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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.

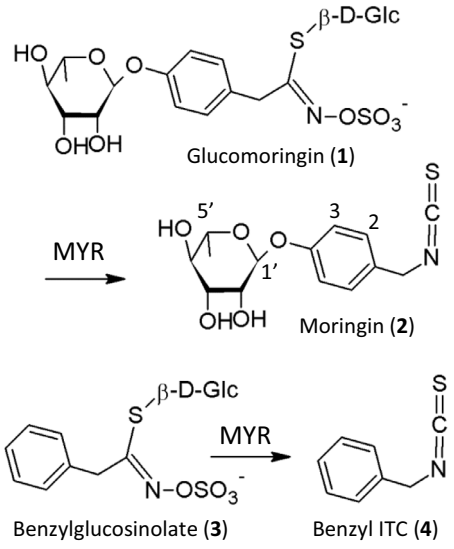

Fig. 1. Investigated glucosinolates (benzylglucosinolate and glucomoringin) and their enzymatic conversion to isothiocyanate products (unbalanced). The NMR-numbering system of moringin is indicated. MYR: myrosinase.

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