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Identification of the major glucosinolate (4-mercaptobutyl glucosinolate) in leaves of *Eruca sativa* L. (salad rocket)

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Abstract

The major and structurally unique glucosinolate (GLS) in leaves of *Eruca sativa* L. (salad rocket) was identified as 4-mercaptobutyl GLS. Both 4-methylthiobutyl GLS and 4-methylsulfinylbutyl GLS were also present, but at lower concentrations. The 4-mercaptobutyl GLS was observed to oxidise under common GLS extraction conditions, generating a disulfide GLS that may be reduced efficiently by tris(2-carboxyethyl) phosphine hydrochloride (TCEP) to reform the parent molecule. The identities of 4-mercaptobutyl GLS and of the corresponding dimeric GLS were confirmed by LC/MS, MS/MS and NMR. Myrosinase treatment of an enriched GLS fraction or of the purified dimer GLS generated a mixture of unique bi-functional disulfides, including *bis*-(4-isothiocyanatobutyl) disulfide (previously identified elsewhere). TCEP reduction of the purified dimer, followed by myrosinase treatment, yielded only 4-mercaptobutyl ITC. GLS-derived volatiles generated by autolysis of fresh seedlings and true leaves were 4-mercaptobutyl ITC (from the newly identified GLS), 4-methylthiobutyl ITC (from 4-methylthiobutyl GLS) and 4-methylsulfinylbutyl ITC (from 4-methylsulfinyl-butyl GLS); no unusual bi-functional disulfides were found in fresh leaf autolysate. These results led to the conclusion that, in planta, the new GLS must be present as 4-mercaptobutyl GLS and not as the disulfide found after extraction and sample concentration. This new GLS and its isothiocyanate are likely to contribute to the unique odour and flavour of *E. sativa*.

Keywords: *Eruca sativa*; 4-Mercaptobutyl glucosinolate; 4-Mercaptobutyl isothiocyanate; *bis*-(4-Isothiocyanotobutyl) disulfide; LC/MS; MS/MS; GC/MS; NMR

1. Introduction

Glucosinolates (GLS) are amino acid-derived secondary metabolites found in crucifer species (Daxenbichler et al., 1991; Rosa et al., 1997; Fahey et al., 1997). Upon tissue disruption they are hydrolysed by the enzyme myrosinase (thioglucosidase, EC 3.2.3.1) to yield a mixture of hydrolysis products. Several GLS hydrolysis products, particularly 4-methylsulfinylbutylisothiocyanate (sulforaphane, SFN) and indole-3-carbinol, are believed to confer beneficial effects on human health by a variety of mechanisms (Mithen et al., 2000; Steinkeller et al., 2001; Bonnessen et al., 2001; Kong et al., 2001; Smith, 2001).

Leaves of salad rocket (*Eruca sativa* L.) are increasingly eaten by humans either alone or as part of mixed salads, and are also used in herbal remedies (Yaniv et al., 1998; Mahran et al., 1991). Previous publications have shown that the seeds are a good source of 4-methylthiobutyl GLS (glucoerucin, MTB) (Iori et al., 1999). The unusual GLS breakdown product *bis*-(4-isothiocyanotobutyl) disulfide has previously been identified as a major component in dichloromethane extracts of *E. sativa* leaves (Cerny et al., 1996). It was proposed that this disulfide was an artefact formed by oxidative dimerisation of myrosinase-generated 4-mercapto ITC

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during the extraction process. SFN, a breakdown product of 4-methylsulfinylbutyl GLS (glucoraphanin, MSB), was also found (Cerny et al., 1996). The chain elongation pathway for MTB has recently been investigated in leaves of *E. sativa* by tracer incorporation and analysis of purified desulfo-MTB (Graser et al., 2000). Other desulfo-GLS were present but were discarded, so their identities and label incorporation were not reported (Graser et al., 2000).

This paper describes the identification of the most abundant GLS in seedlings (aerial parts) and leaves of *E. sativa* using previously validated LC/MS, LC/MS/MS and NMR methods (Mellon et al., in press; Kiddle et al., 2001). Data obtained by GC/MS analysis of volatiles produced by autolysis of fresh *E. sativa* tissues and by myrosinase treatment of various *E. sativa* leaf GLS fractions are also reported.

2. Results and discussion

GLS were extracted from freeze-dried young leaves of *E. sativa*. An enriched GLS fraction (F3) was prepared. This fraction was used for (1) analysis of intact GLS by both negative ion Electrospray (−ESI) LC/MS and LC/MS/MS, (2) positive ion atmospheric pressure chemical ionisation (APCI+) LC/MS after desulfation of the GLS, (3) for purification of the new GLS, and (4) for analysis of volatile and semi-volatile products by electron ionisation (EI) GC/MS produced after myrosinase treatment.

Nine GLS were detected in the F3 fraction using ion-pair LC/MS and LC/MS/MS (Mellon et al., in press) (Fig. 1a). These were 4-methylthiobutyl GLS (peak 5), 4-methylsulfinylbutyl GLS (peak 1), a trace of 4-methoxy-3-indolylmethyl GLS (peak 7), and five previously

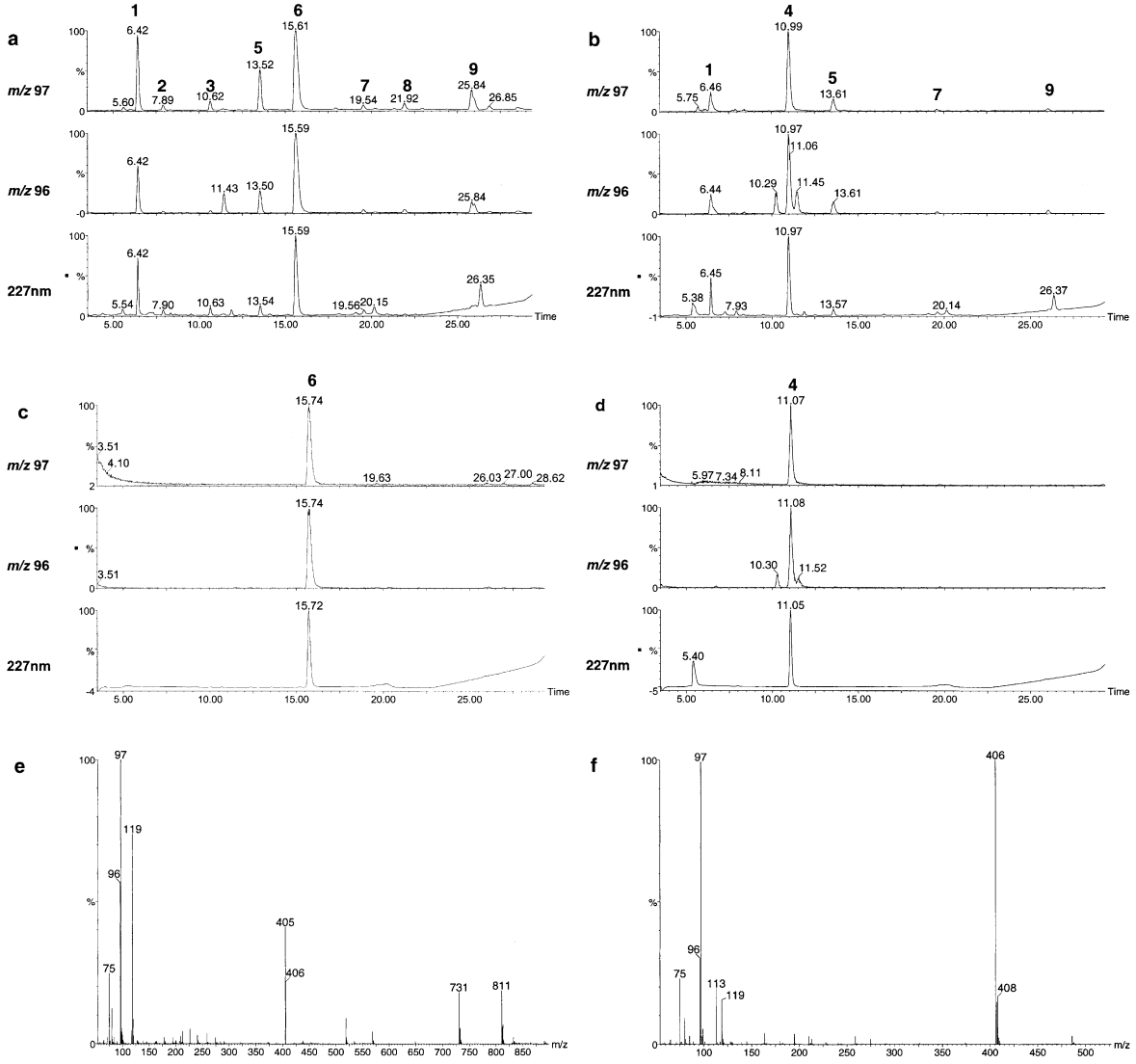

Fig. 1. Negative ion ESI chromatograms of intact GLS in F3 fraction untreated (a) and TCEP-treated (b), purified dimer GLS untreated (c) and TCEP-treated (d), and mass spectra of the GLS dimer (e) and product of TCEP reduction (4-mercaptobutyl GLS) (f). Peak 1 = MSB, 2, 3, 8 and 9 = unidentified aliphatic GLS, 4 = 4-mercaptobutyl GLS, 5 = MTB, 6 = dimeric GLS, 7 = 4-methoxy-3-indolylmethyl GLS.

unidentified GLS (1 major, 4 minor) (Fig. 1a). The most abundant GLS (peak 6) had not been previously identified. Negative ion ESI indicated that it had a molecular weight of 812 (ions at *m/z* 811, $[M-H]^-$ and 405, $[M-2H]^{2-}$). This was supported by APCI+ LC/MS of the desulfo-GLS derived from this GLS, which had a mass of 652 (ion at *m/z* 653, $[M+H]^+$). Supporting data, using a sample of the purified dimer, were also obtained by MS/MS of the $[M-2H]^{2-}$ (*m/z* 405) and of the $[M-H-SO_3]^-$ (*m/z* 731) ions (Table 1). MS data obtained on the intact and desulfo-GLS are summarised in Table 1. Our initial conclusion was that the unusual ITC, identified by Cerny et al. (1996), was derived directly from a new GLS, rather than by the proposed mechanism of dimerisation of 4-mercapto-ITC. The mass spectrometric data suggested an empirical formula for the putative intact GLS of $C_{22}H_{40}O_{18}N_2S_6$, suggesting that it has the structure of a symmetrical dimer, a precursor of the previously identified ITC. However, subsequent investigations led us to conclude that the dimer was an artefact generated by dimerisation of 4-mercapto GLS (see below).

The new GLS was purified (see experimental section for details, Fig. 1c) and ^1H and ^{13}C NMR spectra were obtained. The key features of the spectra were the simplicity that was indicative of a symmetrical molecule and also the absence of sharp singlets that correspond to S–CH_3 groups. The results of these spectra led to the conclusion that the new GLS had a dimeric disulfide structure: δ_H (300 MHz, ^2H_2O) 1.95 (4H, *br*, *s*, CH_2-2′ and CH_2-3′), 2.75–2.9 (4H, *m*, CH_2-1′ and CH_2-4′), 3.45–3.7 (4H, *m*, H-2, 3, 4, 5), 3.79 (1H, *dd*, $J_{5,6a}=5$, $J_{6a,6b}=12.8$ Hz, H-6a), 3.95 (1H, *d*, $J_{6a,6b}=12.8$ Hz, H-6b), 5.09 (1H, *d*, $J_{1,2}=9.6$ Hz, H-1); δ_C (75.47 MHz, ^2H_2O) 23.5 (SCH_2), 25.5 (C-2′), 29.8 (C-3′), 29.8 (C-1′), 35.6 (C-4′), 58.65 (C-6), 67.1 (C-4), 70.1 (C-2), 75.1 (C-5), 78.2 (C-3), 79.9 (C-1), 162.5 (C-7).

Both the enriched GLS fraction F3 (containing MTB, MSB and a high concentration of the dimer GLS) and a sample of the purified dimer GLS were treated with

Table 1
Summary of LC/MS data for the major intact GLS and desulfo-GLS from *Eruca sativa* leaves[a]

GLS (in order of RT)	MS mode	$[M-H]^-$	$[SO_3H^-]^-$	$[SO_3^-]^-$	Other significant ions
4-Methylsulfinylbutyl GLS	ESI−	436 (100%)	97 (69)	96 (22)	
4-Mercaptobutyl GLS (purified dimer post-TCEP)	ESI−	406 (100%)	97 (99)	96 (30)	
4-Methylthiobutyl GLS	ESI−	420 (98%)	97 (100)	96 (28)	
Purified dimer GLS	ESI−	811 (19%)	97 (100)	96 (58)	731 (6, $[(M-H)-SO_3]^-$), 569 (3, $[(M-H)-SO_3-Glc]^-$), 405 (40, $[M-2H]^{2-}$)
Dimer GLS: product ions of *m/z* 731 (cone voltage induced $[M-H-SO_3]^-$)	ESI−MS/MS	Not applicable	97 (100)	96 (26)	731 (24%, $[(M-H)-SO_3]^-$), 535 (6, $[(M-H)-SO_3-GlcSH]^-$), 275 (8, $[GlcSH+SO_3]^-$), 259 (17 $[GlcSO_3]^-$), 241 (14, $[AnhydroGlc+SO_3]^-$), 195 (8, $[GlcS]^-$)
Dimer GLS: product ions of *m/z* 405	ESI−MS/MS	405 (0.2%) ($[M-2H]^{2-}$)	97 (100)	96(51)	535 (0.8, $[M-2H]^{2-}-SO_3-GlcSH+H]^-$), 519 (1.6, $[M-2H]^{2-}-SO_4-GlcSH+H]^-$, 437 ($[GlcSC(=NSO_4)(CH_2)_4SS^{\bullet}]^-$), 372 ($[GlcSC(=NSO_4)(CH_2)_3CH=CH_2]^-$), 275 (0.8, $[GlcSH+SO_3]^-$), 259 (1.5, $[GlcSO_3]^-$), 242 (0.7, $[AnhydroGlc+HSO_3]^-$)
Desulfo-GLS		$[M+K]^+$	$[M+Na]^+$	$[M+H]^+$	Other ions
4-Methylsulfinylbutyl d-GLS	APCI+	396 (24%)	380 (100)	358 (2)	196 (5, $[aglycone+H]^+$),
4-Methylthioburtyl d-GLS	APCI+	380 (15%)	364 (47)	342 (89)	180 (100, $[aglycone+H]^+$)
Dimer d-GLS	APCI+	691 (13%)	675 (100)	653 (3)	529 (14, $[aglycone+K]^+$), 479 (11, $[aglycone-S,+Na]^+$)
Dimer d-GLS (high cone voltage, 75eV)	APCI+	691 (19%)	675 (100)	653 (1)	529 (3, $[aglycone+K]^+$), 513 (39, $[aglycone+Na]+$), 479 (26, $[aglycone-S,+Na]^+$)

Glc = glucose, AnhydroGlc = anhydroglucose.

[a] N.B. The identities of 4-methylsulfinylbutyl (intact and d-GLS) and 4-methylthiobutyl (intact and d-GLS) were also confirmed with the aid of purified standards.

Table 2
Summary of GC/EI MS data for glucosinolate-derived hydrolysis products from *Eruca sativa* tissues and purified GLS[a]

Hydrolysis product (in order of RT)	RT (min)	M	Other ions
4-Methylsulfinylbutyl ITC (SFN)	6.4	(177)	113 (57, C_5H_7NS), 85 (9, C_3H_3NS), 72 (100, C_2H_2NS)
4-Mercaptobutyl ITC	15.1	147 (4%)	114 (100, C_5H_8NS), 87(55, C_4H_7S), 72 (60, C_2H_2NS), 55 (54, C_4H_7)
4-Methylthiobutyl ITC (ERN)	17.1	161 (100%)	146 (52, $C_5H_8NS_2$)
bis (Cyanatobutyl) disulfide	30.3	228 (16%)	114 (17, C_5H_8NS), 87 (35, C_4H_7S), 82 (100, C_5H_8N), 55 (92, C_4H_7)
4-Isothiocyanatobutyl, 4′-cyanatobutyl disulfide	34.2	260 (1%)	146 (2, $C_5H_8NS_2$), 114 (71, C_5H_8NS), 87(15, C_4H_7S), 72 (73, C_2H_2NS), 55 (100, C_4H_7)
bis (Isothiocyanatobutyl) disulfide	38.0	292 (2%)	146 (4, $C_5H_8NS_2$), 114 (99, C_5H_8NS), 87(25, C_4H_7S), 72 (100, C_2H_2NS), 55 (66, C_4H_7)

[a] N.B. SFN is unstable to EI MS and produces 3-butenyl ITC as the major fragment. Pure SFN and ERN standards also run.

S. alba myrosinase. Following incubation the samples were extracted with CH_2Cl_2 and the organic phase was analysed by Electron Ionisation (EI) GC/MS (Table 2). The identities of 4-methylthiobutyl ITC and 4-methylsuflinylbutyl ITC were further confirmed by injection of commercial standards. The treatment of F3 produced a number of unique hydrolysis products (Fig. 2a). Myrosinase treatment of a less concentrated sample of the purified dimer mainly generated 4-mercaptobutyl ITC and *bis*-(4-isothiocyanatobutyl) disulfide. Traces of the other disulfides were also observed (data not shown). In contrast, analysis of volatiles released by autolysis of fresh tissues (both seedlings and leaves) yielded a very different pattern. The volatiles produced by fresh 5-day old seedlings (Fig. 2b) or young leaves from mature plants (Fig. 2c) did not include any of the previously identified dimeric disulfides. The CH_2Cl_2 extracts of the autolysed fresh tissues contained 4-methylsulfinylbutyl ITC (leaf extract), 4-methylthiobutyl ITC (seedling and leaf extracts) and 4-mercaptobutyl ITC (seedling and leaf extracts). A small amount of *bis*-(4-isothiocyanatobutyl) disulfide was detected in the young leaves, but could have been formed as an artefact from the high amount of 4-mercaptobutyl ITC in this sample. These data lead us to conclude that the dimer GLS is an artefact hence the derived bifunctional disulfides are also artefacts.

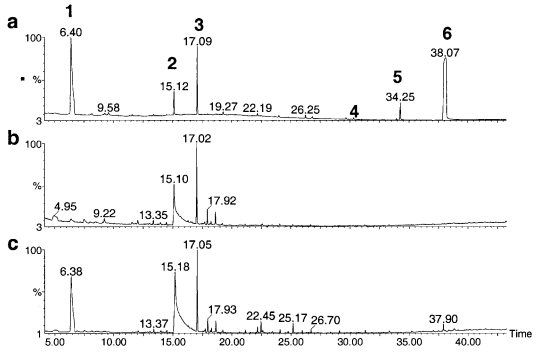

Fig. 2. Representative GC/MS chromatograms of volatiles released by exogenous myrosinase treatment of the F3 fraction (a), by autolysis of fresh *Eruca sativa* seedlings (b), and by autolysis of fresh young leaves (c). Peak 1 = 4-methylsulfinylbutyl ITC, 2 = 4-mercaptobutyl ITC, 3 = 4-methylthiobutyl ITC, 4 = *bis* (cyanatobutyl) disulfide, 5 = 4-isothiocyanatobtyl, 4′-cyanatobutyl disulfide, 6 = *bis* (isothiocyanatobutyl) disulfide.

Further structural data were obtained by TCEP treatment of either the F3 fraction or the purified dimer. The disulfide GLS was reduced to 4-mercaptobutyl GLS but no effects were observed on the other GLS (Fig. 1b, d and f). Myrosinase treatment of the new GLS post-TCEP reduction and subsequent GC/MS analysis of the CH_2Cl_2 extracts showed that only 4-mercaptobutyl ITC was generated (data not shown).

These results demonstrate that, in planta, the major *E. sativa* glucosinolate in leaves and seedlings is 4-mercaptobutyl GLS (Fig. 3). Extraction and concentration induces this molecule to oxidise forming a dimeric disulfide GLS artefact. This dimer GLS can be reduced efficiently by TCEP. The production of 4-mercaptobutyl ITC as a major volatile from fresh tissues may be responsible for the characteristic flavour and odour of *E. sativa*.

3. Experimental

3.1. General

All solvents were of HPLC grade, and all water was of ultra-pure grade. 4-Methylthiobutylisothiocyanate was obtained from LKT Laboratories (St Paul, Minnesota,

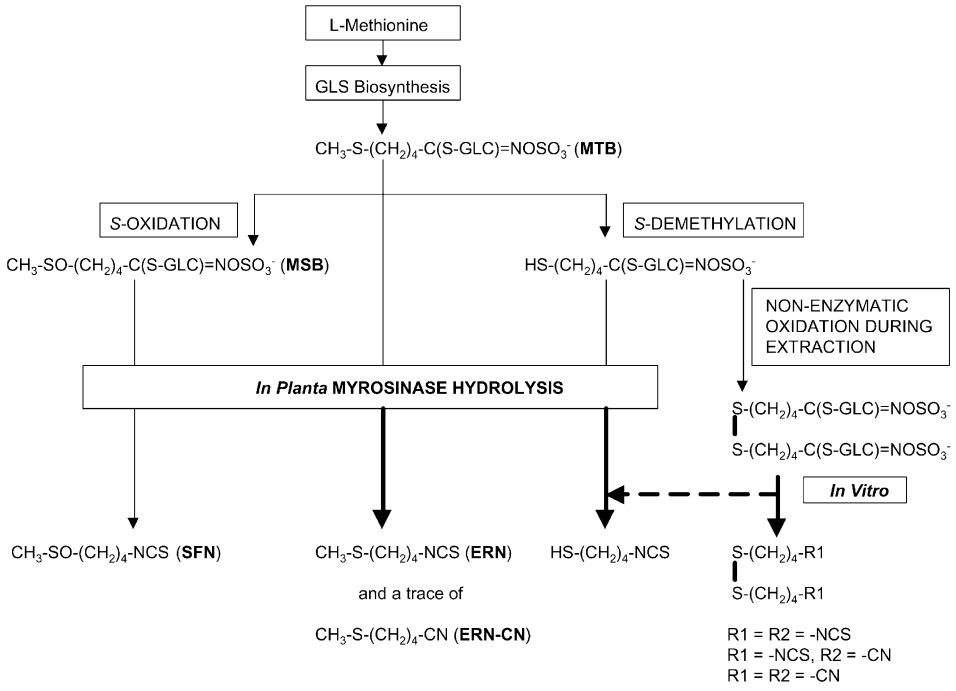

Fig. 3. *Eruca sativa* glucosinolate biosynthesis and hydrolysis in planta (endogenous myrosinase) and in vitro (exogenous myrosinase) showing formation of the artefact GLS and its hydrolysis products.

USA) and 4-methylsulfinylbutylisothiocyanate from ICN Pharmaceuticals Ltd. (Basingstoke, UK).

3.2. Plant material and extraction of glucosinolates

Seeds of *E. sativa* L. were obtained from E.W. King and Co Ltd, Colchester, UK. Young leaves were collected from 28-day-old plants and flash frozen in liquid N_2, freeze-dried and milled to a fine powder. The freeze-dried tissue was extracted in 70% MeOH (14 ml g^{-1} dry weight of tissue) at 70 °C for 20 min with vortex mixing every 5 min. After cooling, the samples were centrifuged (7000 *g*, 15 min, 4 °C) and the supernatants were combined (F1). The sample was concentrated by rotary evaporation at 40 °C. The concentrate (F2) was treated with 75 μl ml^{-1} of a 1:1 mixture of 0.1M lead and barium acetate and left at 4 °C for 30 min to precipitate protein. The sample was centrifuged (7000 *g*, 15 min, 4 °C) and the supernatant filtered (0.2 μm Target® PVDF filter); this filtered sample is the F3 fraction.

3.3. Ion-pair ESI LC/MS and LC/MS/MS

Intact GLS were analysed using previously validated LC/MS and LC/MS/MS methods (Mellon et al., in press). The only variations from the previously reported procedure were: (1) an optimum collision energy of 32.5 V (instead of 30 V) was used to obtain MS/MS spectra of the doubly negatively charged ion in the −ESI spectrum of the intact GLS dimer; (2) a cone voltage of 50 V was used to optimise the height of the *m/z* 731 peak ($[M-H-SO_3]^-$) in the −ESI spectrum of the GLS dimer for MS/MS analysis of the product ions of this peak. Desulfo-GLS were analysed by previously published methods (Kiddle et al., 2001). Mass spectrometric data are presented for all intact and desulfo-GLS, with the exception of two minor aliphatic GLS. The identities of 4-methylthiobutyl GLS and 4-methylsulfinylbutyl GLS were also confirmed by comparison with pure GLS standards. No aromatic or indole GLS were detected in leaves of *E. sativa*.

3.4. Purification of the new GLS and subsequent NMR

The new GLS was purified using Ecteola cellulose (EC23); 1.5 ml columns were prepared by adding 3 ml 1:1 EC23 in water to each column. A 400 μl sub-fraction of F3 was added to each column and samples were eluted sequentially with 3 ml volumes of water, 0.1% K_2SO_4, and 1% K_2SO_4. Fractions were checked for purity and content by −ESI LC/MS (14). The water fraction contained mainly MSB and traces of MTB and dimer, the 0.1% K_2SO_4 contained traces of MSB and dimer, and the 1% K_2SO_4 fraction only contained the dimer (90% of the dimer GLS loaded onto the columns was recovered in this fraction). Multiple EC23 columns were eluted and the 1% fractions were combined and freeze-dried. NMR was performed on the purified dimer GLS using standard methods (Kiddle et al., 2001); NMR spectra were run on a Varian Gemini spectrophotometer (^1H, 300 MHz; ^{13}C, 75.76 MHz).

3.5. Analysis of volatiles derived from E. sativa leaf GLS

Sub-samples (200 μl) of the enriched *E. sativa* GLS fraction (in order of concentration: new GLS > MSB > MTB) were treated with 3U of *Sinapis alba* myrosinase (Sigma) and incubated in a sealed screw-top Eppendorf at 20 °C for 30 min. CH_2Cl_2 (200 μl) was added and the samples were vortex mixed and then centrifuged (17,000 *g*, 12 min, 10 °C). The lower CH_2Cl_2 phase was removed with a glass syringe and transferred to vials for GC/MS analysis. In addition, fresh tissues of 5-day-old *E. sativa* seedlings (cotyledon and stem, in duplicate, 2 × 90 mg FW tissue) and leaves (leaf and petiole from a commercial source, in triplicate, 3 × 90 mg FW tissue) were crushed in tubes to induce autolysis, and were then extracted with 300 μl CH_2Cl_2. The samples were vortex mixed and centrifuged (17,000 *g*, 12 min, 4 °C) and the organic phases transferred to vials containing 10 mg of anhydrous $MgSO_4$ to remove residual water from the plant tissues. The samples were filtered (0.2 μm PTFE micro-filter) and analysed by EI GC/MS using A Trio 1-S mass spectrometer (Thermo Finnigan, Hemel Hempstead, UK), coupled to a Hewlett-Packard (Agilent Technologies UK Ltd., Stockport, UK) 5890 Series II gas chromatograph. Samples (1 μl solutions) were injected in splitless mode onto a 30 m long, 0.25 mm i.d., 0.25 μm film thickness J&W DB-5MS GC column (Agilent Technologies UK Ltd., Stockport, UK). The GC conditions were: Carrier gas (helium) flow Injector temperature 280 °C, column temperature 40 °C for 5 min, program to 300 °C at 6 °C/min, then back to 40 °C at −30 °C/min. and equilibrate for 1 min. Mass spectra were obtained at a rate of 1s per scan over the mass range m/z 35–500, with an interscan time of 0.1 s. The ion source temperature was maintained at 200 °C and samples were ionised in EI mode at 70 eV electron energy.

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