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Brassica vegetables as sources of epithionitriles: Novel secondary products formed during cooking

Franziska S. Hanschen[a,*], Martin Kaufmann[b], Franziska Kupke[c], Thomas Hackl[d], Lothar W. Kroh[b], Sascha Rohn[c], Monika Schreiner[a]

[a] Plant Quality and Food Security, Leibniz Institute of Vegetable and Ornamental Crops, Theodor-Echtermeyer-Weg 1, 14979 Grossbeeren, Germany
[b] Department of Food Chemistry and Analysis, Institute of Food Technology and Food Chemistry, Technische Universität Berlin, TIB 4/3-1, Gustav-Meyer-Allee 25, 13355 Berlin, Germany
[c] Institute of Food Chemistry, Hamburg School of Food Science, University of Hamburg, Grindelallee 117, 20146 Hamburg, Germany
[d] Institute of Organic Chemistry, Hamburg School of Food Science, University of Hamburg, Martin-Luther-King-Platz 6, 20146 Hamburg, Germany

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ABSTRACT

The epithionitriles, 1-cyano-2,3-epithiopropane, in particular, and 1-cyano-3,4-epithiobutane, are important, but yet underestimated glucosinolate hydrolysis products that are released instead of the cancer preventative isothiocyanates in *Brassica* vegetables, such as cabbage, broccoli, or pak choi. Here, we characterized the reactivity of 1-cyano-2,3-epithiopropane under aqueous heat treatment conditions and compared our findings to those of the related epithionitriles 1-cyano-3,4-epithiobutane and 1-cyano-4,5-epithiopentane. In contrast to the other epithionitriles, 1-cyano-2,3-epithiopropane is highly reactive. As a result, 2-aminothiophene and dimeric 1,4-dithiane-2,5-diacetonitrile were identified as main products and a reaction mechanism is proposed. Formation of 2-aminothiophene was also observed in cooked white cabbage samples. Moreover, three novel compounds were identified as derivatives of the related epithionitriles. The results imply that apart from isothiocyanates, process-derived compounds should be considered in regards to cancer preventative *Brassica* vegetable related bioactivity.

1. Introduction

The allylic 2-propenyl glucosinolate (sinigrin) (**1**) (Fig. 1) is considered to be the most abundant aliphatic glucosinolate (GLS) in the human diet as it is widely distributed in the botanical order Brassicales (Daxenbichler et al., 1991; Fahey, Zalcmann, & Talalay, 2001) and daily intake is estimated to be 1.7 and 2.5 mg for men and women in Germany, respectively (Steinbrecher & Linseisen, 2009). Enzymatic hydrolysis of GLS induced by, e.g. chewing or food processing, leads primarily to the formation of electrophilic isothiocyanates (ITCs). Those volatile hydrolysis products have a controversially discussed pharmacological potential such as antimicrobial, anti-inflammatory, antithrombotic, as well as cancer preventive properties (Ku & Bae, 2014; Singh & Singh, 2012; Veeranki, Bhattacharya, Tang, Marshall, & Zhang, 2015; Zhang, 2012). Consequently, studying the fate of GLSs and the formation of their breakdown products has a global importance as *Brassica* vegetables, such as cabbage (*B. oleracea* var. *capitata*), broccoli (*B. oleracea* var. *italica*), or pak choi (*B. rapa* ssp. *chinensis*), are regularly consumed all over the world. Their pharmacological potential is discussed to be exploited as remedies in cancer therapy (Novío, Cartea, Soengas, Freire-Garabal, & Núñez-Iglesias, 2016). Frequently, *Brassica* vegetables release epithionitriles (EPTs) and nitriles instead of ITCs upon enzymatic hydrolysis (Hanschen et al., 2015; Klopsch, Witzel, Börner, Schreiner, & Hanschen, 2017; Kyung, Fleming, Young, & Haney, 1995; Rungapamestry, Duncan, Fuller, & Ratcliffe, 2006). This is due to the presence of the so-called epithiospecifier proteins (ESPs). These proteins interact with the labile thiohydroximate-*O*-sulfate GLS aglucon, which is formed by the plant endogenous enzyme myrosinase when plant cells are disrupted. Thus, alkenyl GLS aglucons are rearranged to EPTs and the spontaneous degradation to ITCs is prevented (Matusheski et al., 2006). While ITCs are known to have health-promoting effects, for the EPTs, such as 1-cyano-2,3-epithiopropane (**2**) (other names: 3,4-epithiobutanenitrile, thiiraneacetonitrile), the situation is more controversial. In detail, compound **2** was reported to induce necrosis in both cancerous and healthy liver cells (Hanschen et al., 2015). Moreover, oral administration of a single dose of 50–125 mg/kg body weight (BW) of EPTs to rodents induced nephrotoxic effects that were accompanied by karyomegaly, kidney lesions, and necrosis

* Corresponding author.
E-mail addresses: hanschen@igzev.de (F.S. Hanschen), martin.kaufmann@tu-berlin.de (M. Kaufmann), franziska.kupke@chemie.uni-hamburg.de (F. Kupke), hackl@chemie.uni-hamburg.de (T. Hackl), lothar.kroh@tu-berlin.de (L.W. Kroh), rohn@chemie.uni-hamburg.de (S. Rohn), schreiner@igzev.de (M. Schreiner).

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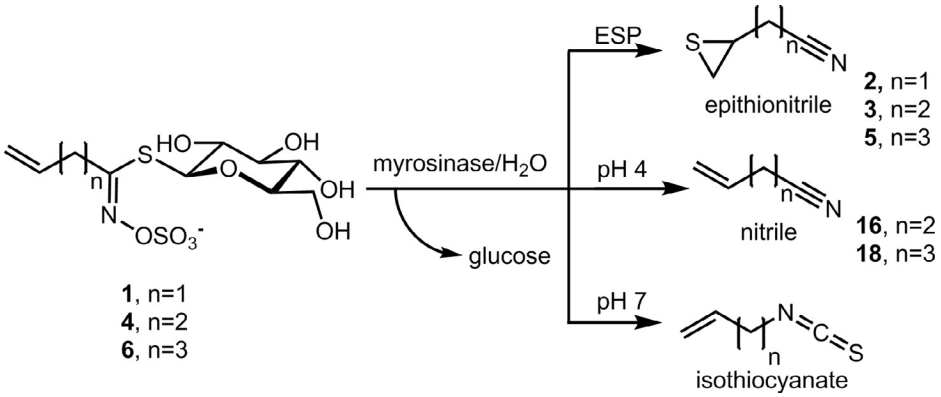

Fig. 1. Enzymatic hydrolysis of glucosinolates and formation of epithionitriles **2**, **3**, and **5** investigated in this study.

(Gould, Gumbmann, & Daxenbichler, 1980; Nishie & Daxenbichler, 1980; VanSteenhouse, Fettman, & Gould, 1989; Wallig, Gould, Fettman, & Willhite, 1988). In contrast, compound **2** was observed to have chemopreventative effects such as the ability to induce phase-II enzymes in a similar mode of action compared to the related isothiocyanate (Kelleher et al., 2009), thereby indicating that EPTs might have cancer preventative properties to some extent. However, *Brassica* vegetables are often not eaten raw, but are cooked prior to consumption – a process that inhibits enzymes as well as leaches and degrades GLSs (Hanschen, Lamy, Schreiner, & Rohn, 2014; Sarvan, Verkerk, & Dekker, 2012). Currently, literature on the reactivity of EPTs is scarce. As being thiiranes, sulfur analogs of epoxides, EPTs were reported to polymerize at room temperature (RT) due to the ring opening of the epithiogroup (Fokin & Kolomiets, 1976; Lüthy & Benn, 1980). Covalent binding to DNA and mutagenicity has also been reported for some EPTs (Lüthy et al., 1981). However, there has been no comprehensive study on the chemistry of these natural compounds in foods or under cooking conditions. Herein, we report a study on the reactivity of compound **2**, the main GLS hydrolysis product of cabbage (Hanschen & Schreiner, 2017), and focus on its follow-up reactions. The reactivity of the three EPTs, **2**, 1-cyano-3,4-epithiobutane [**3**; 4,5-epithiopentanenitrile, a product of 3-butenyl GLS (gluconapin) (**4**)], and 1-cyano-4,5-epithiopentane [**5**; 5,6-epithiohexanenitrile, a product of 4-pentenyl GLS (glucobrassicanapin) (**6**)] (Fig. 1) during aqueous heat treatment was studied in model solutions as well as in a vegetable matrix ('cooking').

2. Materials and methods

2.1. Chemicals and buffers

Benzonitrile (≥99.9%), KH_2PO_4, Na_2HPO_4, CH_3COOK, and methylene chloride-d_2 (≥99.9%) were purchased from Sigma-Aldrich Chemie GmbH, (Steinheim, Germany). Methylene chloride (GC Ultra Grade), imidazole (p.A.), HCl (37%, p.A.), and acetic acid (100%) were obtained from Carl Roth GmbH (Karlsruhe, Germany). $NaSO_4$ (≥99%) was purchased from VWR International GmbH (Darmstadt, Germany). Compound **2** (≥97.6%) was purchased from Taros Chemicals GmbH Co. KG (Dortmund, Germany). Compound **3** (≥99%) and compound **5** (≥99%) were purchased from ASCA GmbH Angewandte Synthesechemie Adlershof (Berlin, Germany). 2-Aminothiophene × HCl (compound **7**, ≥97%) was obtained from Sirius Fine Chemicals SiChem GmbH, Bremen, Germany. Acetonitrile (LC-MS grade) was purchased from Th. Geyer GmbH & Co. KG (Renningen, Germany). All solvents were of LC-MS grade and water was of Milli-Q quality.

2.2. Studies of epithionitrile reactivity and sample preparation

2.2.1. Epithionitrile reactivity in model systems

In a first approach, 1 mL solutions of 1 mM compound **2** with or without 1 mM glutathione (GSH) containing 800 µL of 0.1 M potassium acetate buffer (pH 5; CH_3COOK/CH_3COOH) or imidazole buffer (pH 7.8; 0.2 M imidazole/0.1 M HCl) were sealed into 20 mL headspace vials and stored at RT for 6 h or 24 h (only without GSH) or treated (without GSH) at 100 °C for 30 or 60 min in a thermoshaker (type MHR 11; Digitabis AG, Pforzheim, Germany) under continuous shaking. After treatment, samples were cooled on ice and subjected to analysis by GC–MS and LC-ESI-TOF-MS. Each treatment was carried out in triplicate.

Experiments on thermal reactivity were performed using 1 mL solutions of 1 mM epithionitrile (compound **2**, **3**, or **5**) containing 800 µL of 1/15 M phosphate buffer (pH 5, pH 7, or pH 8; KH_2PO_4/Na_2HPO_4) closed in a 20 mL headspace vial. Samples were treated for 5, 15, 35, 65, and 125 min (only pH 5) at 100 °C in the thermoshaker as described above. A come-up time of 5 min to reach 100 °C is included in the treatment times.

2.2.2. Epithionitrile reactivity in Brassica vegetable matrices

White cabbage (forms compound **2**) (*Brassica oleracea* var. *capitata* f. *alba* cv. Tolsma F1; Rijk Zwaan, Blankensee, Germany) and pak choi (forms compounds **3** and **5**) (*B. rapa* ssp. *chinensis* cv. Black Behi) seeds were sown on water soaked fleece in aluminum trays filled with perlite and water. Using a water sprayer, seeds were atomized daily until germination and sprouts were grown in a greenhouse and harvested at 8 days old. At harvest, sprouts were mixed with water (1:1) and homogenized within 3 min using a vibratory mill (MM400 Retsch GmbH, Germany) at 30 Hz and this homogenate again was diluted with water to result in a plant homogenate containing 200 mg plant/mL. The pH was then adjusted to pH 5.0 or pH 7.0 by adding 1/15 M phosphate buffer (KH_2PO_4/Na_2HPO_4) or water was added for **2** at pH 5.4, and the final concentration of plant material was 0.1 g/mL. Of this homogenate, 2 mL were added into a 20 mL headspace vial and the vial was tightly shut and treated for up to 2.25 h at 100 °C in the thermoshaker as described above. Each experiment was carried out three times.

2.3. Sample preparation for NMR measurements

For compound **2**, 20 mg were dissolved in 200 mL of water and filled into ten 20 mL headspace vials and then treated for 1 h (7 vials) or 1.5 h (5 vials) in the thermoshaker as described above. The contents of the vials for each treatment were then combined and cooled to RT. After which, 200 mL were added to a 5 g CROMABOND® C18 ec column (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) pre-conditioned with 20 mL of acetonitrile and 20 mL of water. The column was then washed twice with 10 mL of water, and several 10 mL volumes of 10%, 25%, and 40% acetonitrile were used for elution and fractions were collected. The monomeric product **7** was detected in the 200 mL eluate and the first 10% acetonitrile fractions (brownish), and these fractions were combined. Moreover, fractions containing the dimeric compound **8** (25% and 40% acetonitrile fractions, yellow color) were