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Derivatization of isothiocyanates and their reactive adducts for chromatographic analysis

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ABSTRACT

Isothiocyanates form adducts with a multitude of biomolecules, and these adducts need analytical methods. Likewise, analytical methods for hydrophilic isothiocyanates are needed. We considered reaction with ammonia to form thiourea derivatives. The hydrophilic, glycosylated isothiocyanate moringin, 4-(α-L-rhamnopyranosyloxy)benzyl isothiocyanate, was efficiently derivatized to the thiourea derivative by incubation with ammonia. The hydrophobic benzyl isothiocyanate was also efficiently derivatized to the thiourea derivative. The thiourea group provided a UV absorbing chromophore, and the derivatives showed expectable sodium and hydrogen adducts in ion trap mass spectrometry and were suitable for liquid chromatography analysis. Reactive dithiocarbamate adducts constitute the major type of reactive ITC adduct expected in biological matrices. Incubation of a model dithiocarbamate with ammonia likewise resulted in conversion to the corresponding thiourea derivative, suggesting that a variety of matrix-bound reactive isothiocyanate adducts can be determined using this strategy. As an example of the application of the method, recovery of moringin and benzyl isothiocyanate applied to cabbage leaf discs was studied in simulated insect feeding assays. The majority of moringin was recovered as native isothiocyanate, but a major part of benzyl isothiocyanate was converted to reactive adducts.

1. Introduction

Glucosinolates are characteristic constituents of plants of the order Brassicales, including cabbages and many other wild and cultivated plants (Agerbirk and Olsen, 2012). Upon tissue disruption, e.g. due to chewing by animals, glucosinolates are converted to isothiocyanates (or sometimes other products) due to endogenous enzymes, myrosinases (β-thioglucoside glucohydrolases; E.C. 3.2.1.147). Isothiocyanates (ITCs) are reactive with nucleophiles such as cysteine and proteins (Nakamura et al., 2009; Brown and Hampton, 2011; Hanschen et al., 2012), and this reactivity is thought to be a major reason for their toxicity to many organisms (Holst and Williamson, 2004; Winde and Wittstock, 2011). Some ITC adducts are themselves reactive with nucleophiles, and might be equally toxic (Nakamura et al., 2009; Brown and Hampton, 2011). This heterogeneous group is collectively called "reactive adducts" in the following, and would be expected to mainly include thiol adducts and similar adducts derived from oxidative scission of disulfide bonds (Kawakashi and Kaneko, 1985). In addition, more stable adducts with amines can be formed (Nakamura et al., 2009; Brown and Hampton, 2011), while biological adducts with alcohols are mainly known from intramolecular reactions (Agerbirk et al., 2014; Agerbirk and Olsen, 2015). A final type of matrix binding is non-covalent binding of lipophilic ITCs to, e.g., serum proteins (Ji et al., 2005).

Insects are potential targets of ITCs (Winde and Wittstock, 2011). There is some uncertainty in actual levels of ITCs offered in various published insect feeding tests either topically applied to other leaf disks or otherwise incorporated into diet, as discussed elsewhere (Müller et al., submitted for publication), due to the possibility of loss by evaporation and loss by chemical reaction with matrix components. In order to be able to quantify remaining ITCs in insect feeding assays after solvent evaporation, a non-volatile ITC would be useful for comparison with usually tested volatile ITCs like allyl ITC and benzyl ITC. We expected the rare class of side chain-glycosylated glucosinolates (Kjær et al., 1979; Olsen and Sørensen, 1979; Olsen et al., 1981; Gueyrard et al., 2000; Bennett et al., 2003; Kim et al., 2004) to form particularly non-volatile ITCs. A representative glucosinolate from this structural class is

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4-(α-L-rhamnopyranosyloxy)benzylglucosinolate (**1**) (Kjær et al., 1979; Gueyrard et al., 2000), also known as glucomoringin (Fig. 1). Glucomoringin occurs naturally in species of the tropical plant genus *Moringa* and in at least one other plant species (De Graaf et al., 2015; Förster et al., 2015a,b). The corresponding ITC is 4-(α-L-rhamnopyranosyloxy)benzyl ITC (**2**), with the suggested common name moringin (Müller et al., submitted for publication).

As a representative volatile ITC, we chose benzyl ITC (**3**), the natural hydrolysis product of benzylglucosinolate (**2**) that occurs naturally in garden cress (*Lepidium sativum*), white mustard (*Sinapis alba*), maca (*Lepidium meyenii*) and many wild plants from the Brassicales order (Burow et al., 2007; Agerbirk et al., 2008, 2010; Esparza et al., 2015) (Fig. 1). Recently, benzylglucosinolate was also transferred to a non-Brassicales, tobacco, by metabolic engineering (Møldrup et al., 2012).

In order to test experimentally the expected higher recovery of the hydrophilic moringin compared to benzyl ITC, and whether any native benzyl ITC or corresponding reactive adducts would remain after solvent evaporation, a suitable analysis method was needed. In addition to direct analysis of the native ITC (Müller et al., submitted for publication), another option was the classical derivatization with dilute ammonia in alcohol known to give thiourea derivatives detectable by UV spectroscopy or paper chromatography (e.g. Appelquist and Josefsson, 1967; Harborne, 1973; Olsen and Sørensen, 1979). A seemingly realistic hope was to include reactive adducts in analysis based on thiourea derivatives. In case of a hydrophobic ITC, adaption to contemporary liquid chromatography with MS/MS detection had been demonstrated (Ji and Morris, 2003).

The aims of this work were (1) to examine the conversion of ITCs and dithiocarbamates to thiourea derivatives, (2) to characterize and quantitate the thiourea derivatives using NMR, ion trap MS/MS and liquid chromatography, and (3) to critically examine the recovery of ITCs and any reactive adducts under simulated insect feeding assay conditions. We show that both ITCs and a dithiocarbamate are converted to thioureas at mild conditions, and characterize the thioureas. Applying this derivatization, we show that a non-volatile ITC including reactive adducts can be recovered after incubation at feeding assay conditions, and that the majority is present as the intact ITC. In contrast, the vast majority of a volatile ITC spiked to leaf discs is lost by evaporation, but significant amounts of free ITC and reactive adducts remain on cabbage leaf discs even after extended evaporation in simulated feeding assays.

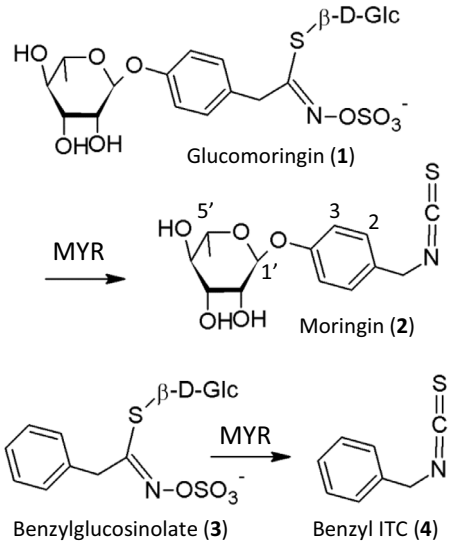

Fig. 1. Investigated glucosinolates (benzylglucosinolate and glucomoringin) and their enzymatic conversion to isothiocyanate products (unbalanced). The NMR-numbering system of moringin is indicated. MYR: myrosinase.

2. Results and discussion

2.1. Isothiocyanates react quantitatively with ammonia to form thiourea derivatives

Conversion of moringin to the thiourea derivative **5** under mild conditions (5% NH_3 in 80% aq. MeOH at room temperature overnight) was tested. Analysis of the crude remnant by ^{1}H NMR and HPLC after evaporation of the solvent showed complete conversion essentially to a single product with good chromatographic peak shape in HPLC-MS and HPLC-PDA: moringin thiourea (**5**). The UV spectrum of **5** showed the expected thiourea chromophore with an absorption band near 240 nm (Appelquist and Josefsson, 1967), suitable for routine detection in HPLC-PDA.

Benzyl ITC (**4**) also gave essentially a single reaction product under these derivatization conditions, benzyl thiourea (**6**) (Section 3.7), which likewise showed good chromatographic peak shape and a characteristic and useful UV absorbance band around 240 nm. In general, this adaptation of the classical 'thiourea' method for ITC analysis by liquid chromatography is attractive: all ITCs can be expected to react and as the UV absorptivity of the thiourea group will be the same, quantification is possible by UV detection and comparison with a commercially available ITC as standard (except if the remaining part of the molecule contains conjugated systems with overlapping UV absorbance). Although volatile ITCs are well suited for gas chromatographic determination, inclusion of polar, non-volatile ITCs would be possible using the thiourea method.

The identity of moringin thiourea (**5**) was further confirmed by MS/MS and NMR spectroscopy after HPLC isolation. Ion trap mass spectra of the sodium and hydrogen adducts of **5** were characteristic and in agreement with the expected structure (Fig. 2). In ^{1}H NMR, a sharp singlet (2H) at 4.7 ppm from the benzylic CH_2-group was observed for moringin but was absent from the spectrum of the thiourea **5**. Instead, a broad signal with three maxima between 4.2 and 4.7 ppm was observed from the benzylic CH_2-group in **5**, interpreted as a result of tautomerism in the thiourea moeity with a rate of conversion in the "NMR time scale". A similar signal from the benzylic CH_2 group was observed for the simpler benzyl thiourea **6**, suggesting that this signal shape is a general feature of a monosubstituted thiourea. The chemical shift of the thiourea carbon in **5** could not be observed in ordinary ^{13}C NMR. Neither could C1 (identified in HMBC), in both cases lack of detection was probably because of their quaternary nature. The thiourea C was expected near 180 ppm) (Lu et al., 2015). Unfortunately, at the time of the measurement of the HMBC spectrum, the chemical shift range was set at 175 ppm, so it is uncertain whether the chemical shift of the thiourea C could be identified in this spectrum by correlation with the CH_2 protons, or whether the broad shape of the latter signal would interphere. However, the presence of the thiourea functional group was obvious from the *m*/*z* value and an observed loss of 76 (thiourea) in MS2 (Fig. 2b + c). Indeed, loss of thiourea in MS/MS is known for *N*-substituted thioureas (Ji and Morris, 2003). Comprehensive NMR analysis provided the remaining NMR spectral characteristics (Section 3.6).

2.2. A dithiocarbamate also reacts quantitatively with ammonia to form the thiourea derivative

The dithiocarbamate adduct **7** was synthesized, characterized by NMR and ion trap MS/MS (Section 3.8) and subjected to the

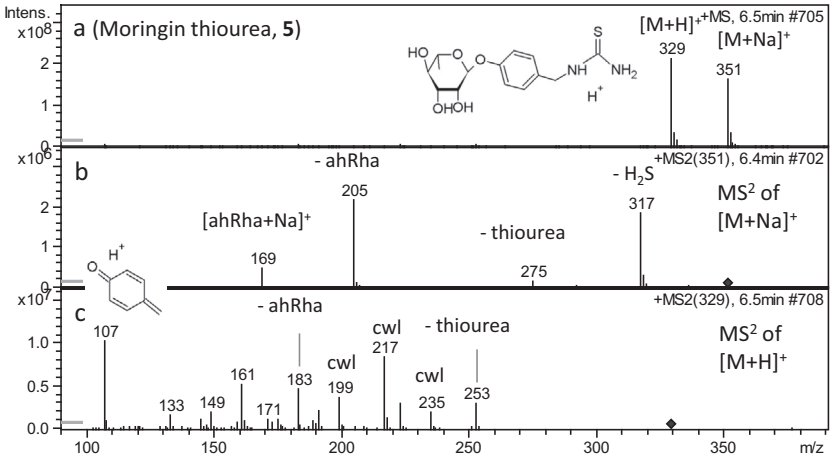

Fig. 2. Ion trap mass spectrometry of moringin thiourea (a–c). a: ESI mass spectrum of moringin thiourea at the HPLC-MS peak apex showing hydrogen and sodium adducts at approximately equal intensity from NaCl doped eluent. b + c: MS2 ion trap spectra of the sodium adduct (b) and the hydrogen adduct (c). Losses of either H_2S, thiourea or anhydroRha (ahRha) from the molecular adducts explaining the formation of each fragment ion is indicated. In addition, some other fragments in panel c are explained by consecutive water loss (cwl) and a quinone-like proton adduct.

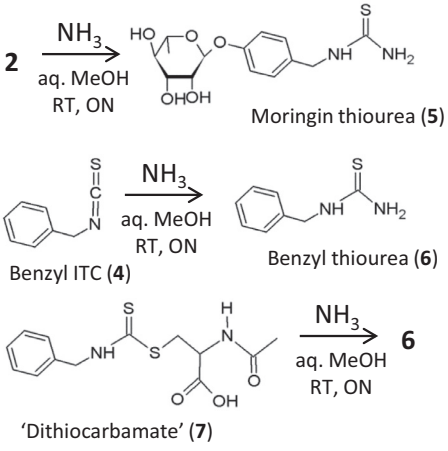

Fig. 3. Tested reactants and observed products at the investigated derivatization conditions. ITC, isothiocyanate; RT, room temperature; ON, over night.

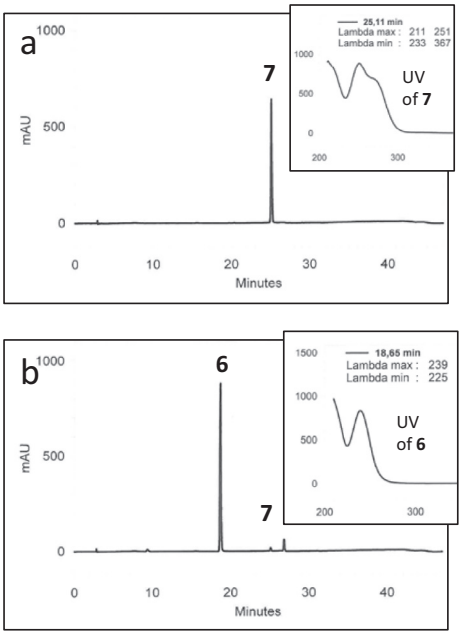

Fig. 4. HPLC-PDA data showing conversion of the dithiocarbamate (**7**) to benzylthiourea (**6**). Chromatograms (240 nm) are shown with UV spectra of major peaks as inserts. a: Dithiocarbamate (**7**) incubated in MeOH (control), dried and redissolved in 80% aq. MeOH (1.00 mL). b: Products of dithiocarbamate (**7**) (same amount as in a) incubated in 5% NH_3 in 80% aq. MeOH, dried and re-dissolved in 80% aq. MeOH (1.00 mL), showing the thiourea (**6**) as the dominating product.

same mild derivatization conditions over a range of concentrations. Analysis by HPLC showed dominant conversion of this expected type of adduct under the derivatization conditions (Fig. 4): of six replicates in two blocks, ranging from 0.1 to 1 μmol/assay, the disappearance of **7** was 98–100% in five experiments and 52% in the remaining (outlier) experiment. In accordance with the disappearance of **7** upon incubation with 5% NH_3, **6** was formed with HPLC peak area comparable to the missing peak area of **7** in each case. Hence, the ITC analysis method based on derivatization with ammonia at mild conditions would include intact ITCs as well as other reactive adducts such as dithiocarbamates (Fig. 3).

2.3. Recoveries of isothiocyanates and reactive adducts in simulated feeding assays

With the purpose of measuring the combined recovery of ITCs and a range of potentially bioactive ITC adducts, the devised 'thiourea derivative analysis method' (Fig. 3) was applied at realistic feeding assay-like conditions used in tests of insect feeding. Therefore, ITCs were applied on cabbage leaf discs (that neither contain glucomoringin nor benzylglucosinolate) and the solvent was allowed to evaporate, followed by immediate incubation in closed Petri dishes with moist filter paper but without insect larvae present.

At both high and intermediate levels of spiked moringin, the corresponding thiourea **5** was the dominant peak in the HPLC-PDA chromatograms at 240 nm after derivatization with NH_3, while no significant interfering peaks were observed in blanks (Fig. S1). A clear difference in the recovery was observed for the non-volatile moringin versus the volatile benzyl ITC (Figs. 5 and 6). There was no systematic decline in moringin levels with time (results not shown), in contrast to the situation with the volatile benzyl ITC (Fig. 6). Notably, the major loss of benzyl ITC happened during the 30 min solvent evaporation, i.e. before or close to "time zero" in any insect feeding experiment (Fig. 6).

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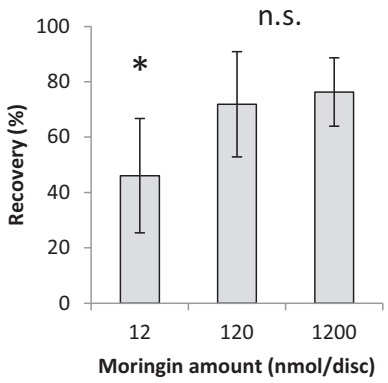

Fig. 5. Recovery of moringin after application on *Brassica oleracea* leaf discs at simulated feeding assay conditions at either 0 h, 1 h, 2 h, 3 h, 4 h or 5 h ($N = 1$ per time point) after the complete evaporation of the solvent. Means (±1 SD) are given for each amount of spiked moringin, as there was no systematic time dependency. The analysis method, based on thiourea derivatives, included free isothiocyanates and reactive adducts. Statistical significance: n.s., not significantly different (*t*-test, $P = 0.64$); *, significantly different from remaining recoveries (*t*-test, $P < 0.05$).

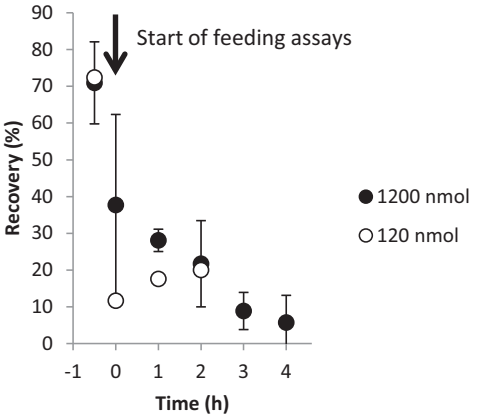

Fig. 6. Recovery of benzyl isothiocyanate at different time-points after application on *Brassica oleracea* leaf discs at simulated feeding assay-conditions. Amounts applied per leaf disc were either 1200 nmol ($N = 2–3$, ±1 SD) or 120 nmol ($N = 1$) as indicated. The analysis method, based on thiourea derivatives, included free isothiocyanates and reactive adducts. Time 'zero' indicates the time when the solvent had disappeared allowing start of insect feeding assays as indicated with an arrow.

The mean total recovery of moringin and reactive adducts in simulated bioassays was generally high at the high and intermediate level tested: 74% (SD 15%, $N = 12$) for the pooled 120 nmol and 1200 nmol recoveries, that were not significantly different (Fig. 5). At the low level tested, 12 nmol/leaf disc, the recovery was lower: 46% (SD 21%, $N = 6$).

During the actual simulated bioassay, the recovered mean total of benzyl ITC (**4**) and reactive adducts varied between 38% and 6% of the applied amount, with a decreasing trend (Fig. 6). Since the analysis included the most likely type of reaction product in plant tissue (dithiocarbamate adducts), the different recovery of the two ITCs was attributed to the difference in volatility due to the presence or absence of a rhamnosyloxy group.

A quite large natural variation in recovery of moringin was apparent (Fig. 5), perhaps due to biochemical differences between the leaf discs. The variation was mainly attributed to biological rather than technical variation, because two subsequent control experiments on separate days showed 2.1 and 5.6-fold higher absolute standard deviations of recoveries from leaf discs compared to recoveries from glass vials after solvent evaporation and 3 h incubation. Mean recoveries from glass vials were also consistently higher, as expected if leaf disc enzymes were involved in the reduced, variable recovery.

*2.4. Recovered moringin (**2**) and benzyl isothiocyanate (**4**) equivalents include the free compound*

To test whether recovered moringin equivalents, measured after derivatization with ammonia, included the free ITC, a recovery experiment with determination of the free moringin by direct HPLC was carried out using the usual evaporation of solvent followed by incubation at feeding assay-like conditions for 3 h (Müller et al., submitted for publication). Two levels tested, 1200 and 120 nmol per leaf disc, did not give significantly different relative recoveries, and the mean relative recovery for the combined experiment was 63% (SD 21%, $N = 11$). Comparison with the, at most, moderately higher recovery measured after derivatization with ammonia (Fig. 5) allowed us to conclude that moringin was relatively stable after spiking to leaf discs and that the majority of recovered moringin-equivalents were accounted for by free, non-conjugated moringin.

In order to test whether residual benzyl ITC (**4**) in simulated feeding assays was present in the free form or as a reactive adduct (such as a dithiocarbamate like **7**), a variation of the analysis aimed at distinction of apolar **4** and polar adducts of **4** was carried out: extraction in apolar solvent (hexane) before derivatization of the extracted ITC (Appelquist and Josefsson, 1967; Ji and Morris, 2003). A moderate reduction (ca. 30%) in yield of the derivative **6** due to extraction and inclusion of hexane was observed in control experiments. Taking this reduction into account, there was still a lower recovery of hexane-extractable **4** compared to total **4**-equivalents including polar adducts: spiking with 1.2 µmol **4** resulted in recovery after 2 h in the simulated feeding assay of 0.23 µmol of total **4** equivalents (SD 0.11 µmol, $N = 5$) and 0.08 µmol hexane extractable (intact) **4** (SD 0.06 µmol, $N = 5$), which was significantly different by a *t*-test ($P = 0.02$). This result suggested that approximately half of the recovered **4**-equivalents after 2 h was actual intact **4** despite the considerable time allowed for evaporation and possible reaction with the leaf disc matrix, while another half might be in the form of reactive adducts with nucleophilic plant constituents.

2.5. Perspectives

An analysis method capable of quantifying the sum of free ITCs and their reactive adducts was devised, including dithiocarbamates and possibly other reactive adducts. Preliminary results suggest that a significant part of recovered volatile benzyl ITC was in the form of reactive adducts (possibly with macromolecules), while the majority of the non-volatile, hydrophilic moringin was in the free form even after extended incubation on leaf discs. Hence, classical and recent investigations of deterrence and/or repellence of ITCs to glucosinolate-adapted insects (Li et al., 2000; Agrawal and Kurashige, 2003; Bejai et al., 2012) should be reconsidered, as reactive adducts may be responsible for the effects, at least in part.

If ITC reaction products rather than the ITCs as such are responsible for some effects of spiking ITCs to experimental diets, this would not be unprecedented. For example, an aphid antifeedant glucosinolate product was identified as a conjugate with Cys (Kim et al., 2008). On the other hand, common ITC detoxification products with glutathione likewise constitute reactive adducts as defined here, but may still serve to detoxify the ITCs by rendering them prone to secretion (Vanhaelen et al., 2001; Francis et al., 2005; Schramm et al., 2012; Angelino and Jeffery, 2014).

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The surprising recovery of significant amounts of benzyl ITC and activated adducts after prolonged evaporation from leaf surfaces opens the possibility of testing the related phenethyl ITC in simple leaf disc feeding assays (although formation of adducts is a problem for interpretation). Contrasting deterrence of this ITC and oxazolidine-2-thione type products of related glucosinolates have been proposed to explain differences in generalist insect resistance among chemotypes of the crucifer *Barbarea vulgaris* (van Leur et al., 2008; Agerbirk and Olsen, 2015), which is an emerging eco-model plant (Wei et al., 2013), but testing of the proposed active compounds is lacking.

Recently, antibiotic properties as well as promising physiological activities in mammals have been reported for moringin (Brunelli et al., 2010; Park et al., 2011; Galuppo et al., 2013; Waterman et al., 2014; Giacoppo et al., 2015). Diverse predicted reactive adducts with peptides and proteins in serum and cell extracts (Nakamura et al., 2009; Brown and Hampton, 2011; Angelino and Jeffery, 2014) may be quantifiable as a group using the devised derivatization with ammonia.

3. Experimental

3.1. Chemicals and general conditions

The HPLC-MS/MS instrument was an Agilent 1100 Series HPLC (Agilent Technologies, Germany) coupled to a Bruker HCT-Ultra ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) fitted with a Zorbax C18 column (Agilent; 1.8 μm, 2.1 × 50 mm). The oven temperature was maintained at 35 °C, eluents were H_2O with 0.1% (v/v) HCOOH and 50 μM NaCl (A) and acetonitrile with 0.1% (v/v) HCOOH (B), and the gradient program was 0 to 0.5 min, isocratic 2% B; 0.5–7.5 min, linear gradient 2–40% B; 7.5–8.5 min, linear gradient 40–90% B; 8.5–11.5 isocratic 90% B; 11.60–17 min, isocratic 2% B. The flow rate was 0.2 mL/min but increased to 0.3 mL/min in the interval 11.2–13.5 min. The mass spectrometer was run in positive electrospray mode. Ion trap MS/MS was carried out using the SmartFrag procedure with instrument default settings (isolation width 4 *m*/*z*, MS/MS fragmentation amplitude 1V, start amplitude 30%, end amplitude 200%, acquisition time 40 ms). Instruments for HPLC-PDA and NMR were as previously described (Agerbirk and Olsen, 2012). The HPLC-PDA instrument was equipped with a Luna phenylhexyl column (5 μm, 250 × 4.5 mm) (Phenomenex) with PDA detection at 210–370 nm, gradient programmes and detection wavelength for quantitation (band width 8 nm) depended on the specific analyte as detailed in the relevant sections. Chemical shifts in NMR for D_2O as solvent are relative to those of dioxane ($\delta_H = 3.75$, $\delta_C = 67.4$) and for CD_3OD as solvent are relative to those of TMS at 0.0 ppm. Glucomoringin and moringin were isolated and identified as described elsewhere (Müller et al., submitted for publication). Benzyl isothiocyanate (98%) was from Aldrich (Schnelldorf, Germany) and *N*-acetylCys (p.a.) was from Merck Eurolab (Darmstadt, Germany).

3.2. Derivatization with ammonia to form thiourea derivatives

Samples (spiked or control leaf discs as well as the pure compounds **2**, **4** and **7**) were added to 1 mL of 5% NH_3 in 80% aq. MeOH (MeOH: 25% aq. NH_3 20/80). The mixture was incubated at room temperature over-night (16–20 h), any leaf disk removed with a clean needle, and remaining solvent and reagent removed by evaporation under a gentle air-stream. The residue was dissolved in 1.00 mL H_2O (**5**) or 1.00 mL 80% aq. MeOH (**6**) and subjected to HPLC analysis.

3.3. Determination of isothiocyanate adducts

Adducts were identified and quantitated by HPLC-PDA adapted for the specific analytes. In all cases the column was a Luna phenylhexyl column (250 mm × 4.6 mm, 5 μm) (Phenomenex, Torrance, CA) and the detection wavelength for quantitation was 240 nm with additional collection of PDA data at 210–370 nm. For moringin thiourea (**5**) and benzyl thiourea (**6**), the solvents were H_2O (A) and MeOH (B). The gradient program for **5** was 0–2 min, isocratic 100% A, 2–30 min, linear gradient from 0% to 60% B, followed by a brief wash with B and 7 min equilibration with water. The t_R for **5** was 21 min (Fig. S1). The gradient program for **6** was 0–2 min, isocratic 20% B, 2–30 min, linear gradient from 20% to 100% B, followed by a brief wash with B and 7 min equilibration with 20% B. The t_R for **6** was 18 min. For combined analysis of the carboxylic acid **7** and the neutral **6**, the gradient program for **6** was slightly modified by spiking eluent A with TFA to 0.1% (vol.), which secured a sharp peak of **7** at 25 min and did not affect the t_R, peak shape or area of **6** (Fig. 4). Concentrations of **7** were tentatively calculated using the same response factor as for **6** at 240 nm, data from conversion experiments suggested this approximation to be reasonable. The injection volume was in general 10 μL but was increased to 100 μL for dilute samples with **5** in H_2O.

3.4. Recovery of moringin and activated adducts

White cabbage (*Brassica oleracea*) was obtained at a Copenhagen grocery store. Leaf discs (1 cm diameter) were treated with 40 μL of moringin solution in 80% aq. MeOH, either 30 mM, 3 mM, 0.3 mM or 0 mM (blank). Calculated concentrations (1200, 120 and 12 nmol, respectively) were based on moringin mass. Recovery-analysis of moringin equivalents (moringin and reactive conjugates) based on derivatization with ammonia (Fig. 3), was carried out (Fig. 5). After evaporation of the solvent (completed in 80 min, judged visually), one leaf disc from each concentration series was immediately taken for analysis, while the remaining were transferred to simulated feeding assays in glass Petri dishes (9 cm diameter, 1.6 cm inner height) containing a small piece of moist filter paper. One leaf disc from each concentration series was removed at 1, 2, 3, 4 and 5 h after the start of the simulated bioassay, and immediately taken for derivatization with NH_3 and subsequent HPLC-PDA analysis of the thiourea derivative (Fig. S1). For quantification, standard curves were also produced, using 0, 10, 20, 30 and 40 μL of each moringin solution, which were immediately taken for derivatization and analysis. In order to compare recoveries from leaf discs and an inert surface, recoveries of 1200 nmol aliquots ($N = 2 + 2$) and 120 nmol aliquots ($N = 3 + 3$) were compared after solvent evaporation and 3 h incubation on either a leaf disc or at the bottom of an open, relatively wide glass vial. In addition, recovery of native moringin was measured (Müller et al., submitted for publication).

For statistical analysis of recoveries (using Microsoft Excel 2010), variance homogeneity was confirmed by *F*-tests and difference of means were estimated by pairwise *t*-tests (two-tailed) of the 120 nmol and 1200 nmol series (not different) and of the 12 nmol series compared to the 120 nmol series, the 1200 nmol series, and both combined ($P < 0.05$ in all cases).

3.5. Recovery of benzyl isothiocyanate

This experiment was in principle similar to the experiment with moringin, except that solvent polarities were adjusted to suit the apolar nature of **4**: solutions for application to leaf discs were prepared in 96% aq. EtOH, the time for evaporation was only 30 min, leaf discs were sampled at the time of application, after solvent-evaporation for 30 min (time zero), and after a further 1, 2, 3 and

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4 h in the simulated bioassay. The experiment was carried out with 1200 nmol per leaf disc in duplicate or triplicate at each time point and with 120 nmol/leaf disc at each time point without replicates. The assymetrical replication is due to pooling data from two experiments as follows. In the first experiment, recovery at both levels was studied at application and at 0 h, 1 h and 2 h after the complete evaporation of the solvent. Using 2 h as the last data point was based on a (wrong) expectation of all ITC being gone by then. As detectable amounts of ITC were observed at 2 h, a second experiment (in duplicate but only considering the highest ITC concentration) studied recovery at application and at hourly intervals between 0 h and 4 h after complete solvent evaporation in simulated bioassays. As the results were similar, data from the two experiments were pooled, resulting in $N = 2–3$ for the various data points for the high level and $N = 1$ for the low level.

An additional experiment was aimed at distinction of free (apolar) **4** and hypothetic polar or insoluble (macromolecule-bound) adducts of **4**. Aliquots (1200 nmol) of **4** were applied to leaf discs as in the 2 h experiment above. At 2 h, half of the leaf discs ($N = 3$) were transferred to 5% NH_3 as above, while the remaining ($N = 3$) were extracted in hexane (3 mL). The hexane was partitioned against H_2O and quantitatively transferred (using an additional 2 × 2 mL hexane) to the usual 5% NH_3 reagent. All samples, either with or without hexane, were incubated over-night, with proper controls for testing the combined effect of hexane extraction and presence of hexane in the reagent solution.

3.6. Moringin thiourea (**5**)

Moringin was converted to the thiourea derivative in 5% NH_3 in 80% aq. MeOH over-night. The liquid was removed under a gentle air stream and the solid remnant dissolved in CD_3OD and subjected to ^1H NMR, showing moringin thiourea as dominant constituent. Analysis by HPLC revealed a dominant product at $t_R = 21$ min (93% of total area at 240 nm) which was isolated by preparative HPLC for spectroscopic characterization. HPLC-UV (λ_{max}/nm): 220 (sh), 238, 279 (sh) (Fig. S1). ^1H NMR (400 MHz) (CD_3OD), δ (assignment, #H, multiplicity, J/Hz): 7.26 (2 + 6, 2H, d, 8.5), 7.03 (3 + 5, 2H, d, 8.5), 5.40 (1′, 1H, d, 1.7), 4.25–4.65 (Ar-CH_2, 2H, br. irregular peak with three maxima at ca. 4.63 ppm (major), 4.58 ppm (intermediate) and 4.33 ppm (minor)), 3.98 (2′, 1H, dd, 3.5/1.8), 3.83 (3′, 1H, dd, 9.3/3.5), 3.62 (5′, 1H, m), 3.45 (4′, 1H, 'tr', 9.5), 1.21 (6′, 3H, d, 6.4). ^{13}C NMR (100 MHz), δ: 157.2 (4), ca. 134 (1 detected in HMBC), 130.0 (2 + 6), 117.7 (3 + 5), 99.9 (1′), 73.9 (4′), 72.3 (2′ or 3′), 72.1 (2′ or 3′), 70.7 (5′), ca. 49 (Ar-CH_2 detected in HMBC), 18.1 (6′). Assignments of glycosidic protons were confirmed by COSY, informative HMBC correlations included 1′ to 4 and 2 to Ar-CH_2. HPLC-MS/MS: see Fig. 2. NMR-spectra: see Fig. S2.

3.7. Benzyl thiourea (**6**)

Benzyl ITC was derivatized with NH_3 as described above and subjected to preparative HPLC followed by ^1H NMR in CD_3OD. ^1H NMR (400 MHz), δ (assignment, #H, peak type): 7.1–7.35 (Ar-H, 5H, m), 4.3–4.75 (br. irregular peak with three maxima at ca. 4.7 ppm (major), 4.6 ppm (minor) and 4.4 ppm (minor)). HPLC-UV (λ_{max}/nm): 239 (Fig. 4b). MS (ESI) 177 $[M+H]^+$.

3.8. N-Acetyl-S-(N-benzylthiocarbamoyl)-cysteine (**7**)

Benzyl ITC (**4**) (0.25 g, 1.68 mmol) was dissolved in 1.68 mL MeOH, *N*-acetylCys (0.29 g, 1.76 mmol) was added and dissolved, and the mixture allowed to react for 3 days at room temperature. HPLC-analysis after 3 days revealed a dominant UV-absorbing product which was isolated by preparative HPLC. The combined fraction (3 mL) was neutralized by addition of 30 µL 1 M sodium bicarbonate, dried under a gentle air stream and subjected to NMR in CD_3OD. From the MS and NMR (^1H, ^{13}C, DEPT, HSQC, HMBC), the isolated product (Fig 4a) was identified as the expected dithiocarbamate-type adduct with the thiol group of *N*-acetylCys (**7**, *N*-acetyl-*S*-(*N*-benzylthiocarbamoyl)-cysteine = 2-(acetylamino)-3-[(benzylcarbamothioyl)sulfanyl]propanoic acid). HPLC-UV (λ_{max}/nm): 251, 270 (sh) (Fig. 4a). ^1H NMR (400 MHz) (D_2O), δ (assignment, #H, multiplicity, J/Hz): 7.2–7.35 (2′–6′, 5H, m), ca. 4.9 (Ar-CH_2a detected in 2D spectra), 4.86 (Ar-CH_2b, d, 14.6), 4.64 (2, 1H, dd, 8.5/4.7), 3.97 (3a, 1H, dd, 14.0/4.7), 3.54 (3b, 1H, dd, 14.0/8.5), 1.93 (2″, 3H, s). ^{13}C NMR (100 MHz), δ: 198.8 (C=S), 174.4 (1″), 173.2 (1), 138.6 (1′), 129.5 (3′ + 5′), 129.1 (2′ + 6′), 128.5 (4′), 54.4 (2), 51.4 (7′), 37.4 (3), 22.6 (2″). Informative HMBC correlations included Ar-CH_2 with C=S and 3a/b with C=S. Ion trap MS: see Fig. 7.

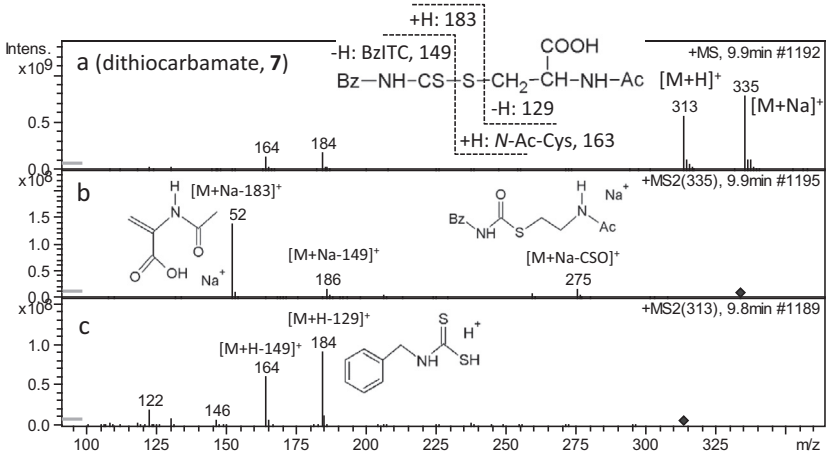

Fig. 7. Ion trap MS of the dithiocarbamate (**7**), showing contrasting MS2 spectra of sodium and hydrogen adducts due to different cleavages and ion-affinities. a: ESI mass spectrum of **7** at the HPLC-MS peak apex. b: MS2 of the sodium adduct, showing dominant cleavage at one side of the bridging S and formation of adducts with the Cys moiety. Depicted adducts are m/z 152 and m/z 275. The minor loss of 60 was tentatively attributed to elimination of CSO, possibly originating from reaction between the thiocarbonyl and the carboxylic acid functionalities. c: MS2 of the proton adduct, showing cleavage at both sides of the bridging S with the proton following the former bridging S. The neutral losses matched 2-(acetylamino)prop-2-enoic acid (129) and the ITC, **4** (149). The depicted adduct is m/z 184.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2015.06.004. These data include MOL files and InChiKeys of the most important compounds described in this article.

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