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Acylated glucosinolates with diverse acyl groups investigated by high resolution mass spectrometry and infrared multiphoton dissociation

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ABSTRACT

With the aim of developing a procedure for detecting and identifying intact acylated glucosinolates (a-GLSs) found in trace quantities in natural plant samples, extracts of *Barbarea vulgaris* seeds were analyzed by reversed-phase liquid chromatography coupled with electrospray ionization and Fourier-transform ion cyclotron resonance mass spectrometry (RPLC-ESI FTICR MS). After a preliminary optimization of fragmentation conditions, based on a non-acylated parent glucosinolate (glucobarbarin) and three previously identified a-GLSs (the 6′-isoferuloyl esters of glucobarbarin, gluconasturtiin and glucobrassicin), infrared multiphoton dissociation (IRMPD) was employed for a tandem MS-based elucidation of the molecular structures of novel a-GLSs. As a result, three acylated derivatives of glucobarbarin, esterified at the thioglucose moiety with a coumaric acid isomer, sinapic acid or an isomer and a dimethoxycinnamic acid isomer, were identified. In addition, a further acylated glucosinolate was tentatively identified as the isoferuloyl ester of an unidentified hydroxylic derivative of glucobarbarin. This is the first demonstration of diversity in the acyl moieties of thioglucose-acylated glucosinolates, which may reflect the substrate specificity of the endogenous acyl transferase. As expected, 6′-isoferuloyl-glucobarbarin was detected as the main acylated GLS in extracts of *B. vulgaris* seeds. A quantitative estimate suggested that non-isoferuloyl substituted glucobarbarins correspond to ca. 0.026% of the level of 6′-isoferuloyl glucobarbarin. The formation of an uncommon distonic radical anion, most likely generated in the gas phase upon methyl radical ($CH_3^{\cdot}$) loss from the isoferuloyl anion, is demonstrated.

Introduction

Glucosinolates (GLSs) are a class of plant secondary metabolites commonly found in Brassicaceae and related families (Agerbirk and Olsen, 2012; Fahey et al., 2001). These compounds are involved in complex biochemical and ecological interactions with herbivores and disease agents (Agerbirk and Olsen, 2012) and are also of considerable nutritional interest (Traka and Mithen, 2009). All glucosinolates share a common structure consisting of a β-thioglucose moiety, a sulfated oxime group, and a variable aglycone derived from an α-amino acid (Agerbirk and Olsen, 2012; Fahey et al., 2001). While a large number of aglycone moieties have been described (Agerbirk and Olsen, 2012; Fahey et al., 2001) only a few investigations have been focused on the modified structure of the β-thioglucose moiety (Agerbirk and Olsen, 2011, 2012; Reichelt et al., 2002). Although the vast majority of known GLSs is not substituted at any thioglucose hydroxyl group, the presence of a few GLSs with substituents on the thioglucose moiety have been reported for seeds of three plant genera, namely *Raphanus sativus* (radish) (Linsheid et al., 1980), the general model plant *Arabidopsis thaliana* (Reichelt et al., 2002) and all investigated species of the genus *Barbarea* (Agerbirk and Olsen, 2011), including *Barbarea vulgaris*, which is a chemical ecology and ecogenetics model plant (Badenes-Pérez et al., 2011; Dalby-Brown et al., 2011; Kuzina et al., 2009, 2011; Wei et al., 2013). Only three thioglucose acyl groups have so far been documented, one in each plant genus, namely sinapoyl in the case of radish (Linsheid et al., 1980), benzoyl in the case of *A. thaliana* (Reichelt et al., 2002) and the rare isoferuloyl (2-hydroxy-3-methoxycinnamoyl) in the case of *Barbarea* (Agerbirk and Olsen, 2011), with no structural diversity within each species documented. As the apparent pattern of a distinct type of acyl group in each genus is striking and believed to reflect biological function and substrate specificity of an endogenous acyl transferase, it is of interest to look for possible diversity in acylation and, if present, to quantify such

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diversity. So far, thioglucose acylated glucosinolates have been identified based on NMR spectroscopy and chromatography of products upon treatment with esterase (Agerbirk and Olsen, 2011; Reichelt et al., 2002). Even trace constituents (as low as 0.04 μmol/g dry wt) were identified with certainty by this strategy, including a GLS in trace amount with a different acyl group (Agerbirk and Olsen, 2011). However, as GLSs with diverse acylation were much less abundant and could not be identified solely with the applied low resolution MS (Agerbirk and Olsen, 2011), alternative methods had to be considered.

Recently, the combination of the tandem mass spectrometry capabilities provided by infrared multiphoton dissociation (IRMPD) and the high resolution/accuracy available with Fourier transform ion cyclotron resonance mass spectrometry (FTICRMS) have been exploited for the structural characterization of glucosinolates (Agneta et al., 2012; Bianco et al., 2012). IRMPD is a slow activation method that yields very similar fragments to low-energy collision-induced dissociation (CID). Indeed, the resonant absorption of a few IR photons with relatively low energy allows the ions of interest to fragment selectively along their lowest energy dissociation pathways; product ions are thus formed from the dissociation of the weakest bonds in the precursor ion (McLuckey and Goeringer, 1997; Sleno and Volmer, 2004). IRMPD is especially well suited for the dissociation of trapped ions in an FTICR cell since no collision gas is required and no translational excitation of ions (as during CID) occurs, thereby minimizing ion losses due to ejection or ion scattering and enabling on-axis fragmentation.

Starting from the considerations made so far, IRMPD-FTICRMS, combined to reversed-phase liquid chromatography (RPLC) through an electrospray ionization (ESI) interface, has been recently adopted in our laboratory for the structural investigation of a group of intact acylated GLSs found in the seeds of *B. vulgaris*. A preliminary study of the fragmentation behaviour, under IRMPD conditions, of two purified GLSs naturally occurring in *B. vulgaris*, viz. glucobarbarin (BAR) and 6′-isoferuloyl-glucobarbarin (6′-isoferuloyl-BAR), and of two previously known acylated glucosinolates, viz. 6′-isoferuloyl-gluconasturtiin (6′-isoferuloyl-NAS) and 6′-isoferuloyl-glucobrassicin (6′-isoferuloyl-GBS) has been performed. Starting from the resulting fragmentation models a partial structural elucidation of hitherto unrecognised acylated GLSs, present in trace amounts in *B. vulgaris* seeds, has been achieved and will be described in the present paper.

Results and discussion

Tandem MS and fragmentation behavior of purified glucobarbarin and 6′-isoferuloyl-glucobarbarin

The most abundant glucosinolates of *B. vulgaris* seeds, BAR and 6′-isoferuloyl-BAR were available as pure compounds. As reported by several authors, fragmentation of GLSs in tandem MS with collision induced dissociation (CID) gives rise to a common set of definite product ions, which represent key features for a correct molecular structure identification (Bialecki et al., 2010; Cataldi et al., 2007, 2010; Fabre et al., 2007; Kokkonen et al., 1991; Lee et al., 2008; Mellon et al., 2002; Millán et al., 2009; Rochfort et al., 2008; Tolra et al., 2000; Velasco et al., 2011). These fragments originate both from the thioglucoside and the side chain moieties and most of these studies report a complete list of common diagnostic fragment ions (Bialecki et al., 2010; Cataldi et al., 2010; Fabre et al., 2007; Kokkonen et al., 1991). Yet, tandem MS by IRMPD FTIRC MS has not been exploited for acylated GLSs and a careful optimization and fragment ion identification was required. Two important parameters can be managed to affect the fragmentation of precursor ions when IRMPD is performed: energy deposition influenced by irradiation time and kinetics, i.e. the time that is available for the ion to release the acquired photon energy.

The IRMPD FTICR MS spectra obtained by irradiating the deprotonated molecule of BAR ($[C_{15}H_{20}NO_{10}S_2]^-$) at *m*/*z* 438 employing two different irradiation times, 100 and 200 ms, are shown in Fig. 1. An irradiation time of 100 ms at 100% laser power does not allow a sufficient fragmentation of the deprotonated BAR (see Fig. 1A) because its $[M-H]^-$ ion is the most intense in the mass spectrum and the only other peak, observed at *m*/*z* 219.02668, is not related to a product ion but it is due to the $3\omega_+$ harmonic frequency of the precursor ion (Mathur and O'Connor, 2009). It is worth pointing out that all the *m*/*z* values reported here have been rounded to the fifth decimal place, although the observed variability is on the fourth decimal place (vide infra). This choice is equivalent to reporting each measurement rounded to the first non significant figure, as recently recommended for reporting high accuracy/resolution mass spectrometric data (Brenton and Godfrey, 2010). Upon increasing the irradiation time up to 200 ms, IRMPD yielded increased fragmentation and a richer mass spectrum (see plot B in Fig. 1). Irradiation times longer than 200 ms, however, did not provide a much richer mass spectrum or a better signal-to-noise ratio. Characteristic *m*/*z* values, evaluated with mass error in the low ppm range, together with their isotopic patterns, supported the unequivocal chemical formulas validation of all product ions observed for BAR, down to that one detected at *m*/*z* 74.99107 ($[C_2H_3OS]^-$), which is normally not explored with 2D and 3D ion trap mass instruments because of their inherent low-mass cut-off (Todd and March, 2005). Known bond cleavage mechanisms and rearrangements may support a tentative structural assignment of all observed product ions, as depicted in Scheme 1.

The generation of a hydrogen sulfate ion, $[HSO_4]^-$ (*m*/*z* 96.96018, mass error +0.83 ppm), from the precursor ion was observed as the major fragmentation pathway, yet several additional peaks, emphasized by signal magnification in Fig. 1B, were detected. The prevailing one, found at *m*/*z* 135.97113, most likely corresponds to N-sulfate ethenimine, $[C_2H_2O_4NS]^-$ generated in the gas-phase (see Scheme 1). Other peaks, at *m*/*z* 138.97081 and 128.93231, corresponding to $[C_2H_3O_5S]^-$ and $[HO_4S_2]^-$, respectively, have been described by Attygalle and coworkers (Bialecki et al., 2010) as common product ions of GLSs. Among minor product ions, the one detected at *m*/*z* 332.01218 $[C_8H_{14}O_9NS_2]^-$, resulting from the neutral loss of benzaldehyde (see Scheme 1), while retaining both the thioglucose and the sulfonated oxime groups, indicates the tendency for structural rearrangements, most likely favoured by the vibrational excitation of the precursor ion. A similar consideration may apply to the product ion at *m*/*z* 301.00618 ($[C_8H_{13}O_8S_2]^-$ and mass error +1.49 ppm) depicted in Fig. 1, plot B, in which rearrangements and bond cleavages occurring in the gas-phase have to be invoked for its formation. More typical MS/MS decompositions of GLSs derived from the thioglucoside moiety, at *m*/*z* 274.99051 $[C_6H_{11}O_8S_2]^-$, 259.01331 $[C_6H_{11}O_9S]^-$ and 241.00276 $[C_6H_9O_8S]^-$, were also detected for BAR (Cataldi et al., 2010). Moreover, fragment ions originated from the side chain, at *m*/*z* 260.02383, 242.01323 and 196.04404 (see underlined values in plot (B) of Fig. 1), corresponding to $[C_9H_{10}O_6NS]^-$, $[C_9H_8O_5NS]^-$ and $[C_9H_{10}O_2NS]^-$, respectively, were observed (see Scheme 1). The occurrence of peaks at *m*/*z* 169.95892 and 153.98186, i.e. product ions $[C_2H_4O_4NS_2]^-$ and $[C_2H_4O_5NS]^-$ correspondingly, completes the IRMPD FTICR mass spectrum of glucobarbarin. In summary, a mass spectrum rich in product ions was produced at the optimized conditions. In contrast, a common problem with collision induced dissociation for large molecules is the lack of fragments, and sequential mass spectrometry (MS^n, with $n > 2$) is required to obtain a complete structural characterization. IRMPD is a slow heating activation tool which entails a single MS/MS