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Characterization of Products from the Reaction of Glucosinolate-Derived Isothiocyanates with Cysteine and Lysine Derivatives Formed in Either Model Systems or Broccoli Sprouts

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ABSTRACT: Glucosinolates, present in *Brassica* vegetables, are thought to contribute to human health prevention because of their enzymatically induced breakdown products, primarily isothiocyanates (ITCs). ITCs are reactive substances that readily react with nucleophilic (food) compounds. The reactivity of allyl-ITC and 4-(methylsulfinyl)butyl-ITC (sulforaphane) toward thiol and amino groups of cysteine and lysine derivatives was studied in buffered model systems as well as broccoli sprouts. The thiol group is the preferred reaction site, and it was demonstrated that even endogenously released sulforaphane is able to react very fast with cysteine in broccoli sprouts. Amino groups reacted slower and only under basic conditions. However, great differences in the reactivity between the different amino compounds were revealed. The aliphatic allylamine reacted very fast with allyl-ITC, forming *N*,*N*′-diallylthiourea, a compound identified as a main thermal degradation product of allyl-ITC.

KEYWORDS: *Amino acid derivatives, reactivity, dithiocarbamates, thiourea, thermal stability*

INTRODUCTION

Consumption of *Brassica* vegetables, primarily broccoli (*Brassica oleracea* var. *italica*), is thought to contribute to a healthy human diet by reducing the risk of developing cancer.[1] Glucosinolates, sulfur-containing glycosides present in these vegetables, are hydrolyzed enzymatically after cell disruption and form isothiocyanates (ITCs) next to several other breakdown products.[2] ITCs, such as allyl-ITC (compound **1**; Figure 1) and especially sulforaphane (**2**) [4-(methylsulfinyl)butyl-ITC; SFN], occurring in broccoli, are able to induce several phase II detoxification enzymes and apoptosis, inhibit the cell cycle, and also show other biological activities, such as anti-inflammatory and antibacterial properties.[3] Because of their structure, they are strong electrophilic substances that are able to react with nucleophilic food compounds, such as thiol, hydroxyl, and amino groups, of, e.g., proteins or amino acids, to form dithiocarbamates, thiocarbamates, or thiourea derivatives.[4] With regard to food consumption, most *Brassica* vegetables are consumed in processed form. However, processing influences the glucosinolate and, hence, the ITC contents of the vegetables because of an enzymatic breakdown of the glucosinolates, leaching of the glucosinolates into the cooking water, or thermally induced degradation of the glucosinolates as well as the ITCs. Further, also, electrophilic attack of the ITCs on nucleophilic food compounds might take place as well.[5,6]

In the past, several studies dealt with reactions of proteins and ITCs. It was shown that especially benzyl- and phenyl-ITC reacted with the amino and thiol side chains of egg white proteins and led to a shift of the isoelectric points to a lower pH value and a decrease in the protein solubility.[7,8] The disulfide bond of oxidized glutathione and cystine in proteins was cleaved by compound **1**, but the latter also reacted with the free amino groups of lysine and arginine protein side chains.[9−11] Because of modifications of proteins with ITCs, their proteolytic digestibility, their bioavailable lysine content, and their bioutilization of the nitrogen decreased.[10,12,13] The reactivity of amino and thiol groups is favored under basic conditions, but disulfide bonds are also able to react in slightly acidic medium (pH 6),[14,15] pH values relevant for plant foods. Literature data regarding the course of the reaction of ITCs with free amino acids are rare or comparatively old, in most cases relying only on spectrophotometric measurements. Cejpek et al. studied the reactivity of compound **1** with alanine, glycine, and di- and tripeptides (containing alanine and/or glycine).[16] They observed the formation of thiourea derivatives, which was favored at higher pH values.[16] Thiol groups are more reactive toward ITCs, but this reaction can be reversible.[17] Podradský et al. studied the reactions of ITCs with cysteine or other thiols and demonstrated that the reaction with the thiol group took place even at moderate acidic pH values.[15] With an increasing pH value, the ITCs also reacted with the amino group of cysteine.[15] When the ITC breakdown pathway and fate under physiological or processing conditions were taken into account, the present study was conducted to evaluate the reactivity of ITCs with different nucleophiles in buffered aqueous/methanolic model systems and

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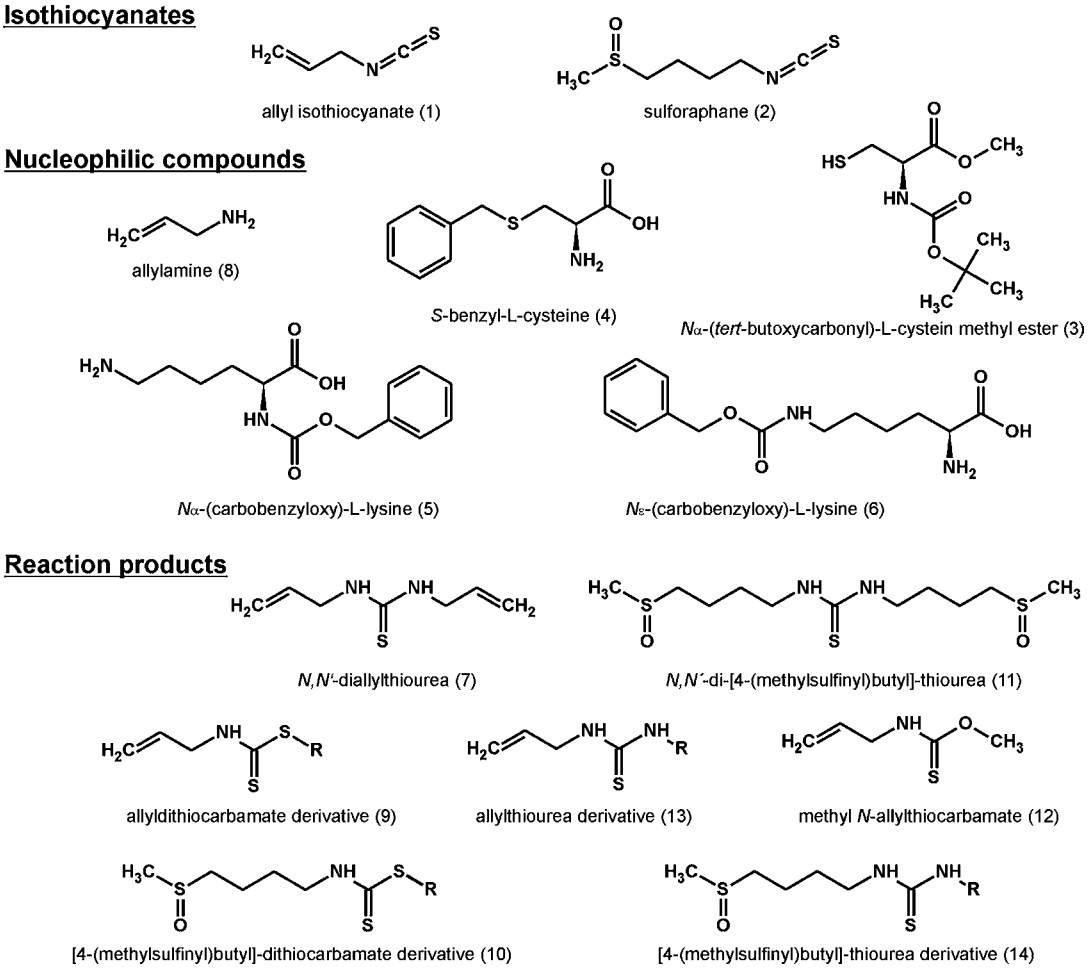

Figure 1. Structures of the ITCs and the nucleophiles used in the study.

Brassica vegetables. Protected derivatives of cysteine and lysine were used to investigate the reactivity of the ITCs toward the α- and ε-amino groups as well as the thiol group of cysteine, separately. The influence of the pH value on these reactions was studied as well. To our knowledge, no data exist about these reactions when ITCs have been endogenously released in *Brassica*-based foods. Therefore, the reactivity between free amino acids (e.g., cysteine) and the enzymatically formed ITCs was tested *in situ* in broccoli sprouts. Additionally, the reactivity of ITCs was evaluated under thermal (food) processing conditions.

MATERIALS AND METHODS

Chemicals. Allyl-ITC (**1**) (≥99%), benzonitrile (99%), *S*-benzyl-L-cysteine (**4**) (97%), N_α-(*tert*-butoxycarbonyl)-L-cysteine methyl ester (**3**) (97%), N_α-(carbobenzyloxy)-L-lysine (**5**) (98%), N_ε-(carbobenzyloxy)-L-lysine (**6**) (≥99%), DEAE-Sephadex A-25, 1,3-diallyl-2-thiourea [*N*,*N*′-diallylthiourea (**7**)], and myrosinase (thioglucosidase, extracted from *Sinapis alba*) were purchased from Sigma-Aldrich (Steinheim, Germany). Arylsulfatase, isolated from *Helix pomatia*, was purchased from Roche-Diagnostics GmbH (Mannheim, Germany). Citric acid monohydrate (≥99%), formic acid (≥98%), methanol, dipotassium hydrogen phosphate (≥99%), potassium dihydrogen phosphate (≥99%), disodium hydrogen phosphate dihydrate (≥99%), and sodium chloride (≥99%) were purchased from Merck (Darmstadt, Germany). Acetic acid (100%), acetonitrile, imidazole (≥99%), and orthophosphoric acid (85%) were purchased from Roth (Karlsruhe, Germany). 1-Isothiocyanato-4-(methylsulfinyl)butane [D,L-sulforaphane, (**2**)] was purchased from Enzo Life Sciences GmbH (Lörrach, Germany). 4-(Methylsulfinyl)butyl glucosinolate potassium salt (glucoraphanin) was purchased from PhytoLab GmbH and Co. KG (Vestenbergsgreuth, Germany). Methylene chloride (≥99.8%) was purchased from VWR (Darmstadt, Germany). Allylamine (**8**) (≥98%) and L-cysteine (99%) were purchased from Acros Organics (Thermo Fisher Scientific, Geel, Belgium). All solvents were of high-performance liquid chromatography (HPLC) grade, and water was of Milli-Q quality.

Plant Material. Broccoli (*B. oleracea* var. *italica*) sprouts cv. Calabrese were cultivated by the Leibniz-Institute of Vegetable and Ornamental Crops Grossbeeren/Erfurt e.V. (Grossbeeren, Germany). Seeds were sown on water-soaked fleece in aluminum trays filled with perlite and water. They were watered daily until germination using a water sprayer, given as needed for optimal sprout growth. Trays were kept in a greenhouse with a temperature regime of 24/20 °C (day/night) and a relative humidity of about 75%. Supplemental photosynthetic active radiation of 150 μmol m^{-2} s^{-1} was provided to give a 16 h photoperiod. Sprouts were harvested after 12 days, lyophilized, and ground to a fine powder (<0.5 mm).

Model Experiments. Solutions with equimolar concentrations of the reactants, i.e., 1 mmol L^{-1} of an amino or thiol compound and 1 mmol L^{-1} of the ITCs (compound **1** or **2**) in buffered aqueous solutions containing 25% methanol, water to make up to 100%, and 50% of a buffer solution at various pH values [pH 5, 10.308 g of citric acid monohydrate and 18.179 g of $Na_2PO_4{\cdot}2H_2O$ L^{-1} of water; pH 7.3, phosphate-buffered saline (PBS) buffer: 8.765 g of NaCl, 0.714 g of K_2HPO_4, and 0.123 g of KH_2PO_4 L^{-1} of water; and pH 8, 0.336 g of KH_2PO_4 and 11.431 g of $Na_2HPO_4{\cdot}2H_2O$ L^{-1} of water] were prepared in vials and stored at room temperature (22 °C). First measurement of the reaction started after 2 min. Sampling times were generally 6, 12, and 18 h. If reactions were very slow (or fast), additional sampling times

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were added, and if reactions were already negligible at a certain pH, these substances were not investigated any further. All experiments were carried out in triplicate.

Thermal Treatment of ITCs. A total of 1 mmol L^{-1} solutions of the corresponding ITCs (compound **1** or **2**) in phosphate-buffered aqueous solutions at various pH values (pH 5.3, 8.828 g of KH_2PO_4 and 0.320 g of $Na_2HPO_4 \cdot 2H_2O$ L^{-1} of water; and pH 7.3, see above) were prepared in vials and sealed. Treatment at 100 °C was performed immediately by placing the vials in an oven (Heraeus Instruments, Type T6). The come-up time of the samples was 5 min. Sampling was performed in triplicate after 30 (only compound **1**), 60, and 120 min (only compound **2**), and the samples were analyzed by a HPLC−diode array detector (DAD), immediately after cooling to room temperature.

HPLC−DAD Analysis of ITC Reactions. Compounds in the reaction mixtures of the model systems were separated by performing HPLC [Shimadzu HPLC pump LC-9A, Shimadzu degasser DGU-4A, Shimadzu gradient mixer FCV-10 AL, JASCO automatic sampler AS-950, and Shimadzu photodiode array ultraviolet−visible (UV−vis) detector SPD-M6A] on a ProntoSIL Spheribond ODS2 column (3 μm, 125 × 4 mm). The volume of 20 μL of the mixture was injected, and analytes were separated with a linear gradient of 1−35% acetonitrile in 0.05% orthophosphoric acid from 2 to 10 min, followed by 70% acetonitrile in 0.05% orthophosphoric acid until 34 min, and holding at this percentage for 6 min. UV detection was conducted at a flow rate of 0.5 mL min^{-1}, a column temperature of 30 °C, and the wavelengths of 214 nm (quantification of amino acids), 240 nm (quantification of ITCs), and 254 nm (quantification of thioureas and dithiocarbamates).

For the separation of the lysine derivatives, modified HPLC parameters were used; the column temperature was set to 35 °C, and flow rate was 0.7 mL min^{-1}. The gradient was 1−10% acetonitrile in 0.05% orthophosphoric acid until 5 min, increasing to 20% acetonitrile until 10 min, and remained in isocratic separation until 15 min. The gradient then was increased to 35% acetonitrile until 22 min and continued to increase to 70% acetonitrile until 46 min. Finally, 6 min of isocratic elution followed.

ITCs and nucleophilic compounds were identified by comparison of their retention times and their characteristic UV spectra. Dithiocarbamates and thiourea derivatives have been identified by liquid chromatography−mass spectrometry (LC−MS). Calibration curves were used for quantification of the educts (compounds **1**−**6**) by a HPLC−DAD. Therefore, compounds **1**−**6** were prepared in aqueous solutions containing 50% of the corresponding buffer solutions, 25% methanol, and water to make up to 100% (c = 0.1−1.25 mmol L^{-1}).

[4-(Methylsulfinyl)butyl]dithiocarbamate (**10**), formed in the broccoli sprouts, was indirectly quantified by incubating defined concentrations of compound **2** (0.2−1 mmol L^{-1}) in a 2 mmol L^{-1} aqueous solution of N_α-(*tert*-butoxycarbonyl)-L-cysteine methyl ester (**3**) at pH 5 and measuring compound **10** after at least 2 h with a HPLC−DAD. The peak areas of the formed dithiocarbamate correlated well with the concentrations of sulforaphane (R^2 > 0.9999). The dithiocarbamate was at least stable over a period of 15 h, and the yield was >98%.

Thermally formed compound **7** and *N*,*N*′-di[4-(methylsulfinyl)-butyl]thiourea (**11**) were quantified as compound **7** with a HPLC−DAD using an external calibration curve. Solutions of compound **7** were prepared in buffer solution (c = 0.005−1.0 mmol L^{-1}).

HPLC−Positive-Ion Electrospray Ionization [ESI(+)]−MS Analysis. Identification of the reaction products was achieved by LC−MS measurements in the total ion current (TIC) mode by the parent ion obtained (M + 1) and the fragmentation pattern. HPLC separation (Thermo Fischer Scientific pump Accela, CTC PAL autosampler) was carried out by injecting mixtures on a ProntoSIL Spheribond ODS2 column (3 μm, 125 × 4 mm) using the gradient described above for the cysteine derivative reaction mixtures but with 0.05% acetic acid instead of the phosphoric acid and a flow rate of 0.5 mL min^{-1}. Ionization (TSQ vantage system with ion max source, H-ESI II probe) in the ESI positive mode followed, using a spray voltage of 3 kV, a vaporizer temperature of 450 °C, a sheath gas pressure of 60 psi, and a capillary temperature of 270 °C. TIC spectra were obtained by setting the scan at m/z 50−1000. Identification of the thermal breakdown products of the ITCs was also performed in the negative ionization mode by identifying the parent ion (M − 1). Software Thermo Excalibur 2.1.0.1139 was used for the identification of the substances.

Reaction of N_α-(*tert*-Butoxycarbonyl)-L-cysteine Methyl Ester with Endogenous Sulforaphane in Broccoli Sprouts. Freeze-dried and ground broccoli sprouts powder cv. Calabrese, containing 49.5 ± 1.5 μmol of glucosinolates g^{-1} of dry weight (DW) [54 ± 0.5% of which was 4-(methylsulfinyl)butyl glucosinolate (glucoraphanin), analyzed according to Hanschen et al.[6]], was incubated in a 5 mmol L^{-1} solution of N_α-(*tert*-butoxycarbonyl)-L-cysteine methyl ester (**3**) containing 0.1 mg mL^{-1} myrosinase for 1 h. The broccoli sprout concentration was 0.1 g mL^{-1} (glucosinolate concentration of 5 mmol L^{-1}). Afterward, the samples were centrifuged at 18620*g* for 5 min, and supernatant solution was injected in HPLC for the analysis of the formed dithiocarbamates.

Analysis of Sulforaphane and the Corresponding Nitrile by a Gas Chromatography−Flame Ionization Detector (GC−FID). Broccoli sprouts were dissolved in water and hydrolyzed with or without the addition of compound **3**, as described above. For the determination of compound **2** being released enzymatically, GC−FID was used,[6] because other substances of the broccoli sprout matrix disturbed the determination of compound **2** via HPLC. A total of 150 mg of freeze-dried broccoli sprouts powder was hydrolyzed as described above with or without the addition of Cys−ME, and enzymatic hydrolysis products were extracted with methylene chloride, as described previously.[6] The volume of 100 μL of a solution of benzonitrile (1 mmol L^{-1}) in methylene chloride was added as an internal standard during the first extraction step.

A GC−FID was performed using Hewlett-Packard 5890 A GC equipped with a BP 5 column (30 m × 0.25 mm × 0.25 μm film), as reported before.[6] The analyte content was calculated with benzonitrile as an internal standard and the response factor of compound **2** relative to benzonitrile. For the corresponding nitrile [5-(methylsulfinyl)-pentylnitrile] resulting from glucoraphanin, the response factor (RF) was calculated according to the effective carbon number concept.[18]

Analysis of Glucosinolates in Broccoli Sprouts by HPLC−DAD. A modified method by Hanschen et al. was used for the HPLC analysis of non-hydrolyzed glucoraphanin and glucosinolate contents of untreated broccoli sprouts.[6,19] After the hydrolysis, samples were treated in a methanol/water mixture (7:3, v/v; t = 75 °C) for 10 min to inactivate myrosinase. After centrifugation at 18620*g* for 5 min, samples were re-extracted twice with a methanol/water mixture (7:3, v/v; t = 75 °C). The supernatants were pooled and applied on a 150 μL DEAE-Sephadex A-25 ion-exchanger column, prepared and washed according to the method by Mewis et al.[20] Next, the volume of 100 μL of a purified arylsulfatase solution was applied to the column and left for 12 h before the desulfoglucosinolates formed were eluted with 1.5 mL of water. Desulfated extracts were separated and analyzed by a HPLC−DAD, as described previously. Briefly, separation was carried out on a ProntoSIL Spheribond ODS2 column (3 μm, 125 × 4 mm) with a gradient of 0−20% acetonitrile in water from 2 to 34 min, followed by 20% acetonitrile in water until 40 min, and then 70% acetonitrile for 10 min until 50 min. The flow rate was 0.7 mL min^{-1}, and glucosinolates were detected at a wavelength of 229 nm.[19]

Statistical Analysis. For the analysis of variance, Tukey's honest significant difference (HSD) test was used to calculate differences at a significance level of $p \leq 0.05$ using Origin Pro 8.0 (OriginLab Corporation, Northampton, MA). Data in figures were reported as the mean ± standard deviation (SD) for 3-fold analysis.

Calculation of Rate Constants. Rate constants (k) for the reaction between ITCs and the nucleophilic compounds were determined with a second-order reaction model.[17] Half-times ($t_{1/2}$) for the reactions were obtained from the ITC reaction curves. For equimolar starting concentrations $[A]_0$, rate constants can be estimated with the rearranged eq 1.

$$t_{1/2} = 1/(k[A]_0) \quad (1)$$

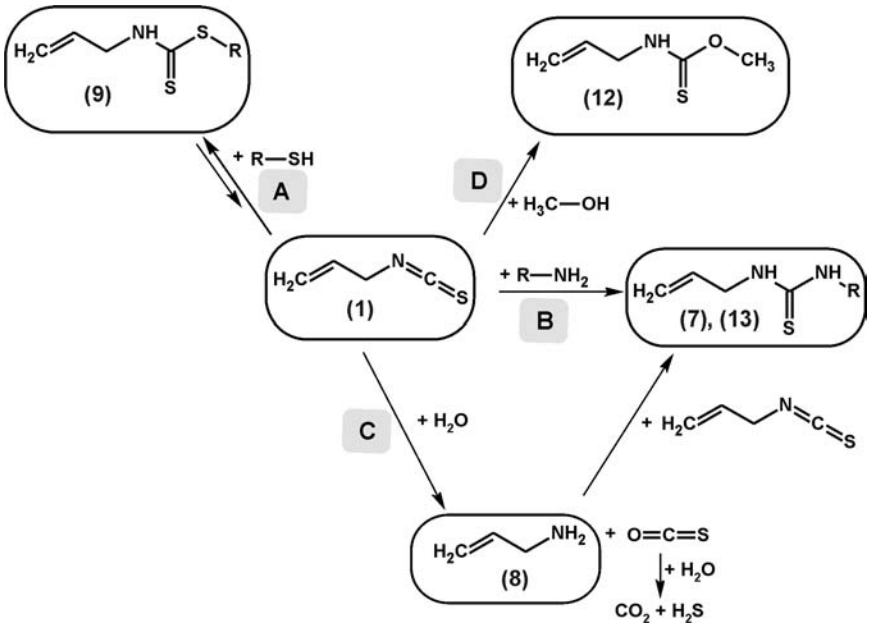

Figure 2. Pathway of the reaction of ITCs with nucleophilic compounds under aqueous conditions shown exemplarily for compound **1**. R = residue of compound **3**, **4**, **5**, **6**, or **8**. For compound numbers, please refer to Figure 1.

RESULTS AND DISCUSSION

To evaluate which nucleophilic group is dominantly reacting with ITCs, several protected and pure cysteine and lysine derivatives have been applied (Figure 1). With regard to the ITCs as the reaction partner, allyl-ITC (**1**), the breakdown product of sinigrin, one of the most widespread occurring glucosinolates, was compared to sulforaphane (**2**), one of the most promising natural cancer preventive agents. It was observed that especially compound **1** was degraded significantly in aqueous medium at room temperature, and the stability of compounds **1** and **2** in the aqueous medium was determined at the selected pH values [remaining content of compound **1** after 18 h: pH 5, 63 ± 13%; pH 7.3, 69 ± 7%; and pH 8, 54 ± 16%]. The degradation curve of the ITCs (R^2 = 0.993−0.999), because of its instability at the selected conditions, was used to correct the concentrations in the reaction mixtures of compound **1** and the nucleophilic compounds. In contrast, compound **2** was comparatively stable in the aqueous medium at room temperature (remaining content after 18 h: pH 5, 98 ± 1%; pH 7.3, 100%; and pH 8, 97 ± 1%).

Reactions of ITCs toward Thiol Groups in Model Systems. The course of the reaction between the ITCs and the thiol group of N_α-(*tert*-butoxycarbonyl)-L-cysteine methyl ester (**3**) is displayed in Figure 2 (pathway A). The corresponding dithiocarbamates were identified as the main reaction products. The HPLC chromatogram and spectral data of this reaction are illustrated in Figure 3A. The reaction with the thiol group was comparatively faster with an increasing pH value (Figure 4 and Table 1). At pH 5, the reaction was comparatively slower compared to pH 7.3 and 8. After 2 min of the reaction at pH 5, only 1.4% of compound **2** reacted compared to 57% at pH 7.3 and 74% at pH 8 (Figure 4A-2). To test whether the reactivity of compound **3** was comparable to pure cysteine, compound **2** was treated similarly with cysteine at pH 7.3. The reaction toward cysteine was slightly but significantly slower, with 60% of compound **2** remaining after 2 min and 22% after 6 h compared to 43 and 11%, respectively, when reacting with the cysteine derivative (for observed rate constants, see Table 1). Data concerning the reaction of ITCs and the thiol group of cysteine are very rare. Podhradský et al. determined the rate constants for the reaction of cysteine and the cysteine methyl ester with several ITCs spectrophotometrically.[15] With the rate constant given for the second-order reaction between phenyl-ITC and cysteine at pH 5 (k = 9.59 M^{-1} s^{-1}),[15] after 104 s, 50% of the ITC should have reacted with cysteine, assuming that both reactants were equimolar. This is more or less comparable to the results obtained in the present study with unprotected cysteine and compound **2** at pH 7.3 (k = 6.04 ± 0.41 M^{-1} s^{-1}). They were similar to the literature data but were achieved at higher pH values. Therefore, the reaction seems to be slower in the present study. It is hypothesized that compound **2**, as a result of its alkylic structure, reacts more slowly compared to phenyl-ITC, because the aromatic ring activates the ITCs, as a result of its negative inductive effect. ITC are more reactive with a decreasing electron density of the carbon atom of the functional group NCS.[21] In the study by Podhradský et al., the cysteine methyl ester reacted 7.5 times slower with phenyl-ITC compared to pure cysteine at pH 4.5. The authors state that the reactivity of the thiol groups is proportional to their pK_a values.[15] Accordingly, in the present study, compound **3** (pK_{SH} = 9.29)[22] reacted slightly faster compared to pure unprotected cysteine (pK_{SH} = 8.29).[15] Therefore, the butoxycarbonyl group somehow increases the basicity of the thiol group and enhances the reactivity of the thiol group.

It was observed that compound **1** was more reactive toward the thiol group compared to compound **2** (Figure 4A-2 and Table 1), with only 7% of compound **1** remaining after 2 min of the reaction at pH 8 compared to 26% of compound **2**. However, it was less stable in water, and the formation of the corresponding *N*,*N*′-diallylthiourea (**7**) was observed. Under aqueous conditions, compound **1** is hydrolyzed by a base-catalyzed mechanism, forming allylamine (**8**), which can react with a second molecule of compound **1** to form compound **7** (pathway C in Figure 2).[17,23] Its higher reactivity is probably due to its smaller molecule size and could also be caused by a smaller positive inductive effect of the alkyl chain compared to the chemical structure of compound **2**.

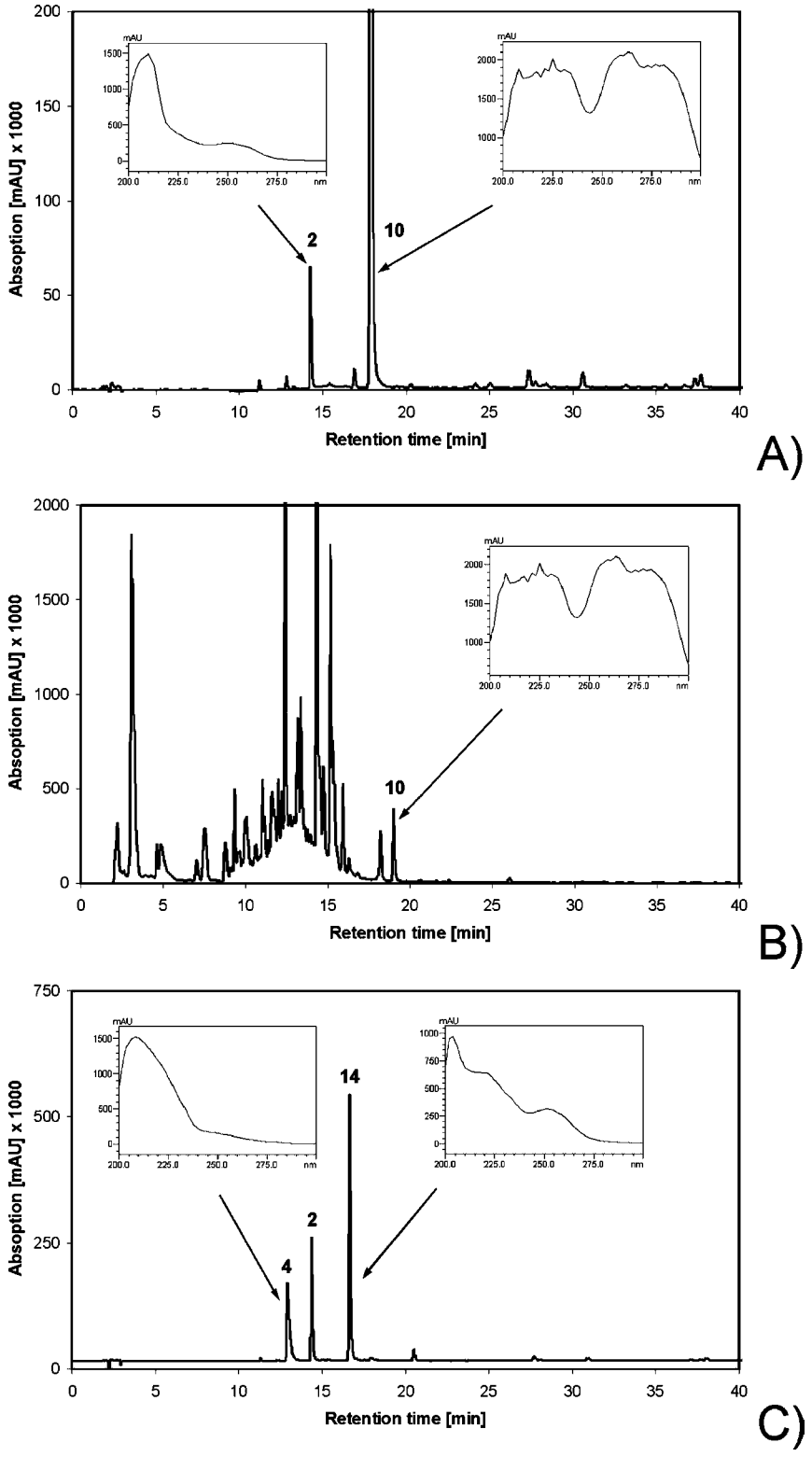

Figure 3. (A–C) Chromatograms and DAD spectra of substances in reaction mixtures of compound **2** and nucleophilic compounds at 254 nm. (A) Compounds **2** and **3** after 2 min at pH 8.0 in the model system. (B): Reaction between endogenous compound **2** and compound **3** in broccoli sprouts. (C) Compounds **2** and **4** after 12 h at pH 8.0 in the model system. For compound numbers, please refer to Figure 1.

The formation of corresponding dithiocarbamates correlated with the decrease of the ITCs (Figure 4B). At pH 5, the formation of the dithiocarbamates was not finished after 18 h. Peak areas of the dithiocarbamates were still lower than the highest obtained at the basic pH values. On the other hand, at pH 7.3 and 8, the concentrations of the dithiocarbamates decreased after 2 min and the allyldithiocarbamate derivative (**9**) formed significantly decreased more compared to the [4-(methylsulfinyl)butyl]dithiocarbamate derivative (**10**). The decrement is probably due to a base-catalyzed demethylation

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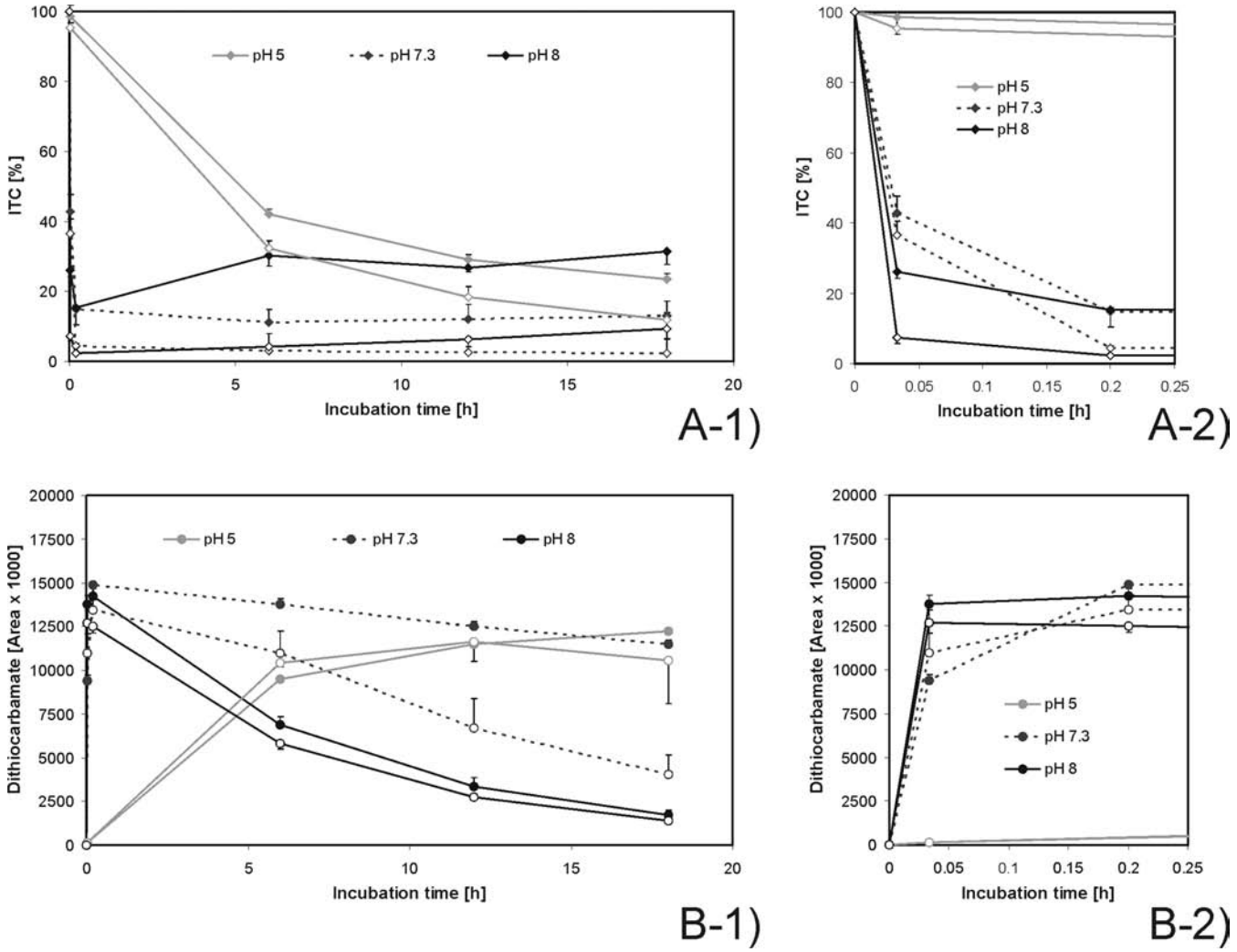

Figure 4. (A and B) Course of the reaction of ITCs with compound **3** at different pH values and corresponding formation of compounds **9** and **10** in model systems. (A) ITC concentrations during the reaction time: (A-1) 0−18 h and (A-2) 0−0.2 h. (B) Peak area of the dithiocarbamates (**9** and **10**) formed: (B-1) 0−18 h and (B-2) 0−0.2 h. Hollow symbols represent compound **1**, and filled symbols represent compound **2** or their corresponding adducts (compounds **9** and **10**). For compound numbers, please refer to Figure 1.

Table 1. Rate Constants of the Reactions between the ITCs and Nucleophilic Compounds[a]

		k (M^{-1} s^{-1})	
nucleophilic compound	pH	**1**	**2**
3	5	0.077 ± 0.005	0.058 ± 0.002
3	7.3	12.16 ± 1.38	10.23 ± 1.31
3	8	32.34 ± 2.4	16.18 ± 0.81
cysteine	7.3		6.04 ± 0.41
4	8	$5.42 \times 10^{-3} \pm 2.54 \times 10^{-3}$	$5.61 \times 10^{-3} \pm 0.19 \times 10^{-3}$
6	8	$1.49 \times 10^{-3} \pm 0.17 \times 10^{-3}$	$1.53 \times 10^{-3} \pm 0.14 \times 10^{-3}$
8	7.3	0.044 ± 0.007	

[a]For compound numbers, please refer to Figure 1.

of the methyl ester group, because the demethylated dithiocarbamates were found in samples treated at pH 8. It could also be observed at pH 8 that the concentration of compound **2** was significantly higher after 6 h compared to an early stage (12 min), indicating the instability of compound **10** (Figure 4A-1). Therefore, the decomposition of compound **10** to the original substrates (**2** and **3**) seems to take place (Figure 4A-1). However, compounds **1** and **3** did not recur from compound **9** or **10**, respectively. Probably, after their potential rerelease, these substances are further degraded because of their instability at pH 8. The instability of the dithiocarbamates and re-release of ITCs has been reported before.[17] Additionally, exchange reactions from thiol to thiol or even to amino groups can also take place.[24−26] Some studies described the release of ITCs from their corresponding glutathione and cysteine conjugates, when incubated under physiological conditions,[27,28] as observed in the present work at pH 8. Further studies hypothesized the ITCs release to be one of the main reasons for the anticarcinogenic

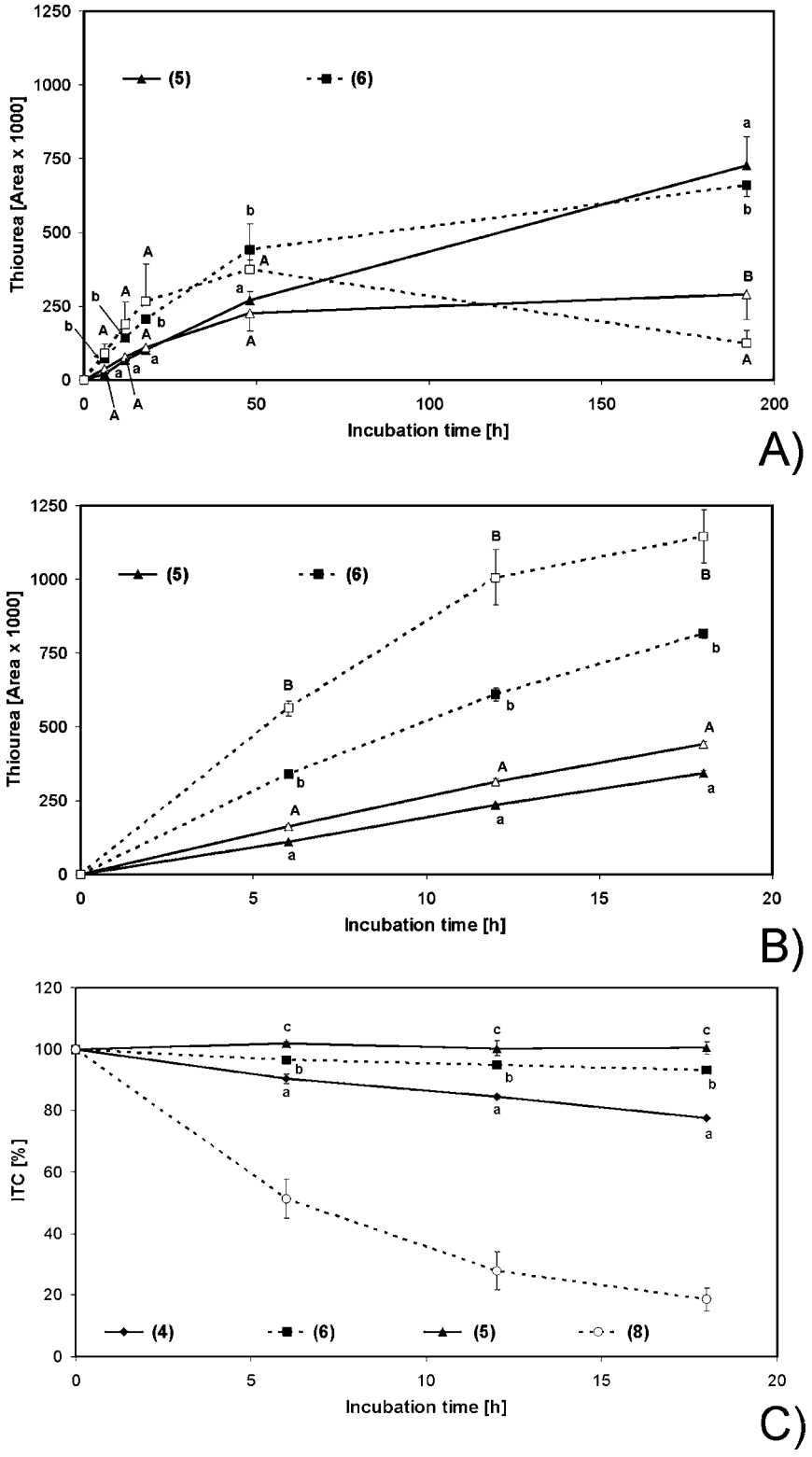

Figure 5. (A–C) Course of the reaction of ITCs with selected amino groups of compounds **4**, **5**, and **6** in model systems. (A) Formation of thiourea derivatives with compounds **5** and **6** at pH 7.3. (B) Formation of thioureas with compounds **5** and **6** at pH 8. (C) ITC concentrations during the course of the reaction with the different amino compounds (compounds **4**, **5**, and **6** at pH 8 and compound **8** at pH 7.3). Hollow symbols represent compound **1**, and filled symbols represent compound **2** or their corresponding adducts (**13** and **14**). For compound numbers, please refer to Figure 1. Different letters in each panel indicate significant differences between the thiourea content, tested for each ITC and for each reaction time separately (panels A and B: compound **1**, capital letters; compound **2**, small letters), or between the concentration of compound **2** after treatment with compounds **4**–**6**, tested for each reaction time separately (panel C) ($p \leq 0.05$ by Tukey's HSD test).

activity of such thiol adducts, with the glutathione adducts being intermediate transport vehicles for the ITCs *in vivo*.[27,29−31]

Dithiocarbamate Formation in Broccoli Sprouts. To evaluate whether ITCs might be able to react with free thiol

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groups present in a plant matrix, broccoli sprouts were incubated in water in the presence of compound **3** and exogenous myrosinase was added to ensure the formation of ITCs from glucosinolates. Broccoli sprouts had a pH value of 5.3 and contained 27.6 μmol of glucoraphanin g^{-1} of DW. After 1 h, compound **10** was detected, resulting from the reaction of compound **2** with compound **3** (Figure 3B). The adduct was quantified and gave a concentration of 1.9 ± 0.2 μmol of compound **2** g^{-1} of broccoli sprouts (DW). With the GC−FID methodology, "free" compound **2** that did not react at all was quantified. A total of 7.2 ± 0.9 μmol of compound **2** g^{-1} of broccoli sprouts (DW) has not reacted. Without presence of compound **3**, the concentration of compound **2** was 9.1 ± 1.2 μmol of compound **2** g^{-1} of broccoli sprouts (DW). In addition to compound **2**, 8.3 ± 0.6 μmol of 5-methylsulfinylpentylnitrile g^{-1} of broccoli sprouts (DW) resulted from the hydrolysis of the glucosinolate glucoraphanin. After 1 h of hydrolysis, glucoraphanin was hydrolyzed to >99%, as shown by HPLC. After 2 h of incubation, the dithiocarbamate concentrations already decreased. Approximately one-fifth of compound **2** reacted with compound **3** to compound **10**. Further reactions with the food matrix are probably the reason that only 63% of breakdown products could be recovered after hydrolysis of glucoraphanin. In other food matrices, such as eggs, with a slightly basic pH value in the egg white (pH 9−9.5),[12] the ITC−protein reaction might be accelerated. The reactivity of ITCs with egg white proteins has been demonstrated by Kroll et al.[7,8] Additionally, Luciano et al. revealed that compound **1** reacted with cysteine and glutathione present in meat to form compound **9** correspondingly.[32] However, the reactivity between ITCs and amino acids in *Brassica* plants was, to our knowledge, not studied before. Cejpek et al. investigated the reactions of sulfite with ITCs in mustard paste and observed a decrease in the ITC levels.[33] Several further studies investigated the reaction of ITCs with isolated proteins, revealing the preferential reaction with free thiol groups prior to further reactions with free amino groups.[34,35] These conjugates showed changed physicochemical as well as technofunctional properties, such as a modified heat aggregation, foaming and emulsifying properties, decreased tryptic degradation and solubility, and shifted isoelectric points.[7,34−36] Therefore, reactions of ITCs with free amino acids, peptides, or proteins of plant origin, food matrices, reduce the levels of essential amino acids, the bioutilization of nitrogen, and the deposition of energy, respectively, as reported before.[12]

Reactions of ITCs with Amino Groups in Model Systems. The reactivity of the ITCs toward the amino groups was much slower compared to the reactions with the thiol group (pathway B in Figure 2 and Table 1). Between the amino groups of the three amino acid derivatives applied, considerable differences in the reactivity were observed. At pH 5, the ITCs did not react with any of the amino compounds. In physiological buffer (pH 7.3), adduct formation was detectable, although the reduction in the concentrations of either amino group containing amino acids or ITCs did not significantly take place. In panels A and B of Figure 5, the formation of thiourea derivatives resulting from the reaction of the corresponding ITCs and the two protected lysine derivatives is shown. At higher pH values, the reaction with the amino groups was accelerated. Because both lysine derivatives show the same detector response, owing to their more or less similar chemical structure, the peak areas of the thioureas with the same ITCs are comparable. The α-NH_2-group adducts, deriving from the reaction with N_ε-(carbobenzyloxy)-L-lysine (**6**) and compound **2** were significantly formed more rapidly at pH 7.3 (Figure 5A) and at pH 8.0 (Figure 5B) compared to the ε-NH_2-group adducts deriving from the reaction with N_α-(carbobenzyloxy)-L-lysine (**5**). However, in the course of the reaction at pH 7.3 (Figure 5A), the concentration of the ε-NH_2-group adducts further increased, reaching higher values after 48 h compared to the α-NH_2-group adducts. In contrast, the thiourea deriving from the reaction of compound **1** with compound **6** showed decreased concentrations after reacting for 192 h. Probably, the thiourea derivatives with the α-NH_2 group are less stable compared to those with the ε-NH_2 group. Thioureas deriving from the reaction of α-amino acids and ITCs are known to produce the corresponding 2-thiohydantoins in a proton-catalyzed reaction. Their cleavage is base-catalyzed and leads, besides the thioureas, to amino acids, carbon disulfide, and amines.[16] In the present study, the formation of these substances was not observed. However, the cyclization of the ε-thioureas is very unlikely. Therefore, this could be the reason for the increasing "ε-thiourea" concentrations over the whole reaction period compared to the decreasing/stagnating "α-thioureas".

The decrease of the concentration of compound **2** as a result of the thiourea formation caused by the reaction with different amino compounds at pH 8 is displayed in Figure 5C. Additionally, compound **8** was treated with compound **1** at pH 7.3 to determine the reactivity toward the aliphatic amine, deriving from the compound **1** itself at physiological pH (pathway C in Figure 2). The decrease of compound **1** because of this reaction can be seen in Figure 5C, too. At pH 8, the amino acids reacted faster with the ITCs compared to pH 7.3. A total of 22% of compound **2** reacted with *S*-benzyl-L-cysteine (**4**) after 18 h (chromatographic and spectral data in Figure 3C), whereas only 7% of compound **2** reacted with compound **6**. However, no decrease of either compound **2** or the concentration of compound **5** could be detected under these conditions. The reactions with compound **1** showed similar results. Compound **8** reacted very fast at pH 7.3 with compound **1** and formed compound **7**. Only 19% of compound **1** remained after 18 h.

In summary and in accordance with the literature, the reaction with amino groups was in general much slower compared to the thiol group (Table 1).[21,26] Because thiol and amino groups only react in their dissociated form, higher pH values favor such reactions.[17] The order of reactivity in the present study was as follows: compound **8** ≫ compound **4** > compound **6** > compound **5**. One possible explanation for the great differences in the reactivity can be found in the basicity level of the amino groups (compound **4**, pK_{NH_2} = 8.63; compound **5**, pK_{NH_2} = 10.63; compound **6**, pK_{NH_2} = 9.92; and compound **8**, pK_{NH_2} = 9.53).[22] With a decreasing basicity of the amino group, the reactivity toward ITCs should decrease.[17] However, in the present study, the reactivity increases with a decreasing basicity within the three amino acids. Cejpek et al. could also not explain the reactivity of different amino acids and peptides with their pK_a values.[16] They concluded that the differences in reactivity of the amino compounds at these pH values (6−10) are determined by steric hindrance and electrical effects.[16] Probably, at low pH values, more acidic amino groups are able to react faster, because the nucleophilic NH_2 form will be present in higher amounts. More basic amino groups will predominantly be present in the non-reactive NH_3^+ form. This explains the observed higher reactivity of the α-NH_2 group, which is more acidic compared to the ε-NH_2 group. The higher reactivity of compound **8** is probably attributed to its smaller size and less sterical hindrance.

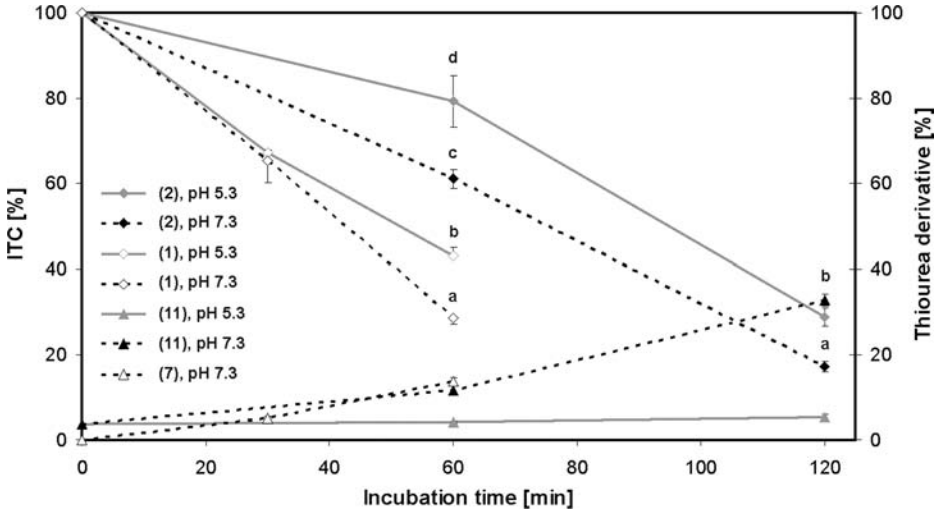

Figure 6. Thermally induced degradation of ITCs (**1** and **2**). Hollow symbols represent compound **1**, and filled symbols represent compound **2** or their corresponding adducts (**7** and **11**). Thiourea derivative (%) = percentage of the formed concentration of the thiourea derivative (quantified as compound **7**) × 2 (2 mol of ITC is necessary for 1 mol of thiourea derivative) in relation to the initial concentration of the ITCs. For compound numbers, please refer to Figure 1. Different letters in the panel indicate significant differences between the concentrations of the ITCs at 60 or 120 min ($p \leq 0.05$ by Tukey's HSD test).

Thermal Degradation of Allyl-ITC and Sulforaphane. When reactions between ITCs and nucleophilic compounds in food systems are expected, an important influential factor is the temperature. In most cases, plant foods are not consumed raw but treated at comparatively high temperatures. Therefore, the thermally induced reactivity/stability of the ITCs was studied to evaluate their behavior relevant under cooking conditions. Both ITCs were treated at 100 °C and a pH value of 5.3 or 7.3. In Figure 6, the breakdown of compounds **1** and **2** and the formation of compound **7** and *N*,*N*′-di[4-(methylsulfinyl)butyl]-thiourea (**11**) during the heat treatment is presented. Degradation of compound **1** was more pronounced in comparison to compound **2**, and for example, after 1 h at pH 5.3, approximately 43% of compound **1** remained, in contrast to 79% of compound **2**. Both ITCs were significantly more stable at a pH value of 5.3 (20% degradation of compound **2** after 1 h) compared to pH 7.3 (39% degradation of compound **2** after 1 h). Under physiological pH conditions (pH 7.3), the formation of *N*,*N*′-di(alkyl)thiourea (compound **7** or **11**) could be observed for both ITCs, with increased concentrations depending upon the treatment period (in comparison to pathway C in Figure 2). At this pH, the thioureas were the main breakdown products. Assuming that compound **11** had the same detector response as compound **7**, approximately 40% of the degraded compound **2** was obtained as compound **11** after 2 h of heat treatment and 19% of degraded compound **1** was obtained as compound **7** after 1 h. In this context, less compound **11** was produced at pH 5.3 and no compound **7** was found. When methanol was present, the formation of *O*-methyl(alkyl)thiocarbamate was observed as another reaction product (pathway D in Figure 2). Further products could not be identified by either LC−MS or GC−MS. The formation of both *N*,*N*′-di(alkyl)thiourea and *O*-methyl-(alkyl)thiocarbamate was also observed in the reaction mixtures at room temperature (especially at pH 8) as well as after comparatively long reaction times, being more pronounced for compound **1** compared to compound **2**. Because only part of the ITCs resulted in *N*,*N*′-di(alkyl)thiourea as a result of thermal degradation, it is hypothesized that the formation of other, non-identified compounds plays also a great role in the degradation of ITCs. In general, the heat treatment seems to accelerate the base-catalyzed amine formation. In the past, some other investigations have been conducted to study the degradation of compounds **1** and **2** in aqueous medium. Chen and Ho found only 29% of compound **1** after 1 h of boiling at pH 7, with results being more or less similar to the present results (also 29% after 1 h of heat treatment at pH 7.3).[37] A higher pH value also led to an accelerated thermally induced breakdown in that study.[37] The main non-volatile breakdown product was identified to be compound **7**.[37,38] Additionally, Chen and Ho identified several volatile breakdown products with garlic-like smell, such as diallyl sulfide and diallyl disulfide, and cyclic compounds (e.g., 5-methyl-1,2,3,4-tetrathiane and 4*H*-1,2,3-trithiin).[37] Pecháček et al. stored compound **1** at 80 °C at different pH values for 80 min.[39] In that study, compound **1** was much less stable, with only 2% left after 1 h at pH 6 or 8. At pH 8, the main breakdown products were compound **8** and allyl dithiocarbamate.[39] Degradation of compound **2** at 100 °C in distilled water also led to the corresponding *N*,*N*′-di(alkyl)thiourea (**11**), next to volatile breakdown products, such as dimethyl diulfide, *S*-methyl methylthiosulfinate and -sulfonate, and 4-isothiocyanato-1-(methylthio)-1-butene, resulting from a dehydration of compound **2**.[40] Unfortunately, the degree of degradation of compound **2** was not given in that study. In the present study, it was shown that the reaction between compounds **1** and **8** is unexpectedly very fast at physiological pH values, because the reactions with the amino groups of the amino acids were comparatively very slow at this pH and still slower at pH 8 (Figure 5C and Table 1).

In the intestinal tract, several bacteria exist that produce amines by amino acid decarboxylation, resulting in the formation of substances, such as ethylamine, histamine, or phenylethylamine.[41] Therefore, these substances might also be able to react with ITCs deriving from the bacterial hydrolysis of glucosinolates in the intestine.[42]

In summary, the present study aimed at the characterization of possible pathways that ITCs might undergo when being released during a (enzymatically or thermally induced) breakdown of glucosinolates present in *Brassica* vegetables (Figure 2). It was

shown that ITCs react very fast with the thiol group of cysteine even at pH 5, representing the pH value common for *Brassica* vegetables. The formation of dithiocarbamates by the reaction of endogenously released compound **2** with the thiol group of cysteine was demonstrated in broccoli sprouts. These findings illustrate the relevance of these reactions during preparation of food containing *Brassica* vegetables, because they might result in modified proteins and influence indispensable amino acids.

The reaction of the ITCs with amino groups was comparatively slower and did not occur under acidic conditions. However, great differences in the reactivity of different amino groups were revealed, resulting from surrounding functional groups that influenced the basicity of the amino groups. ITCs reacted much faster with the α-NH_2 group of lysine compared to the ε-NH_2 group, whereas the formed ε-NH_2-thioureas were more stable. On the other hand, the aliphatic compound **8** reacted fast with compound **1** at physiological pH values, forming compound **7**, also being a main thermal degradation product of compound **1** at pH 7.3. Therefore, the relevance for this reaction should be further investigated in detail, and the physiological behavior of the thioureas formed during the reaction needs to be evaluated.

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Notes

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ABBREVIATIONS USED

DW, dry weight; ITC, isothiocyanate

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