Novel transformation products from the glucosinolate breakdown products isothiocyanates and thioglucose formed during cooking

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Abstract

Glucosinolates are secondary plant metabolites occurring in Brassicaceae plants. Upon tissue disruption these compounds can be enzymatically hydrolyzed into isothiocyanates. The latter are very reactive and can react with nucleophiles during food processing such as cooking. Here, a novel type of glucosinolate degradation product was identified resulting from the reaction of the isothiocyanates sulforaphane and allyl isothiocyanate with thioglucose during aqueous heat treatment. The cyclic compounds were isolated and their structure elucidated by NMR spectroscopy and high-resolution mass spectrometry as 4-hydroxy-3-(4-(methylsulfinyl)butyl)thiazolidine-2-thione and 3-allyl-4-hydroxythiazolidine-2-thione. Based on experiments with isotope-labeled reagents, the determination of the diastereomeric ratio and further reactions, a reaction mechanism was proposed. Finally, the formation of the two 3-alk(en)yl-4-hydroxythiazolidine-2-thiones was quantified in boiled cabbage samples with contents of 92 pmol/g respectively 19 pmol/g fresh weight using standard addition method.

Keywords

Glucosinolate, Isothiocyanate, Thioglucose, Cabbage, 4-Hydroxythiazolidine-2-thione, Retro-aldol reaction

1. Introduction

Glucosinolates (GLSs) are found in *Brassica* vegetables such as cabbage, Brussels sprouts, cauliflower or broccoli (van Poppel, Verhoeven, Verhagen, & Goldbohm, 1999). When plant tissue is injured, myrosinase (a β-D-thioglucosidase) and GLS come in contact and the glucosinolate is hydrolyzed to glucose and an unstable thiohydroximate-*O*-sulfonate. The latter can decompose spontaneously to isothiocyanates (ITCs) and at low pH values to nitriles, or if additional proteins are present and depending on the structure of the thiohydroximate-*O*-sulfonate, can be converted to nitriles, epithionitriles or thiocyanates (Burow & Wittstock, 2009). Health-promoting effects from the occurrence of GLSs in foods are especially attributed to the release of ITCs: sulforaphane [4-(methylsulfinyl)butyl isothiocyanate], a breakdown product of glucoraphanin [4-(methylsulfinyl)butyl glucosinolate], has chemopreventive functions thereby showing promising anti-cancer effects like for lung cancer (Palliyaguru, Yuan, Kensler, & Fahey, 2018). Similarly, allyl isothiocyanate (AITC) shows anti-cancer activity in animal models and cultured cancer cells (Rakariyatham et al., 2019). Due to its high electrophilicity the ITC functional group is very reactive and leads to react with nucleophilic substituents such as amino-, hydroxy- and especially thiol groups (Drobnica, Kristián, & Augustín, 1977; Hanschen, Brüggemann, et al., 2012). Given that many *Brassica* vegetables are cooked before consumption, the stability of ITCs should be noted. During heating in aqueous solutions ITCs are labile and degraded depending of treatment time and pH value, whereby among others reaction products such as *N,N'*-bis[alk(en)yl] thioureas can be detected (Jin, Wang, Rosen, & Ho, 1999; Pecháček, Velíšek, & Hrabcová, 1997). Compared to ITCs, GLSs are more stable (Song & Thornalley, 2007), but can thermally decompose as well (Hanschen, Rohn, Mewis, Schreiner, & Kroh, 2012). Using aqueous model systems

with sinigrin (allyl glucosinolate) it was shown that for example thioglucose can be released, which was accompanied with nitrile formation (Hanschen, Bauer, et al., 2012). Thioglucose and nitrile formation can also be the result of iron-(II)-induced GLS degradation (Bellostas, Sørensen, Sørensen, & Sørensen, 2008). Moreover, during boiling of vegetables GLS release high amounts of nitriles (Hanschen, Kühn, Nickel, Rohn, & Dekker, 2018). That said, a concomitant formation of thioglucose appears likely. Thioglucose contains a nucleophilic thiol-function which easily might react with electrophilic compounds such as ITCs. Thereby, it might lead to a reduction of ITC contents present in boiled *Brassica* vegetables. It is currently not known whether thioglucose reacts with the two health promoting isothiocyanates AITC and sulforaphane during cooking and thus could affect their levels or lead to novel reaction products. To our knowledge, here, we investigate reactions of thioglucose with the two selected isothiocyanates AITC and sulforaphane for the first time. These two ITCs were selected because of their presence in larger quantities in some vegetables and the fact that for these two ITCs their health-promoting effect is described in literature. The main products, which were not reported as natural products before, formed during boiling were isolated and identified using NMR spectroscopy and high-resolution mass spectrometry. Moreover, several reactions were carried out to postulate a plausible reaction mechanism for the formation of the cyclic product.

2. Material and methods

2.1 Chemicals

AITC (stabilized ≥ 95%), $NaH_2PO_4 \cdot H_2O$ (> 99%, puriss. p.a.), NaOH (≥ 97%, ACS reagent), thioglucose (1-thio-β-D-glucose sodium salt, ≥ 98%), thiourea (≥ 99%,

ReagentPlus®), sodium metabisulfite ($Na_2S_2O_5$, ≥ 99%, ReagentPlus®), benzaldehyde dimethyl acetal (99%), *p*-toluenesulfonic acid monohydrate (PTSA, ≥ 98% ReagentPlus®), deuterium oxide (D_2O, 99.9 atom % D), water-^{18}O ($H_2{}^{18}O$, 97 atom % ^{18}O, for PET), allylamine (≥ 99%), CS_2 (≥ 99%) chloroacetaldehyde solution (~50 wt. % in H_2O), (*S*)-(+)-2-phenylbutyric acid (99%), *N*,*N*'-dicyclohexylcarbodiimide (DCC, 99%), 4-(dimethylamino)pyridine (DMAP, ≥ 99%, ReagentPlus®), pyridine (99.8%, anhydrous), trifluoroacetic anhydride (LiChropur™, ≥ 99.0%, for GC derivatization), *N*-[2-(dansylamino)ethyl]maleimide (≥ 99.0%, BioReagent, suitable for fluorescence), 1,4-dithiane-2,5-diol (mercaptoacetaldehyde dimer, 97%) and $NaHCO_3$ (≥ 99.7%, ACS reagent) were from Sigma-Aldrich (Steinheim, Germany); CD_3CN (Eurisotop ®, 99.8% D, Euriso-Top GmbH, Saarbrücken, Germany); ethyl β-D-thioglucopyranoside (≥ 98%, Biosynth Carbosynth, Berkshire, United Kingdom); dimethylformamide (DMF, > 99.5%, Tokyo Chemical Industry Co., Ltd, Tokyo, Japan); $K_2HPO_4 \cdot 3\ H_2O$ (> 99%, GR for analysis) was purchased from Merck KGaA (Darmstadt, Germany); sulforaphane, (D, L-sulforaphane, ≥ 95%), 2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranosyl bromide (90%) and $^{13}C_6$-thioglucose (1-thio-β-D-glucose-13C6 sodium salt dihydrate, ~ 90%) were from Toronto Research Chemicals Inc. (North York, Canada). For HPLC-DAD measurements and as solvent for reactions Milli-Q water obtained by using PURELAB flex (ELGA LabWater, Celle, Germany) system and for LC-MS measurements water (LiChrosolv ®, LC-MS grade, Merck KGaA, Darmstadt, Germany) was used. As organic solvent respectively mobile phase for measurements acetonitrile (ACN, CHEMSOLUTE ® > 99.95%, LC-MS grade) and methanol (MeOH, CHEMSOLUTE® ≥ 99.95%, for LC-MS) from Th. Geyer GmbH & Co. KG (Renningen, Germany) was used and dichloromethane (DCM, ROTISOLV® ≥ 99.9%, GC Ultra Grade), acetone (ROTISOLV® ≥ 99.9%, UV/IR Grade), α-D-(+)-Glucose monohydrate (≥ 99.5%, Ph.

Eur.), K_2CO_3 (≥ 99%, p. a. ACS), NaCl (≥ 99.8% with anti-caking agent), sinalbin (4-hydroxybenzyl GLS, ≥ 99%), and HCl (ROTIPURAN ® 37%, p.a., ACS, ISO) were from Carl Roth GmbH + Co. KG (Karlsruhe, Germany).

2.2 Boiling experiments of thioglucose and isothiocyanate

AITC respectively sulforaphane were boiled in presence of thioglucose for 1 h at pH values of 6.0, 7.0 and 8.0 and concentrations of 1 mmol/L by using a thermoshaker MHR 23 (Hettich Benelux B.V, Netherlands) at 100 °C and 300 rpm in closed GC vials. The buffers for these tests and experiments in further sections consisting of 3.57 g/L $NaH_2PO_4 \cdot H_2O$ and 9.31 g/L $K_2HPO_4 \cdot 3\ H_2O$ adjusted to the appropriate pH value by 1 M HCl respectively 1 M NaOH. The pH value was determined by pH meter (Metrohm 691 pH meter, Filderstadt, Germany). The samples were measured after transfer into LC vials by a UPLC-DAD system from Agilent Technologies Germany (Waldbronn) with Agilent 1290 Infinity (binary pump, autosampler, degasser, column oven) and Agilent 1260 Infinity (DAD, FLD detector) with HPLC method 1 (gradient of A = water and B = ACN, 0 to 2.5 min 5% B, then a linear increase to 8 min up to 98% B, holding at 98% B until 10 min, decreasing to 5% B (10.5 min) and re-equilibration with 5% B for 4 mins), flow rate: 400 µL/min, oven temperature: 30 °C and injection volume of 10 µL. As column Agilent Zorbax Eclipse Plus C8, RRHD 3 x 100 mm; 1.8 µm with Agilent pre-column EC-C18, 2.1 x 5 mm; 2.7 µm was used. The DAD signals at 200 nm, 220 nm, 240 nm, 260 nm, and 274 nm were analyzed using software Agilent Openlab CDS ChemStation version 2.3.54.

2.3 Characterization of the new reaction products by high-resolution mass spectrometry

The sum formulas of the novel reaction products were determined by high-resolution ESI MS. Therefore, HPLC method 1 was used at a HPLC-DAD-ESI-qToF system (Agilent 1290 Infinity II for binary pump, Agilent 1290 Infinity for autosampler, degasser, column oven, Agilent 1260 Infinity for DAD detector as well as Agilent 1260 Infinity II for isocratic pump for adding reference mass solution) and an Agilent ESI-Q-ToF (G6530B) with Dual AJS ESI source measuring in positive polarization mode with VCap 3500 V, Nozzle Voltage 1000 V, Fragmentor 100 V, Skimmer 1 65 V, OctopoleRFPeak 750 V, mass range *m/z* 30–1000, gas temperature 300 °C, gas flow 10 L/min, nebulizer 35 psig, sheath gas temperature 320 °C, sheath gas flow 12 L/min and using reference mass solution Agilent HP 921 with purine (reference *m/z* 121.0509 and *m/z* 922.0098). For Data evaluation the software Agilent MassHunter Qualitative Analysis 10.0 was used.

2.4 Isolation of the new reaction products

To characterize the new formed compounds by NMR spectroscopy an isolation of the pure compounds was necessary. Therefore, in several approaches 10 mg thioglucose and 50 µL AITC (100 g/L in ACN) dissolved in 15 mL phosphate buffer (pH = 8, see section 2.2.) were boiled for 1 h at 100 °C and 300 rpm in 20 mL head-space vials. Afterwards, the reaction mixtures were extracted using 3 x 10 mL DCM. The combined organic phases were evaporated to dryness under a stream of nitrogen. Then, the residue was dissolved in 2 mL of water/ACN (50/50, v/v %) and purified by using preparative HPLC. The preparative HPLC system was an Agilent 1260 Infinity (binary

pump, autosampler, degasser, DAD detector and fraction collector) coupled to a preparative Agilent Zorbax SB-C18; 21.2 x 250 mm, 7 µm Prep HT column. The used HPLC method 2 was a gradient of A = water and B = ACN, 0 to 4.5 min 15% B, an increase to 22 min to 98% B, decreasing to 15% B (22.1 min) and re-equilibration with 15% B for 7.9 mins), flow rate: 15 mL/min at room temperature, by using an injection volume of 500 µL. For peak detection the DAD signal at 220 nm and 250 nm were used. The collected eluate in the time range of 13–14 min was concentrated in nitrogen stream down to approximately 5 mL. Afterwards, the concentrate was extracted using 3 x 2 mL DCM and dried under a stream of nitrogen. The obtained product showed an impurity up to 20% by peak area in its LC-UV chromatogram at 220 nm using HPLC method 1. Therefore, the product was purified a second time by preparative HPLC using the same column but a method with water/ACN for 0–2.5 min 25% B, linear increasing to 45% B at 18 min, within 0.1 min decreasing back to 25% B and holding for 7 min at 25% B. The eluate was collected in the time range of 11.6–12.6 min and then concentrated and extracted as described above.

To obtain the purified reaction product of sulforaphane and thioglucose, several approaches of 4 µL sulforaphane and 8 mg thioglucose dissolved in 20 mL phosphate buffer (pH = 8) were boiled at 100 °C and 300 rpm for 1 h in 20 mL head-space vials. 5 g of NaCl were dissolved in cooled down reaction mixture. The resulting solution was extracted using 3 x 8 mL ACN. The combined organic phases were dried under reduced pressure at 30 °C. Then, the residue was re-dissolved using 4 mL of ACN whereby two phases were obtained. Therefore, a second liquid-liquid-extraction step using 3 x 2 mL ACN was carried out. The ACN phases were combined, dried in a stream of nitrogen and the residue was dissolved in 2 mL of water/ACN (50/50, v/v %) and purified by preparative HPLC. The used HPLC method 3 was a gradient of

A = water and B = ACN, 0 to 3 min 10% B, to 16 min an increase to 98% B, holding for 7 min, a decrease to 24 min back to 10% B and holding for 6 min on 10% B. Additional parameters were identical to HPLC method 2. The time range of the collected eluate was 9.0–9.8 min. The eluate was dried under reduced pressure at 40 °C. As the product showed impurities of up to 10% in LC-UV chromatogram (at 220 nm), it was purified a second time by using the same parameters as before but using a different gradient of 0–5 min 20% B, at 12 min 50% B, increase to 98% B (13 min), holding for 4 min, decreasing within 1 min to 20% B and holding for 7 min at 20% B. In the time range of 6.5–7.5 min the eluate was collected and dried under reduced pressure at 40 °C.

2.5 Nuclear magnetic resonance spectroscopy (NMR)

NMR data were obtained by using a Bruker AVANCE III 600 MHz NMR spectrometer (Bruker Corporation, Rheinstetten, Germany) operating at 600.2 MHz (^{1}H NMR) respectively 150.9 MHz (^{13}C NMR). The samples were dissolved in CD_3CN and measured at 25 °C at concentrations of approximately 50 mM by 1D and 2D NMR (^{1}H, ^{13}C, H,H-COSY, HSQC, HMBC). The spectra were referenced to the following solvent resonances: CH_3CN (δH 1.94 ppm) respectively CD_3CN (δC 118.26 ppm) (Fulmer et al., 2010). Software TopSpin 3.6.2 was used for data evaluation.

2.6 Deciphering the reaction mechanism between thioglucose and isothiocyanate during boiling

2.6.1 Reactions of different derivatives of thioglucose

Different derivatives of thioglucose were purchased respectively synthesized. To test necessity of a thiol group in the reaction mechanism, an ethyl group protected thioglucose (ethyl β-D-thioglucopyranoside) respectively thioglucose was boiled at 100 °C with AITC for 1 h on concentrations of 1 mM in phosphate buffer (pH = 8) at 300 rpm. Samples in this section were measured by mass spectrometry (AB Sciex Germany, Darmstadt, Sciex 6500 QTRAP) QTRAP method 1 (ESI positive, CUR 50 psi, TEM 500 °C, IS 5500 V, GS1 50 psi, GS2 60 psi, DP 30 V, EP 10 V) using HPLC method 4: gradient of A = water and B = ACN, 0 to 5.1 min 2% B, followed by a linear increase to 14 min to 98% B, holding at 98% B until 19.4 min, decreasing to 2% B (20 min) and re-equilibration with 2% B for 4.5 mins), flow rate: 300 µL/min, oven temperature: 35 °C, Eclipse Plus C8 column (above).

A second approach was using thioglucose in which the hydroxy groups are protected by benzyl ether groups (2,3,4,6-tetra-*O*-benzyl-1-thio-α/β-D-glucopyranose (**BnSGlc**)). **BnSGlc** was prepared based on Johnston et al. (Johnston & Pinto, 2000). 4 mg 2,3,4,6-tetra-*O*-benzyl-α-D-glucopyranosyl bromide and 1 mg thiourea were dissolved in 300 µL dry acetone and boiled under reflux for 1 h. Afterwards, acetone was removed using a stream of nitrogen and the residue dissolved in 300 µL of water/DCM (33/67, v/v %). 2 mg $Na_2S_2O_5$ were added, and the mixture boiled for 2 h under reflux. The solvent was removed by nitrogen stream and the residue dissolved in 1 mL of water/ACN (50/50, v/v %). The sample was measured by mass spectrometry (Sciex 6500 QTRAP) to assign *m/z* ratios for the obtained signals in LC-UV chromatogram. Two signals which showed the expected *m/z* $[M+NH_4]^+$: 574, $[M+Na]^+$: 579 and $[M+K]^+$: 595 were isolated by using preparative HPLC. The obtained products of **BnSGlc** respectively thioglucose were boiled at 100 °C for 0.5 h, 1 h and 2 h in phosphate buffer (pH = 8) at 300 rpm in presence of AITC with concentrations of 1 mM as well as

in a second approach with an addition of ACN to a content of 50% of the reaction solution.

In a separate approach another derivative of thioglucose was synthesized based on Chen et al. (Chen, Okafor, Garcia, & Wang, 2018). This thioglucose derivative, has a benzylidene acetal protective group on the hydroxy groups linked to C4 and C6 carbon atom (4,6-*O*-benzyliden-1-thio-D-glucopyranose (**PhCHSGlc**)). 10 mg thioglucose, 18 µL benzaldehyde dimethyl acetal and 2 mg PTSA were dissolved in 100 µL DMF and were shaken for 5 h at 60 °C at 300 rpm. The identification of the desired product *m/z* $[M+H]^+$: 285 was performed by using Sciex 6500 QTRAP and Zorbax SB-C18 column. Purification was done by preparative HPLC and the solvent of the eluate was removed under reduced pressure at 25 °C. **PhCHSGlc** respectively thioglucose was heated for 1 h in the presence of AITC (1 mM) in phosphate buffer (pH = 8) at 300 rpm and 100 °C in closed vials.

2.6.2 Reactions using isotope-labeled reagents

Three different approaches using isotope-labeled reagents were used in order to test, if the reagents are included in the reaction product. First, approximately 0.1 mg $^{13}C_6$-thioglucose respectively thioglucose were heated with approximately 0.05 mg AITC for 1 h at 100 °C in 200 µL phosphate buffer (pH = 8). In a second experiment approximately 0.1 mg thioglucose and approximately 0.05 mg AITC were heated for 1 h at 100 °C in 200 µL D_2O respectively Milli-Q water. In an additional experiment 0.1 mg thioglucose and approximately 0.05 mg AITC were heated for 1 h at 100 °C in 100 µL $H_2{}^{18}O$ respectively Milli-Q water. All samples were measured by using HPLC

method 1 and QTRAP method 2 (ESI positive, CUR 50 psi, TEM 400 °C, IS 5500 V, GS1 50 psi, GS2 60 psi, DP 60 V, EP 10 V).

2.6.3 Determination of the enantiomeric ratio

A racemic mixture of the reaction product of thioglucose and AITC during boiling (3-allyl-4-hydroxy-1,3-thiazolidine-2-thione) was prepared based on Stalling et al. (Stalling, Saak, & Martens, 2013). 8.5 mg K_2CO_3, 15 µL allylamine, 22.5 µL CS_2 and 10 µL chloroacetaldehyde solution were dissolved in 0.75 mL of water and stirred at room temperature for 2 h. Afterwards, the reaction mixture was extracted with 3 x 0.5 mL of DCM. The combined organic phases were dried in a nitrogen stream and the residue was dissolved in 1 mL of water/ACN (50/50, v/v %). The product was purified using preparative HPLC using HPLC method 2. The collected eluate in the time range of 13–14 min was treated like described above in section 2.4. The racemate was converted into two diastereomers by derivatization with (*S*)-(+)-2-phenylbutyric acid in the following way: 0.3 mg 3-allyl-4-hydroxy-1,3-thiazolidine-2-thione (racemate) respectively the obtained purified boiling product of AITC and thioglucose, 0.27 mg (*S*)-(+)-2-phenylbutyric acid, 0.4 mg DCC and 1.7 mg DMAP were dissolved in 167 µL of DCM and stirred for 24 h at room temperature. The solvent was removed under a stream of nitrogen and the residue dissolved in 1 mL of water/ACN (50/50, v/v %). The signal of the desired product was assigned by QTRAP (*m/z* 322) and the product was isolated by preparative HPLC using HPLC method 5 (gradient of A = water and B = ACN, 0 to 3 min 50% B, a linear increase to 98% B at 16 min, holding for 4 min and decreasing back to 50% B within 1 min and re-equilibration with 50% B for 5 mins), flow rate: 15 mL/min at room temperature. The eluate in the time range of 15–16 min was collected and measured by QTRAP method 2 and using Reprosil Fluosil 60, PFP,

4.6 x 250 mm, 3 µm column from Dr. Maisch GmbH (Ammerbuch, Germany) and HPLC method 6 (A = water and B = MeOH, isocratic 30/70 (A/B) for 95.5 min, flow rate: 500 µL/min, oven temperature: 8 °C) for separation of the two diastereomers. In case of the purified boiling product of AITC and thioglucose the reaction mixture was measured after exchange of the solvent, as well as a control in which the purified boiling product were treated with all reagents except (*S*)-(+)-2-phenylbutyric acid.

2.6.4 Retro-aldol reaction of thioglucose – the origin of the C_2H_4SO building block

The formation of mercaptoacetaldehyde from thioglucose was tested by using *N*-[2-(dansylamino)ethyl]maleimide. A solution of 1 mM *N*-[2-(dansylamino)ethyl]maleimide in ACN as well as 1 mM thioglucose respectively mercaptoacetaldehyde dimer dissolved in phosphate buffer (pH = 8) with 10% ACN were prepared. 100 µL *N*-[2-(dansylamino)ethyl]maleimide solution and 100 µL thioglucose respectively the mercaptoacetaldehyde dimer solution were added to 800 µL of phosphate buffer (pH = 8) and heated for 5 min at 100 °C at 300 rpm. The reaction mixtures were measured by using HPLC method 7 (gradient of A = water and B = ACN, 0 to 3 min 2% B, followed by a linear increase to 14 min to 98% B, holding at 98% B until 19.4 min, decreasing to 2% B (20 min) and re-equilibration with 2% B for 4.5 mins, flow rate: 300 µL/min, oven temperature: 35 °C) with Eclipse Plus C8 column with HPLC-DAD analytical system described in section 2.3 coupled to Agilent ESI-qToF (G6546A) with Dual AJS ESI source in positive polarization mode with VCap 3500 V, Nozzle Voltage 0 V, Fragmentor 175 V, Skimmer 1 65 V, OctopoleRFPeak 750 V, mass range *m/z* 100–950, gas temperature 200 °C, gas flow 8 L/min, nebulizer 35 psig, sheath gas temperature 350 °C, sheath gas flow 12 L/min and using reference mass solution

306 Agilent HP 921 with purine (reference *m/z* 121.0509 and *m/z* 922.0098). In a second
307 experiment AITC and mercaptoacetaldehyde dimer respectively thioglucose were
308 dissolved at concentrations of 0.5 mM in phosphate buffer (pH = 8) containing 7% ACN
309 and boiled for 1 h at 300 rpm. The reaction mixtures were measured by QTRAP
310 method 1 and HPLC method 4 using Eclipse Plus C8 column.

311

312 **2.7 Quantification of contents of glucosinolates, isothiocyanates and boiling**
313 **reaction products of isothiocyanate and thioglucose in red and white cabbage**

314 For quantification of the reaction products (3-allyl-4-hydroxy-thiazolidine-2-thione and
315 4-hydroxy-3-(4-(methylsulfinyl)butyl)thiazolidine-2-thione) after boiling of cabbages a
316 conventional 1.938 kg heavy white cabbage (*Brassica oleracea* var. *capitata* f. *alba*)
317 and 1.588 kg heavy red cabbage (*Brassica oleracea* var. *capitata* f. *rubra*) were bought
318 in a supermarket. Approximately 150 g of fresh cabbage were cut into approximately
319 5 x 5 x 5 mm pieces and pooled in a beaker. 2 g of red respectively white cabbage
320 were given to 8 mL of soda water (pH = 8; 2 g/100 mL $NaHCO_3$), like it is used to
321 prepare blue cabbage, and heated for 1 h at 300 rpm at 100 °C in closed 20 mL head-
322 space vials. After cooling down, the suspension of cabbage pieces in the cooking
323 solution was centrifuged (3 min at 4000 *g*, with Thermo Scientific™ Megafuge 16R)
324 and 200 µL of the supernatant were taken as sample for measuring. Afterwards, the
325 purified analyte standard was added to the rest of the centrifuged cooking suspension
326 in an amount of 4–9 times higher than the original amount in the samples. To obtain a
327 realistic distribution of the spiked analyte amount the suspension was vortexed and
328 centrifuged again before taking the spiked sample from the supernatant. All samples
329 were prepared as triplicates and measured by HPLC method 8 (gradient of A = water
330 and B = ACN, 0 to 1.5 min 5% B, linear increase to 10.5 min to 80% B, followed by a

linear increase to 98% B (11 min), holding for 1 min at 98% B, decrease back to 5% B within 0.3 min and re-equilibration with 5% B for 4.7 mins), flow rate: 400 µL/min at 30 °C and an injection volume of 20 µL. Mass spectrometry parameters based on QTRAP method 1 with additional CXP 11 V and individual parameters for 3-allyl-4-hydroxy-1,3-thiazolidine-2-thione: DP 30 V, EP 10 V for all mass transitions 176 → 100 CE 17 V (quantifier) and 176 → 75 CE 17 V, 176 → 67 CE 25 V and 176 → 41 CE 45 V (as qualifiers). For 4-hydroxy-3-(4-(methylsulfinyl)butyl)thiazolidine-2-thione DP 60 V, EP 15 V was used for all mass transitions 276 → 258 CE 20 V, 276 → 212 CE 17 V, 276 → 200 CE 22 V (as qualifiers) and 276 → 182 CE 22 V (quantifier). For determination of the GLS contents in the cabbage samples, the samples were treated in different ways: no treatment (fresh cabbage cut into 20 x 20 x 50 mm pieces), 1 g of pooled cabbage in 4 mL soda water (see above) and heated up to 100 °C within 8 mins, and 1 g of pooled cabbage in 4 mL soda water boiled for 1 h, all in triplicates. In case of the boiled samples (inactivated myrosinase) the internal standard sinalbin was added after cooling down to room temperature. Regardless of the treatment, the samples were freeze dried and homogenized by using a mixer mill (MM 400, RETSCH GmbH, Haan, Germany). Afterwards, the homogenized freeze dried samples were analyzed as desulfo-GLSs by UHPLC-DAD-ToF-MS like described previously (Hanschen, 2020). To quantify the ITC contents, 1 g of pooled cabbage was given to 4 mL of soda water (see above) and heated up to 100 °C within 8 mins, as well as boiled for 1 h at 100 °C in a second approach, all in triplicates. Afterwards, the samples were analyzed like described in Hanschen (Hanschen, 2020) for determination of GLS breakdown products by GC-MS. To determine the contents of thioglucose 60 min boiled samples were freeze dried and 100 mg cabbage derivatized using 150 µL trifluoroacetic anhydride and 25 µL pyridine in 1 mL DCM based on König et al. (König, Bauer, Voelter, & Bayer, 1973). The samples were measured undiluted as well as 1:10

and 1:100 diluted by GC-MS (Agilent 7683 autosampler, Agilent 7890A GC system, Agilent 7683B injector and Agilent 5975C inert XL MSD) using a SGE BPX5 GC-MS column (30 m × 0.25 mm × 0.25 µm, VWR International GmbH, Darmstadt, Germany), helium as a carrier gas (1.8 mL/min), splitless injection of 1 µL of the sample at 190 °C inlet temperature, an AUX temperature of 270 °C and a temperature gradient starting at 110 °C holding for 6 min, increasing to 170 °C within 20 min and rising up to 300 °C at 30 °C/min. The software Enhanced ChemStation MSD ChemStation E.02.02.1431 was used for instrument control, data acquisition and evaluation.

3. Results

3.1 Boiling experiments of thioglucose and isothiocyanate

As shown in Figure 1A, aqueous heating leads to the degradation of AITC and results in the formation of several novel peaks. The most prominent new peak in the chromatogram at 240 nm originating from AITC was at a retention time of 6.7 min and was identified as *N,N'*-diallylthiourea by UHPLC-DAD-ToF-MS using a reference standard. After boiling a mixture of AITC and thioglucose an additional new intensive peak at 6.8 min occurred. As presented in Figure 1B, this new signal at 6.8 min, which was only obtained in the presence of AITC and thioglucose during boiling, is increasing along with an increase of the pH value. Especially the change from an acidic to a neutral pH environment led to a strong signal increase. In the presence of sulforaphane as an ITC similar results were obtained. Here, the retention time of sulforaphane was 6.4 min and the new signal, which accordingly increased by increasing pH, was detected at 5.8 min (Supplementary Figure S1).

3.2 Characterization of the new reaction products by high-resolution mass spectrometry

In order to identify the new products, originated from thioglucose and isothiocyanates during boiling, the compounds were characterized using different approaches. The obtained UV spectra of the new products by using AITC respectively sulforaphane were very similar with the absorption maximum at 274 nm and local maxima at approximately 210 nm and 245 nm (Supplementary Figure S2). The UV spectra were similar to UV spectra of dithiocarbamate derivates shown in Hanschen et al. (Hanschen, Brüggemann, et al., 2012). Additionally the sum formula was obtained by high-resolution mass spectrometry. For the new reaction product of AITC and thioglucose a sum formula ($C_6H_9NOS_2$) with a given mass accuracy (*m/z*: $[M+H]^+$: measured 176.0197, calculated 176.0198, difference 0.34 ppm) and accordance between measured and calculated isotope pattern was obtained (Supplementary Figure S3). The new compound is related to the sum formula of AITC (C_4H_5NS) but contains an additional C_2H_4SO moiety. By using sulforaphane the analogue with an additional C_2H_4SO moiety was obtained too (Supplementary Figure S4), but here the sodium adduct was the most intensive pseudo molecular ion most probably because of the high affinity of sodium to the sulfinyl group (*m/z*: $[M+Na]^+$: measured 276.0165, calculated 276.0157, difference 2.6 ppm). For a complete structural elucidation, of the reaction product of AITC respectively sulforaphane and thioglucose formed due to aqueous heat treatment, 1D and 2D NMR experiments were conducted with the purified products. Apart from these compounds, new intensive UV signals were obtained for the reaction of AITC respectively sulforaphane and thioglucose at room temperature in buffer using UHPLC-DAD. The obtained signals at 5.3 min respectively 4.8 min showed an UV spectra similar to dithiocarbamate derivate with a maximum at

274 nm (Supplementary Figure S5). The signals were detectable directly after mixing of ITC and thioglucose. The measured masses and accordance between measured and calculated isotope pattern indicated that this compounds are conjugates of AITC respectively sulforaphane and thioglucose (*m/z*: $[C_{10}H_{17}NO_5S_2+H]^+$: measured 296.0622, calculated 296.0621, difference 0.76 ppm respectively *m/z*: $[C_{12}H_{23}NO_6S_3+Na]^+$: measured 396.0564, calculated 396.0580, difference 4.3 ppm) shown in Supplementary Figure S6. The conjugates are most probably formed by a nucleophilic attack of the thiol group with the electrophilic carbon atom of the ITC function. The isolated conjugates were not stable at room temperature. With increasing temperature the signal intensity of the conjugate decreased while the signal intensity of the ITC increased. Most probably increasing temperature lead to the reverse reaction of the conjugate. Therefore, the conjugate was not a precursor for the reaction product during boiling (Supplementary Figure S7).

3.3 Isolation of the new reaction products

The extraction of the two novel reaction products out of the reaction mixture was different because of their different polarity. Using the software ACD/ChemSketch 2020.1.2, log P (partition coefficient in octanol/water) values of 0.47 ± 0.46 for the new reaction product of thioglucose and AITC and -1.12 ± 0.52 for the new reaction product using sulforaphane were calculated. The yields for the products purified twice by preparative HPLC were 2% for the product using AITC and 6% using sulforaphane. Using higher concentrations of the educts did not increase the yields, but lead to larger amounts of by-products.

3.4 Nuclear magnetic resonance spectroscopy (NMR)

The isolated compounds were measured by 1D and 2D-NMR and identified as the cyclic 3-allyl-4-hydroxythiazolidine-2-thione and 4-hydroxy-3-(4-(methylsulfinyl)butyl)thiazolidine-2-thione) (Figure 2). The ^{1}H and ^{13}C spectra are shown in Supplemental Figures S8-S11. The signal assignment was as following.

3-allyl-4-hydroxythiazolidine-2-thione (**1**): ^{1}H NMR (CD_3CN, 600 MHz, 25 °C) δ in ppm: 3.07 (dd, 1H, *J* = 12.3 and 2.1 Hz, C*H*$_2$-S), 3.63 (dd, 1H, *J* = 12.3 and 6.7 Hz, C*H*$_2$-S), 4.02 (dddd, 1H, *J* = 15.6, 6.7, 1.3 and 1.3 Hz, C*H*$_2$-N), 4.76 (dddd, 1H, *J* = 15.6, 4.7, 1.8 and 1.8 Hz, C*H*$_2$-N), 4.81 (d, 1H, *J* = 7.5 Hz, O*H*), 5.22 (dddd, 1H, *J* = 10.0, 1.8, 1.4 and 1.3 Hz C*H*$_2$=CH), 5.25 (dddd, 1H, *J* = 17.0, 1.8, 1.4 and 1.3 Hz C*H*$_2$=CH), 5.63 (ddd, 1H, *J* = 7.5, 6.7 and 2.1 Hz, C*H*-OH), 5.85 (dddd, 1H, *J* = 17.0, 10.0, 6.7 and 4.7 Hz, C*H*=CH$_2$). ^{13}C NMR (CD_3CN, 151 MHz, 25 °C) δ in ppm: 36.5 (*C*H$_2$-S), 49.1 (*C*H$_2$-N), 89.7 (*C*H-OH), 118.6 (*C*H$_2$=CH), 132.5 (*C*H=CH$_2$), 197.8 (*C*=S).

4-hydroxy-3-(4-(methylsulfinyl)butyl)thiazolidine-2-thione (**2**): ^{1}H NMR (CD_3CN, 600 MHz, 25 °C) δ in ppm: 1.74 (m, 2H, C*H*$_2$-CH$_2$-S(O)), 1.86 (m, 2H, C*H*$_2$-CH$_2$-N), 2.50 (s, 3H, C*H*$_3$), 2.72 (m, 2H, C*H*$_2$-S(O)), 3.05 (dd, 1H, *J* = 12.3 and 2.3 Hz, CH-C*H*$_2$-S), 3.58 (m, 1H, C*H*$_2$-N), 3.61 (dd, 1H, *J* = 12.3 and 6.6 Hz, CH-C*H*$_2$-S), 4.00 (m, 1H, C*H*$_2$-N), 5.30 (s, 1H, O*H*), 5.68 (dd, 1H, *J* = 6.6 and 2.3 Hz, C*H*-OH). ^{13}C NMR (CD_3CN, 151 MHz, 25 °C) δ in ppm: 20.6 (20.8) (*C*H$_2$-CH$_2$-S(O)), 26.9 (26.9) (*C*H$_2$-CH$_2$-N), 36.4 (36.5) (CH-*C*H$_2$-S), 38.9 (38.9) (*C*H$_3$), 46.6 (46.7) (*C*H$_2$-N), 54.2 (54.2) (*C*H$_2$-S(O)), 90.5 (90.6) (*C*H-OH), 197.5 (197.5) (*C*=S).

No significant impurities were detected. Pairs of peaks with 1:1 ratio were observed in ^{13}C spectra for product **2**, because compound **2** was a mixture of diastereomers.

3.5 Deciphering the reaction mechanism between thioglucose and isothiocyanate during boiling

3.5.1 Reactions of different derivatives of thioglucose

An ethyl group protected thioglucose (ethyl β-D-thioglucopyranoside) was used to verify if a free thiol group is needed in the precursor to undergo the reaction. Boiling of ethyl β-D-thioglucopyranoside and AITC did not lead to the formation of **1** or its ethyl derivate (Supplementary Figure S12B). This indicates that a free thiol group is necessary for the reaction, probably for a nucleophilic attack on the carbon of the isothiocyanate function. In a second experiment thioglucose protected by benzyl ether (**BnSGlc**) was prepared with a yield of 68%. Two peaks were obtained in LC-UV chromatogram with a ratio of signal intensity of 3:1, which could be the α- and β-anomer. Both anomers were isolated separately and after boiling, the signals for both anomers were observed again with a ratio of 1:1.4 respectively 4:1. That indicated that mutarotation occured. Using the separated **BnSGlc** anomers for boiling with AITC neither the product **1** nor a benzyl ether derivate were observed (Supplementary Figure S12C). The benzyl ether protection groups leads to inability of some reactions like retro-aldol reaction. The absence of the cyclic reaction product shows that free hydroxy groups are necessary to obtain the cyclic reaction product. In a further experiment, thioglucose with a benzylidene acetal protective group on the hydroxy groups linked to C4 and C6 was synthesized (**PhCHSGlc**) to test if the two hydroxy groups have to be free to obtain the cyclic product. **PhCHSGlc** was characterized by high-resolution mass spectrometry (*m/z*: $[C_{13}H_{16}O_5S+H]^+$: measured 285.0790, calculated 285.0791, difference 0.35 ppm) and showed the expected isotope pattern. The purified product contained some minor impurities, suggesting a certain degree of instability of the product during the purification. Using **PhCHSGlc** for boiling with AITC, the desired

product **1** was obtained (Supplementary Figure S12D). Therefore, it was concluded that the hydroxy groups of C4 and C6 most likely are not involved in the reaction of ITC and thioglucose. The formation of thioglucose by cleavage of the benzylidene moiety from **PhCHSGlc** during boiling was not observed.

3.5.2 Reactions using isotope-labeled reagents

To gain insight into the reaction mechanism different isotope-labeled reagents were used for the reaction of thioglucose and AITC. When using deuterium oxide as solvent for the reaction, a mass shift of 2 Da compared to using water was observed (Supplemental Figure S13A). The product was isolated and dissolved in water to exclude the possibility that the shift was caused by D/H exchange of hydroxy functions. The re-measurement still showed a mass shift of 2 Da (Supplemental Figure S14B). Using $^{13}C_6$-thioglucose instead of thioglucose lead to a product which showed a mass shift of 2 Da from *m/z*: $[M+H]^+$: 176 to 178 (Supplemental Figure S14C). This is clear evidence for thioglucose being the source of the carbon atoms C4 and C5 of the 5-membered heterocycle in compound **1** and **2** (Supplemental Figure S13B). In a third experiment the reaction of AITC and thioglucose was investigated in water-^{18}O. The integration of ^{18}O into **1** was observed by a mass shift of 2 Da (Supplemental Figure S13C). A signal of the native form *m/z*: $[M+H]^+$: 176 was detectable with around 3% signal intensity compared to *m/z* 178, which is in good accordance to the purity of the water-^{18}O with 97 atom % (Supplemental Figure S14D). From that it was concluded that the inserted deuterium and ^{18}O into **1** is due to keto-enol tautomerism respectively hydrate formation like shown in Figure 3.

3.5.3 Determination of the enantiomeric ratio

The newly formed 5-membered ring in compound **1** and **2** has a stereocenter on C4. The enantiomeric ratio of compound **1** allows a suggestion about the reaction type (like S_N1 versus S_N2) during the formation of the 5-membered ring. Due to the lack of a method to separate the two enantiomers, an approach via derivatization to diastereomers and their separation by HPLC was chosen. For the development of an appropriate HPLC method access to racemic **1** is required. The three component reaction published by Stalling et al., not being related to food, describes the synthesis of compound **1** as racemate (Stalling et al., 2013). The product was synthesized based on Stalling et al. and had the same retention time, mass spectrum and fragmentation pattern like compound **1**. The route via the synthesis of the reference standard is an additional confirmation of the identification of the compound. After derivatization the two diastereomers could be separated by using a PFP column with retention times of 82.1 min and 84.2 min. The UV signal intensity as well as the signal intensity of the extracted ion chromatogram (EIC) *m/z* 322 showed a signal intensity ratio of 1:1 for the two peaks, as expected. After derivatization of the product obtained from the reaction of AITC and thioglucose, the 2 diastereomers were detected at the expected retention times and with a signal intensity ratio of 1:1 (in EIC *m/z* 322 and LC-UV chromatogram) too. A racemic mixture can obtain from reactions with planar carbon centers in the carbonyl group or carbocations.

3.5.4 Retro-aldol reaction of thioglucose – the origin of the C_2H_4SO building block

The formation of a C_2H_4SO moiety from thioglucose might be possible via a retro-aldol reaction, which is well known for glucose (Yamaguchi & Baba, 2016), but to our knowledge not reported for thioglucose during boiling so far. To obtain further evidence for the retro-aldol reaction of thioglucose, we attempted to quench the formed C_2H_4SO moiety (mercaptoacetaldehyde) from boiling thioglucose with maleimide. The reaction between thiols and maleimide is well known just as the problem of hydrolysis of the succinimide ring (Huang et al., 2019). To minimize interferences with different reaction products, the boiling time of thioglucose and maleimide was reduced and only the non-hydrolyzed product of mercaptoacetaldehyde and maleimide was taken into account. Mercaptoacetaldehyde should be used as reference, but due to the lack of availability of it, the mercaptoacetaldehyde dimer was used. It is reported that this is degraded to mercaptoacetaldehyde even under slightly basic, mild conditions (Baricordi et al., 2012). Boiling of thioglucose or the mercaptoacetaldehyde dimer, respectively, led to the desired product with maleimide. The mass spectrum with given accordance between measured and expected mass (*m/z*: $[C_{20}H_{23}N_3O_5S_2+K]^+$: measured 488.0709, calculated 488.0711, difference 0.51 ppm) as well as the isotope pattern is shown in Supplemental Figure S15. The potassium adduct was the most intensive signal likely because of the use of potassium phosphate buffer. The EIC of a small mass range *m/z* 488.065–488.075 showed for both approaches using thioglucose or mercaptoacetaldehyde dimer two signals at 11.0 min and 11.1 min. The signal intensity by using mercaptoacetaldehyde dimer was than 40 times higher because of the higher yield release of mercaptoacetaldehyde compared to thioglucose (Supplemental Figure S16). The reaction product of thioglucose respectively allyl mercaptan with maleimide resulted in a single peak as expected. When the reaction between maleimide and mercaptoacetaldehyde dimer was performed at room temperature instead of 100 °C the ratio of the two signals shifted from 1:1 to 1:3. This

could be an indication that there are different isomeric forms for mercaptoacetaldehyde, whose ratios change depending on the temperature. The product of thioglucose and maleimide elute at 9.6 min, thus an interference of the maleimide-thioglucose product by insource fragmentation was excluded. These results show that the formation of mercaptoacetaldehyde from thioglucose took place. Additionally, the formation of compound **1** was also observed in the boiled reaction mixture using AITC and mercaptoacetaldehyde dimer.

3.6 Quantification of contents of glucosinolates, isothiocyanates and boiling reaction products of isothiocyanate and thioglucose in red and white cabbage

To study whether the new cyclic reaction products are also formed in *Brassica* foods during cooking, samples of boiled cabbage were analyzed. In Table 1 GLS contents in fresh and boiled cabbage samples are presented. Sinigrin content in fresh red and white cabbage was similar, but red cabbage contained more than 10 times more glucoraphanin compared to white cabbage (Table 1). We next sought to determine the content of thioglucose after 60 min boiling. The estimated limit of detection using external calibration standards was 50 nmol/g FW. Free thioglucose was not detectable in either boiled red or white cabbage. Besides mercaptoacetaldehyde as a product of thioglucose, ITC is the second reagent for the formation of the cyclic reaction product. Therefore the concentrations of AITC and sulforaphane were determined (Table 2). Due to the boiling period the contents of the two ITCs strongly declined. The contents after 60 min boiling were slightly below the limit of quantification and should be regarded as approximated values. In Table 3 the quantified amounts of the new cyclic transformation products **1** and **2** in the boiled cabbages are presented. In red cabbage compound **2** was more than four-times as abundant compared to compound **1**, while

compound **2** could not be detected in boiled white cabbage. The levels of compound **1** are very similar for red and white cabbage. During boiling the cells of the plant material are destroyed and water is released into the cooking solution. The amount of released water is unknown, why a calculation from concentrations to amounts per g FW is not possible. Therefore and because of strong matrix effects during mass spectrometry measurements, the standard addition method was used for quantification.

4. Discussion

In the present study novel thermal glucosinolate degradation products have been detected and identified. The compounds originating from the reaction of AITC or sulforaphane and thioglucose, respectively, have been identified to be 3-allyl-4-hydroxythiazolidine-2-thione (**1**) and 4-hydroxy-3-(4-(methylsulfinyl)butyl)thiazolidine-2-thione (**2**). In future, with the known structure, the synthesis should be performed by using the ITC and 1,4-dithiane-2,5-diol as reported by Kumar et al. (Kumar, Muthusubramanian, & Perumal, 2015). Based on the results of the present study a mechanism for the formation of 3-alk(en)yl-4-hydroxythiazolidine-2-thiones from isothiocyanates and thioglucose during boiling was postulated (Figure 3). The mechanism involves retro-aldol reaction of thioglucose to yield mercaptoacetaldehyde, which then reacts with the electrophilic carbon atom of the ITC function and ring closure by nucleophilic attack of the nitrogen to the carboxylic group of the aldehyde function. To the best of our knowledge this the first time that the formation of retro-aldol reaction fragments is reported for thioglucose during cooking at 100 °C under neutral to slightly basic conditions.

Beside the here presented alk(en)yl-4-hydroxythiazolidine-2-thiones other structurally similar compounds has been reported as transformation product of ITCs: Raphanusamic acid [(4*R*)-2-thioxo-1,3-thiazolidine-4-carboxylic acid] which likely yields from the glutathione conjugate of indol-3-ylmethyl ITC (Bednarek et al., 2009; Blaževic et al., 2020), the cyclic GLS degradation product sativin (1,3-thiazepane-2-thione) which is formed from spontaneous cyclization of 4-mercaptobutyl-ITC (Fechner et al., 2018) or the 1,3-oxazolidine-2-thione derivatives that are formed upon cyclization of 2-hydroxy-alkenyl ITCs (Radulović, Todorovska, Zlatković, Stojanović, & Randjelović, 2017).

In the present study in experiments with cabbage it could be shown that the cyclic 3-alk(en)yl-4-hydroxythiazolidine-2-thiones were also formed in boiled cabbage. Especially cabbages and broccoli are a good source for these ITCs (Hanschen & Schreiner, 2017): In red cabbage amounts up to 1.06 µmol/g FW have been reported to be released from the native GLSs (Wermter, Rohn, & Hanschen, 2020). The obtained amount in this study of 0.07 µmol/g FW sulforaphane for the samples heated up to 100 °C is clearly lower, but myrosinase likely was inactivated by heating up the cabbage immediately as it is inactivated at temperature of 45 °C or higher (Van Eylen, Oey, Hendrickx, & Van Loey, 2007). Additionally, during the heat up period degradation of the ITCs can be expected. For example for sulforaphane a half-live of 0.86 h has been reported in aqueous buffer at pH 6 and 90 °C (Wu, Mao, Mei, & Liu, 2013). During the boiling period of 60 min, AITC and sulforaphane were degraded to approximately 2–6% compared to their initial concentration, after heating up to 100 °C in the cabbage samples, which was similar to the results of the model experiments (Figure 1) and was similar to previous reports (Hanschen, 2020). The presence of ITC even after 60 min

boiling time indicates that the limiting reagent for the formation of the new cyclic reaction product is mercaptoacetaldehyde respectively thioglucose.

Sulforaphane was not quantifiable in treated white cabbage samples, probably due to the lower content of glucoraphanin in white cabbage compared to red cabbage. This finding and the measured contents of approximately 0.27 µmol/g FW sinigrin and 0.87 µmol/g FW glucoraphanin in red cabbage were in accordance to previous studies (Ciska, Martyniak-Przybyszewska, & Kozłowska, 2000; Wermter et al., 2020). However, small amounts of the degradation product sulforaphane nitrile [5-(methylsulfinyl)pentanenitrile] were detected after 60 min boiling in white cabbage. Nitrile formation originating from GLS by releasing thioglucose during thermal treatment, in which iron ions can be involved, was investigated in previous studies (Bellostas et al., 2008; Hanschen, Bauer, et al., 2012). GLS can be thermally degraded whereby during 60 min boiling approximately 130 nmol/g fresh weight (FW) sinigrin and 50 nmol/g FW glucoraphanin in red cabbage respectively 100 nmol/g FW singrin and 20 nmol/g FW glucoraphanin in white cabbage were converted. This conversion of GLS could be the origin of the release of thioglucose, which is necessary for the formation of compound **1** and **2**. The percentage decrease of sinigrin and glucoraphanin during boiling differed for the different cabbages in a range of approximately 10–60%, which is in a range reported by Hanschen et al. for glucoraphanin with more than 40% after 80 min boiling at pH = 8.2 (Hanschen, Rohn, et al., 2012).

In comparison to the degraded total amount of GLS with more than 1000 nmol/g FW in red cabbage during boiling, free thioglucose could not be detected in the present study. Considering the limit of detection it can be only stated that after the boiling period less than 5% of degraded glucosinolate is present as free thioglucose. However, it has

649 to be taken into account that just the concentration after boiling was measured and not
650 the release during the cooking period and just free thioglucose was measureable.
651 Thioglucose which is formed during the boiling and reacting with matrix components
652 or degraded like for the formation of the new cyclic reaction products cannot be
653 detected by this approach. In order to test the applicability of the method, the content
654 of glucose was determined in parallel. The quantified contents of approximately
655 90 mg/g dry mass for glucose in the boiled cabbage samples are lower than in
656 Bhandari et al. with 116–272 mg/g dry mass for fresh freeze-dried white cabbage, but
657 in a similar range (Bhandari et al., 2021). Therefore, the applicability of the method to
658 quantify sugars like thioglucose in cabbage samples is given.

659 The amounts of compound **1** and **2** in boiled cabbage are relatively low, but compared
660 to the ITC the cyclic reaction product seems to be less reactive and therefore less
661 degraded at 100 °C. With longer boiling time the ITC concentration will further
662 decrease (Hanschen, Brüggemann, et al., 2012). For example for sulforaphane a half-
663 live of approximately 7 min at pH 7.3 in heated *Eruca sativa* L. homogenates has been
664 reported (Fechner et al., 2018). Whereby the concentration of the cyclic reaction
665 product will be more constant. After 60 min of boiling, the content ratios between cyclic
666 reaction products to corresponding ITCs were approximately 15% and 3.5% in red
667 cabbage respectively 25% in white cabbage. Therefore, for longer boiling times the
668 new cyclic products were more abundant compared to ITCs. The content ratio of
669 compound **2** to **1** in red cabbage was approximately 5 and therefore in a similar range
670 like the content ratio of sulforaphane to AITC with approximately 20 after the boiling
671 time of 60 min, which indicates that there is no strong difference in the affinity of the
672 different ITCs to form the cyclic reaction product.

The fact that these cyclic products are formed in boiled *Brassica* vegetables raises the question whether these compounds affect human health. The calculated values of log P of compounds **1** and **2** indicate that they are probably suitable for cell uptake. For structurally similar compounds their potential to inhibit the glycogen synthase kinase-3 (Noori et al., 2019) or working as tumor cell specific pyruvate kinase M2 activator was shown (Li et al., 2018). The measured amounts of compounds **1** and **2** in boiled cabbage samples in the present study indicate that with an ordinary meal the consumed amount of 3-alk(en)yl-4-hydroxythiazolidine-2-thiones is in the nanomolar range.

5. Conclusion

In the present study, the formation of a new novel class of thermal GLS degradation reaction products have been shown, that originate from ITCs and thioglucose. Their structure was elucidated by high-resolution mass spectrometry, NMR and via the synthesis of the reference standard. The formation mechanism includes the formation of mercaptoacetaldehyde from thioglucose, which then reacts with ITCs. In boiled cabbages the 3-alk(en)yl-4-hydroxythiazolidine-2-thiones were detected and quantified. In view of the uptake of this compounds by consumption of boiled *Brassica* vegetables their bioavailability and potential as bioactive compounds should be tested in future.

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Figure captions

Figure 1: A) UPLC-DAD chromatogram at 240 nm for samples boiled for 1 h at 100 °C black line: boiled buffer at pH = 7, red line: AITC in buffer at pH = 7 without boiling, green line: AITC in buffer at pH = 7 boiled and blue line: mixture of AITC and thioglucose in buffer at pH = 7 boiled. B) UPLC-DAD chromatogram at 274 nm for samples boiled for 1 h at 100 °C black line: AITC boiled in buffer at pH = 7 and boiled mixture of AITC and thioglucose red line: at pH = 6, green line: at pH = 7 and blue line: at pH = 8.

Figure 2: Structures with marked stereocenters of the two novel isolated reaction products 3-allyl-4-hydroxythiazolidine-2-thione (compound **1**, left) and 4-hydroxy-3-(4-(methylsulfinyl)butyl)thiazolidine-2-thione (compound **2**, right) as products from a 1 h at 100 °C boiled mixture of thioglucose and the isothiocyanate AITC (left) respectively sulforaphane (right) formed due to aqueous heat treatment with assignment of the NMR signals obtained in ^{1}H NMR spectra for protons respectively signals in ^{13}C NMR spectra for carbon atoms (italic).

Figure 3: Proposed reaction mechanism for the reaction of the glucosinolate breakdown products isothiocyanates and thioglucose to form thermally stable 3-alk(en)yl-4-hydroxythiazolidine-2-thiones taking into account the results from section 3.5.

841 **Table 1:** Contents of selected glucosinolates as well total glucosinolates contents in
842 cabbage samples after different treatments

Glucosinolate	Content untreated (nmol/g fresh weight)	Content after aqueous heating up to 100 °C (nmol/g fresh weight)	Content after 60 min of boiling (nmol/g fresh weight)
Red cabbage			
Sinigrin	272 ± 6	185 ± 16	52 ± 11
Glucoraphanin	866 ± 30	635 ± 90	587 ± 44
Total glucosinolates	3470 ± 140	2760 ± 290	1700 ± 180
White cabbage			
Sinigrin	234 ± 73	132 ± 8	31 ± 2
Glucoraphanin	61 ± 22	32 ± 3	12 ± 2
Total glucosinolates	1550 ± 250	1040 ± 80	487 ± 41

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844

845 **Table 2:** Allyl isothiocyanate and sulforaphane levels in plant samples after different
846 heating treatments

Isothiocyanate	Content after heating up to 100 °C (pmol/g fresh weight)	Content after 60 min boiling (pmol/g fresh weight)
Red cabbage		
Allyl isothiocyanate	2080 ± 140	≈ *126 ± 6*
Sulforaphane	69240 ± 410	≈ *2650 ± 60*
White cabbage		
Allyl isothiocyanate	3630 ± 250	≈ *77 ± 6*

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848

849 **Table 3:** Contents of the new cyclic reaction products **1** and **2** after boiling calculated

850 on plant sample fresh weight

New cyclic reaction product	Content after 60 min boiling (pmol/g fresh weight)
Red cabbage	
1	18.8 ± 0.2
2	92 ± 4
White cabbage	
1	19.2 ± 0.8

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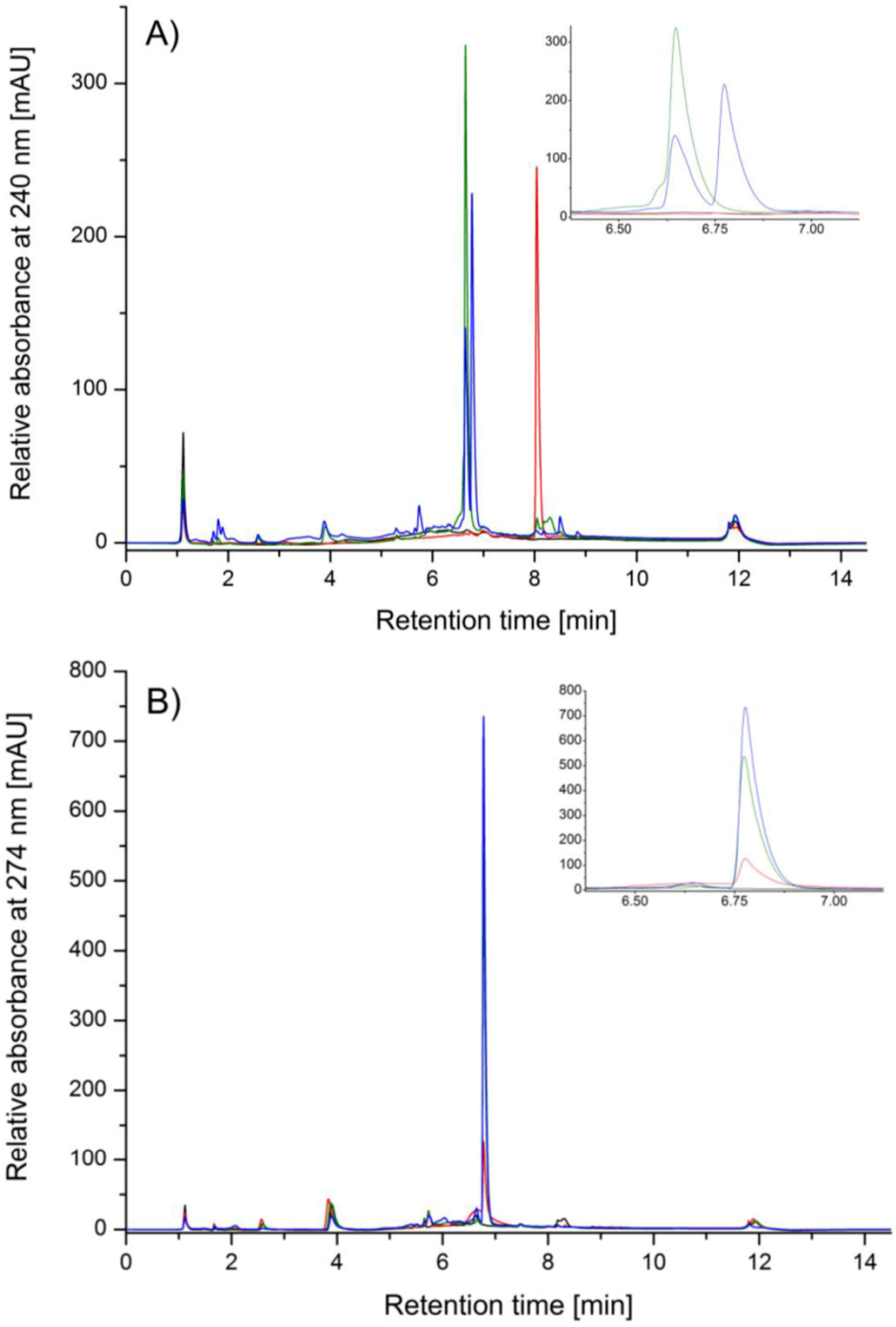

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854 **Figure 1**

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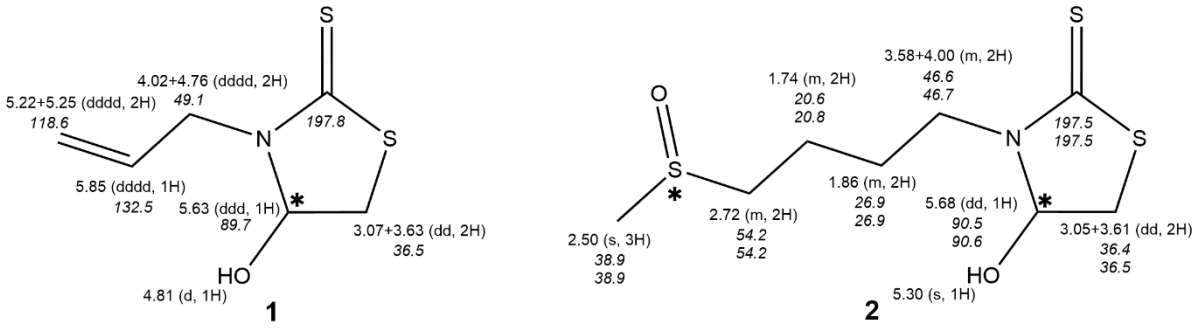

856 3-allyl-4-hydroxythiazolidine-2-thione 4-hydroxy-3-(4-(methylsulfinyl)butyl)thiazolidine-2-thione

857 **Figure 2**

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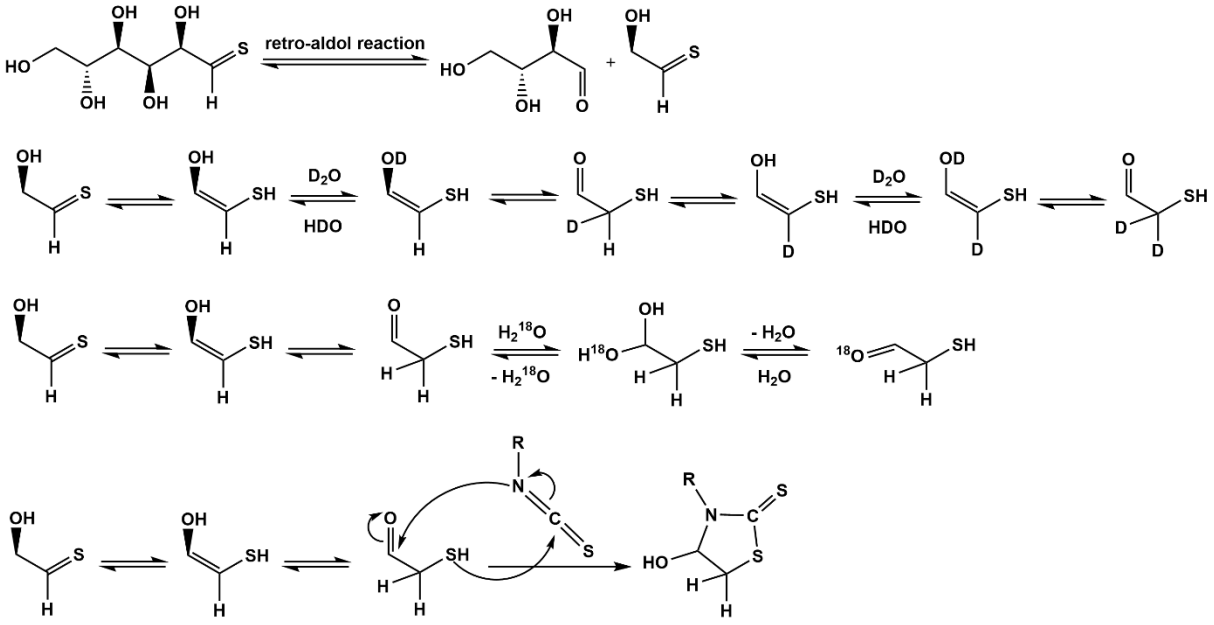

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860 **Figure 3**