a solution of NAC (12.5 mmol) and sodium bicarbonate (13.1 mmol) in 25 mL of aqueous 70% (v/v) ethanol. TLC: NAC $R_F = 0.41$, **10** $R_F = 0.53$. All NAC had reacted within a few hours after phenylethyl isothiocyanate was added. Ethanol was evaporated, the excess of phenylethyl isothiocyanate was extracted with hexane, the remaining mixture was acidified with HCl and the mercapturic acid was extracted with ethyl acetate. Traces of organic solutes were finally evaporated using high vacuum, yielding 9.1 mmol (72.8%) of **10** as white crystals. ^{13}C-NMR: 195.6 (C=S), 172.2 (COOH), 169.5 (C=O), 139.0 (C-1 Ø), 128.8 (C-3 Ø), 128.6 (C-2 Ø), 126.5 (C-4 Ø), 51.9 (CCOOH), 48.3 (ØCCH$_2$), 35.9 (CH$_2$S), 33.5 (ØCH$_2$), 22.6 (CH$_3$).

5.1.2. General procedure for the complete synthesis

Compounds **6**, **7**, **11**, and **12** were synthesised by first adding the corresponding alkyl bromide to phthalimide potassium salt. The obtained *N*-alkyl-phthalimide was hydrazinolysed, yielding the alkylamine, which was subsequently reacted with thiophosgene to produce the isothiocyanate. The diluted isothiocyanate was gradually added to a solution of *N*-acetyl-L-cysteine and sodium bicarbonate which was monitored by TLC with ethyl acetate–water–acetic acid (80:10:8, v/v/v) as eluent.

*5.1.2.1. N-Acetyl-S-(N-3-butenylthiocarbamoyl)-L-cysteine (**6**), mercapturic acid derived from 3-butenyl isothiocyanate.* Phthalimide potassium salt (224.0 mmol) was slowly added to a solution of 25 mL of 4-bromo-1-butene (246.3 mmol) in 75 mL of dimethylformamide (DMF). All phthalimide potassium salt had reacted after overnight stirring. DMF and the excess of 4-bromo-1-butene were evaporated. With heat, the product and by-product (potassium bromide) were dissolved in 400 mL of ethyl acetate–water (1:1, v/v). The ethyl acetate layer was evaporated until dryness and the product was crystallised from boiling diisopropyl ether, yielding 183.0 mmol of *N*-(3-butenyl)-phthalimide as white, needle-shaped crystals. ^{1}H-NMR (in CDCl$_3$, 100 MHz, δ in ppm relative to TMS): 7.78 (m, 4H), 5.82 (m, 1H), 5.07 (dd, 2H), 3.78 (t, 2H), 2.46 (m, 2H). Hydrazine monohydrate (233 mmol) was added to a solution of 179.0 mmol of *N*-(3-butenyl)-phthalimide in 200 mL of

ethanol under nitrogen. After 2 h of reflux at 75 °C, HCl was added and the mixture was further heated for 1 h at 100 °C. The mixture was left to cool overnight, filtered and the residue was washed with water. The filtrate containing 3-butenylamine was extracted twice with an equal volume of diethyl ether, distillated, and dissolved in 100 mL of chloroform. Slowly 1.1 molar equivalent of thiophosgene was added to the mixture with stirring, followed by 3.0 molar equivalents of sodium hydroxide. The reaction was monitored by TLC with chloroform as eluent, a spot was formed at $R_F = 0.60$. The R_F of butyl isothiocyanate was 0.61. The mixture was left overnight at room temperature. The chloroform and thiophosgene were evaporated and 3-butenyl isothiocyanate was obtained by distillation, b.p. 60 °C 12 mmHg (60 °C 12 mmHg [28]; 77.5 °C 28 mmHg [29]), yielding 28.9 mmol. ^{1}H-NMR (in $CDCl_3$, 100 MHz): 5.80 (m, 1H), 5.18 (dd, 2H), 3.57 (t, 2H), 2.45 (m, 2H). 3-Butenyl isothiocyanate (28.2 mmol) was dissolved in 20 mL of ethanol and gradually added to a solution of NAC (27.8 mmol) and sodium bicarbonate (29.2 mmol) in 20 mL of water. TLC: NAC $R_F = 0.49$, **6** $R_F = 0.59$. All NAC had reacted within 1 h after 3-butenyl isothiocyanate was added. Ethanol was evaporated and the excess of 3-butenyl isothiocyanate was extracted with heptane. The mixture was acidified with HCl, the mercapturic acid was extracted with ethyl acetate and ethyl acetate was evaporated yielding 22.9 mmol (10.9%) of **6** as a white sticky product.

5.1.2.2. N-Acetyl-S-(N-4-pentenylthiocarbamoyl)-L-cysteine (7), mercapturic acid derived from 4-pentenyl isothiocyanate. 5-Bromo-1-pentene was used. *N*-4-pentenyl-phthalimide was dissolved in boiling ethanol. ^{1}H-NMR (in $CDCl_3$, 100 MHz, δ in ppm relative to TMS): 7.78 (m, 4H), 5.80 (m, 1H), 4.98 (dd, 2H), 3.70 (t, 2H), 2.00 (m, 4H). The yield of 4-pentenyl isothiocyanate was 24.4 mmol. ^{1}H-NMR (in $CDCl_3$, 100 MHz, δ in ppm): 5.78 (m, 1H), 5.08 (dd, 2H), 3.53 (t, 2H), 2.21 (m, 2H), 1.80 (m, 2H). TLC: NAC $R_F = 0.48$, **7** $R_F = 0.64$. The yield of **7** was 14.6 mmol (12.0%) as an brownish-orange sticky product.

5.1.2.3. N-Acetyl-S-(N-4-methylthiobutylthiocarbamoyl)-L-cysteine (11), mercapturic acid derived from 4-methylthiobutyl isothiocyanate. 1,4-Dibromobutane was used (Scheme 1). *N*-(4-bromobutyl)-phthalimide (103.0 mmol) was yielded as white, needle-shaped crystals. ^{1}H-NMR (in $CDCl_3$, 100 MHz, δ in ppm): 7.79 (m, 4H), 3.73 (m, 2H), 3.45 (m, 2H), 1.88 (m, 4H). *N*-(4-bromobutyl)-phthalimide (14.1 mmol) was slowly added to a solution of sodium methylmercaptide (14.8 mmol) in 10 mL of ice-cooled DMF. The reaction was complete within a few hours. The mixture was pored into 100 mL of ice cold water while stirring, resulting in crystallisation of the product. Crystallisation from diisopropyl ether yielded 9.9 mmol of *N*-(4-methylthiobutyl)-phthalimide as white, needle-shaped crystals. ^{1}H-NMR (in $CDCl_3$, 300 MHz, δ in ppm relative to TMS): 7.83 (m, 2H), 7.70 (m, 2H), 3.71 (t, 2H), 2.54 (t, 2H), 2.09 (s, 3H), 1.80 (m, 2H), 1.66 (m, 2H). Hydrazinolysis yielded 4-methylthiobutylamine which was distilled at reduced pressure, b.p. 72.9 °C (12.0 mmHg), yielding 21.1 mmol. ^{1}H-NMR (in $CDCl_3$, 100 MHz, δ in ppm): 2.72 (t, 2H), 2.52 (t, 2H), 2.11 (s, 3H), 1.59 (m, 4H), 1.16 (s, 2H). After reaction with thiophosgene, 4-methylthiobutyl isothiocyanate was obtained by distillation at 14.5 mmHg, b.p. 135 °C (136 °C at 12 mmHg [34]; 130–140 °C at 9 mmHg [33]), yielding 12.5 mmol of a yellow oil. ^{1}H-NMR (in $CDCl_3$, 100 MHz, δ in ppm): 3.56 (t, 2H), 2.54 (t, 2H), 2.11 (s, 3H), 1.78 (m, 4H). A singlet peak at 2.58 ppm belonging to a methyl adjacent to a sulfinyl group and a singlet at 2.91 belonging to a methyl adjacent to a sulfonyl group were also observed. 4-Methylthiobutyl isothiocyanate (10.5 mmol) was dissolved in 10 mL of ethanol and gradually added to a solution of NAC (9.5 mmol) and sodium bicarbonate (10.0 mmol) in 20 mL of water. TLC: NAC $R_F = 0.45$, **11** $R_F = 0.59$. After acidification and extraction, 7.1 mmol (13.0%) of **11** was yielded as a yellow sticky product.

5.1.2.4. N-Acetyl-S-(N-4-methylsulfinylbutylthiocarbamoyl)-L-cysteine (12), mercapturic acid derived from 4-methylsulfinylbutyl isothiocyanate. To a solution of 4-methylthiobutyl isothiocyanate (10.3 mmol, prepared as described above) in 15 mL of DCM, a solution of *m*-chloroperbenzoic acid (MCPBA, 12.6 mmol) in 15 mL of DCM was gradually added (Scheme 1). The reaction was monitored by TLC with ethyl acetate as eluent (4-methylthiobutyl isothiocyanate $R_F = 0.64$, 4-methylsulfinylbutyl isothiocyanate $R_F = 0.06$). All isothiocyanate was oxidised after 30 min. The DCM layer was separated and evaporated, yielding 10.4 mmol (100%) of 4-methylsulfinylbutyl isothiocyanate as a yellow oil. ^{1}H-NMR (in $CDCl_3$, 100 MHz, δ in ppm relative to TMS): 1.92 (m, 4H), 2.61 (s, 3H), 2.74 (m, 2H), 3.61 (t, 2H). 4-Methylsulfinylbutyl isothiocyanate reacted completely after overnight stirring with NAC. TLC: NAC $R_F = 0.36$, **12** $R_F = 0.09$. The yield of mercapturic acid **12** was 6.3 mmol (69.5%) as white crystals.

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