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Selenoglucosinolates and their metabolites produced in *Brassica* spp. fertilised with sodium selenate

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5-(Methylseleno)pentanenitrile
3-(Methylseleno)propylisothiocyanate
4-(Methylseleno)butylisothiocyanate
5-(Methylseleno)pentylisothiocyanate

ABSTRACT

Glucosinolates are sulphur-containing glycosides found in many *Brassica* spp. that are important because their aglycone hydrolysis products protect the plant from herbivores and exhibit anti-cancer properties in humans. Recently, synthetically produced selenium analogues have been shown to be more effective at suppressing cancers than their sulphur counterparts. Although selenium is incorporated into a number of *Brassica* amino acids and peptides, firm evidence has yet to be presented for the presence of selenium in the glucosinolates and their aglycones *in planta*. In this study broccoli and cauliflower florets, and roots of forage rape, all obtained from plants treated with sodium selenate, were analysed for the presence of organoselenides. GC–MS analysis of pentane/ether extracts identified six organoselenium compounds including selenium analogues of known myrosinase-derived *Brassica* volatiles: 4-(methylseleno)butanenitrile, 5-(methylseleno)pentanenitrile, 3-(methylseleno)propylisothiocyanate, 4-(methylseleno)butylisothiocyanate, and 5-(methylseleno)pentylisothiocyanate. LC–MS analysis of ethanolic extracts identified three selenoglucosinolates: 3-(methylseleno)propylglucosinolate (glucoselenoiberverin), 4-(methylseleno)butylglucosinolate (glucoselenoerucin), and 5-(methylseleno)pentylglucosinolate (glucoselenoberteroin). LC–MS/MS analysis was used to locate the position of the selenium atom in the selenoglucosinolate and indicates preferential incorporation of selenium via selenomethionine into the methylselenyl moiety rather than into the sulphate or β-thioglucose groups. In forage rape, selenoglucosinolates and their aglycones (mainly isothiocyanates), occurred at concentrations up to 10% and 70%, respectively, of their sulphur analogues. In broccoli, concentrations of the selenoglucosinolates and their aglycones (mainly nitriles) were up to 60% and 1300%, respectively of their sulphur analogues. These findings indicate the potential for the incorporation of high levels of selenium into *Brassica* glucosinolates.

1. Introduction

The uptake of selenium (Se) into the sulphur (S) assimilation pathway of edible *Brassica* plants provides a possible route to the increased dietary intake of selenium, which in some countries is low or below the recommended daily intake (Broadley et al., 2006). Selenium deficiency is associated with reduced human fertility, reduced immune and cognitive function, heart disease, hyperthyroidism, and an increased risk of certain types of cancer. There is evidence that these disorders can be suppressed by supplementing the diet with organoselenides (Finley et al., 2000; Ellis and Salt, 2003; Broadley et al., 2006; Rayman, 2008). One very potent anti-cancer agent is methylselenol, which is thought to be biosynthesised from the amino acid derivative Se-methyl selenocysteine and the peptide γ-glutamyl-Se-methyl selenocysteine (Rayman, 2008). Another product of selenium assimilation in plants is selenomethionine, the selenium analogue of methionine (Mounicou et al., 2006; Pedrero et al., 2007), which can be a source of stored selenium in the human body (Rayman, 2008).

A number of methylthioalkyl nitriles, thiocyanates, and isothiocyanates are reported in *Brassica* spp. (Verkerk et al., 2009; Sonderby et al., 2010). Isothiocyanates both prevent the initiation and retard the progression of carcinogenesis (Rose et al., 2005; Hayes et al., 2008; Melchini and Traka, 2010), while synthetic selenium-containing isothiocyanates, seleno-sulphoraphane (4-methylthio) butylisoselenocyanate and several phenylalkylisoselenocyanates are reported to be more potent inhibitors of cancer-cell and tumour growth than their sulphur analogues (Sharma et al., 2009; Emmert et al., 2010). Incorporation of selenium from selenomethionine into the above methylthioalkyl compounds would create methylselenoalkyl nitriles and isothiocyanates similar to the methylseleno aldehydes we have previously identified in genetically modified tobacco (Matich et al., 2009). Methylselenoalkyl nitriles and isothiocyanates have not been reported in nature and it is unknown whether these classes of

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organoselenides also contribute to the anti-cancer activity of *Brassica*. Verification of their presence would provide another group of compounds to be targeted in the study of the anti-cancer activity of *Brassica*.

The above nitriles, thiocyanates and isothiocyanates are biosynthetic hydrolysis products of glucosinolates (Verkerk et al., 2009), characteristic secondary metabolites of *Brassica* which are associated with resistance to herbivores (Halkier and Gershenzon, 2006; Mumm et al., 2008). Glucosinolates (Fig. 1, **22**–**24**) are β-thioglucoside *N*-hydroxysulphates with a side chain derived from one of eight amino acids that may also have undergone chain elongation or modification before glucosinolate formation (Halkier and Gershenzon, 2006). Myrosinase (or thioglucoside glucohydrolase) hydrolysis of glucosinolates yields glucose and an unstable aglycone that spontaneously rearranges to an isothiocyanate. Plants that contain epithiospecifier proteins (ESPs) can alternatively form nitriles and thiocyanates from glucosinolates at the expense of isothiocyanates (Halkier and Gershenzon, 2006; Burow et al., 2007; Mumm et al., 2008; Kissen and Bones, 2009). Trace amounts of selenium-containing glucosinolates (selenoglucosinolates) have been reported in the Brassicaceae plants *Stanleya pinnata* (Prince's plume) and *Nasturtium officinale* (watercress) (Bertelsen et al., 1988; Wielanek et al., 2005). Theoretically, the glucosinolates in *Brassica* might incorporate up to three atoms of selenium: in the sulphate, the modified amino acid (selenomethionine or methylselenocysteine) side chain (Pedrero et al., 2007), or in the β-thioglucose where it has been reported once in nature (Bertelsen et al., 1988). Thus, depending where in the glucosinolate molecule the selenium is incorporated, hydrolysis by myrosinase may produce a number of selenium containing products.

In the present study, we investigated the incorporation of selenium into the florets of the food *Brassica* spp., broccoli and cauliflower (*Brassica oleracea* L. var. *italica* and var. *botrytis*, respectively), and also into the roots of forage rape (*Brassica napus*) since this species has been reported to accumulate very high concentrations of organosulphides (McCully et al., 2008). To assist with the identification of organoselenides that might otherwise be at trace levels, we treated the plants with sodium selenate fertiliser to produce organoselenide concentrations well in excess of potential dietary levels (Finley et al., 2000). Using gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–mass spectrometry (LC–MS), we demonstrate, for the first time, the incorporation of inorganic selenium into the amino acid-derived methylselenyl side chain of *Brassica* selenoglucosinolates.

2. Results and discussion

2.1. GC–MS analysis of volatile organoselenides

Organoselenides are readily identifiable by mass spectrometry in complex plant extracts due to the mass deficiency of elemental selenium (Se^{80} = 79.9165 Da), the distinctive isotope pattern of the six major isotopes and the several characteristic selenium-containing ions (*m*/*z* 93, $CHSe^+$; 95, CH_3Se^+; 96, CH_3Se^+H; and 109, $C_2H_5Se^+$) frequently observed in the mass spectra of organoselenides. The structures of the volatile organoselenides identified in selenium supplemented broccoli and cauliflower florets, and forage rape roots are shown in Fig. 1. Eleven selenium containing volatiles were identified, including two methylseleno-aldehydes (**5** and **8** in Fig. 1), three methylseleno-nitriles (**4b**, **9b**, and **10b** in Fig. 1), and three methylseleno-isothiocyanates (**11b**, **12b**, and **13b** in Fig. 1). For comparison, their sulphur analogues identified herein in these species are also shown. All organosulphides identified in these samples have previously been reported in *Brassica* (Hashimoto et al., 1982; Nakamura et al., 1993; Tulio et al., 2002; de Pinho et al., 2009; Krumbein et al., 2010) as have some of the organoselenides. Dimethylselenosulphide (**2**, CH_3SeSCH_3) has been reported in garlic (Cai et al., 1994) and *Brassica juncea* (Indian mustard) (Meija et al., 2002; Kubachka et al., 2007) watered with selenium fertilisers. Dimethyldiselenide (**3**, $CH_3Se_2CH_3$) is frequently reported in *Brassica* (Meija et al., 2002; Kubachka et al., 2007), while $CH_3S_2SeCH_3$ (**7**) has been identified in genetically modified bacteria and tobacco (Swearingen et al., 2006; McKenzie et al., 2009) and in selenium-fertilised green onions (*Allium fistulosum*) (Shah et al., 2007). These mixed sulphur–selenium compounds are probably products of the exchange reaction between $CH_3Se_2CH_3$ (**3**), $CH_3S_2CH_3$ (**1**), and $CH_3S_3CH_3$ (**6**) (Chasteen, 1993), all of which we identified in the *Brassica* extracts (Table 1).

2.1.1. Selenium volatiles not previously reported in Brassica spp.

Methylselenomethanenitrile (**4b**, CH_3SeCN) was identified from its mass spectrum (Fig. 2) (Yang et al., 2004) and was found in only one of the cauliflower extracts. This compound has not previously been reported in nature, although its sulphur analogue, methylthiomethanenitrile (**4a**, CH_3SCN), is reported in broccoli (Jacobsson et al., 2004; Vidal-Aragón et al., 2009; Krumbein et al., 2010) and was present in all three *Brassica* species (Table 1). The sulphur compound 2-(methylthio)ethanal has been reported in tomatoes (Birtic et al., 2009) and heated onion sprouts (*Allium cepa* L.) (Takahashi and Shibamoto, 2008). We recently reported its selenium analogue, 2-(methylseleno)ethanal (**5**), in genetically modified tobacco overexpressing a selenocysteine methyltransferase transgene as a possible metabolite of methylselenocysteine (Matich et al., 2009). In the present study we detected traces of **5** in both broccoli and forage rape, but only in solvent extracts stored for several weeks at −20 °C, and not in extracts stored for shorter periods of time, suggesting this compound may be a storage artefact.

2.1.2. Selenium-containing aldehydes

To our knowledge, the other organoselenides listed in Table 1 (**8**, **9b**, **10b**, **11b**, **12b**, and **13b**) have not previously been reported. Aldehyde **8** was tentatively identified by its fragmentation pattern and its distinctive selenium isotope pattern which was repeated for the clusters of ions around *m*/*z* 192 (M^+), 177 (M^+-CH_3), 163 ($M^+-C_2H_5$), and 135 ($M^+-C_2H_5-CO$) (Fig. 3). The fragments *m*/*z* 163 and 135 were previously used as identifiers of 4-(methylseleno)-2-alkenals (Matich et al., 2009). Herein, CH_3Se^+H (*m*/*z* 96) indicates the loss of C_6H_8O (hexa-2,4-dienal) and the distinctive *m*/*z* 81 ($CH_2C^+CHCHCHO$) the loss of C_2H_7Se. 4-(Methylseleno)hex-*E*2-enal (**8**) was synthesised by the acid hydrolysis of (2,2-dimethoxyethyl)(methyl)selane in the presence of hex-*E*2-enal, as used previously to prepare 4-(methylseleno)non-*E*2-enal and 4-(methylseleno)nona-*E*2,*Z*6-dienal (Matich et al., 2009). In the present study, however, the product yield was poor and we were unable to purify **8**, which did, however, have the same mass spectrum and GC retention time as the compound in the *Brassica* extracts. We did not identify the sulphur analogue (4-(methylthio)hex-*E*2-enal) of compound **8** in our solvent extracts, and could not find it reported in the literature. Previously, we suggested that methylseleno aldehydes with structures similar to **8** were artefacts or arose from spontaneous chemical reactions within the plant (Matich et al., 2009). In the present study aldehyde **8** occurred at low concentrations, and was a minor component of the organoselenides found in the broccoli and cauliflower samples.

2.1.3. Selenium-containing nitriles

The molecular ion ($M^{+\cdot}$) of compound **9b** (4-(methylseleno)butanenitrile) was *m*/*z* 162.9901 (calcd. for C_5H_9NSe, 162.9900) and its fragmentation pattern (Fig. 2) was the most diagnostic of the two nitriles identified, viz. *m*/*z* 123 (M^+-CH_2CN), *m*/*z* 109 ($M^+-C_2H_4CN$ to give $CH_3SeCH_2^+$), and *m*/*z* 95 (CH_3Se^+). 5-

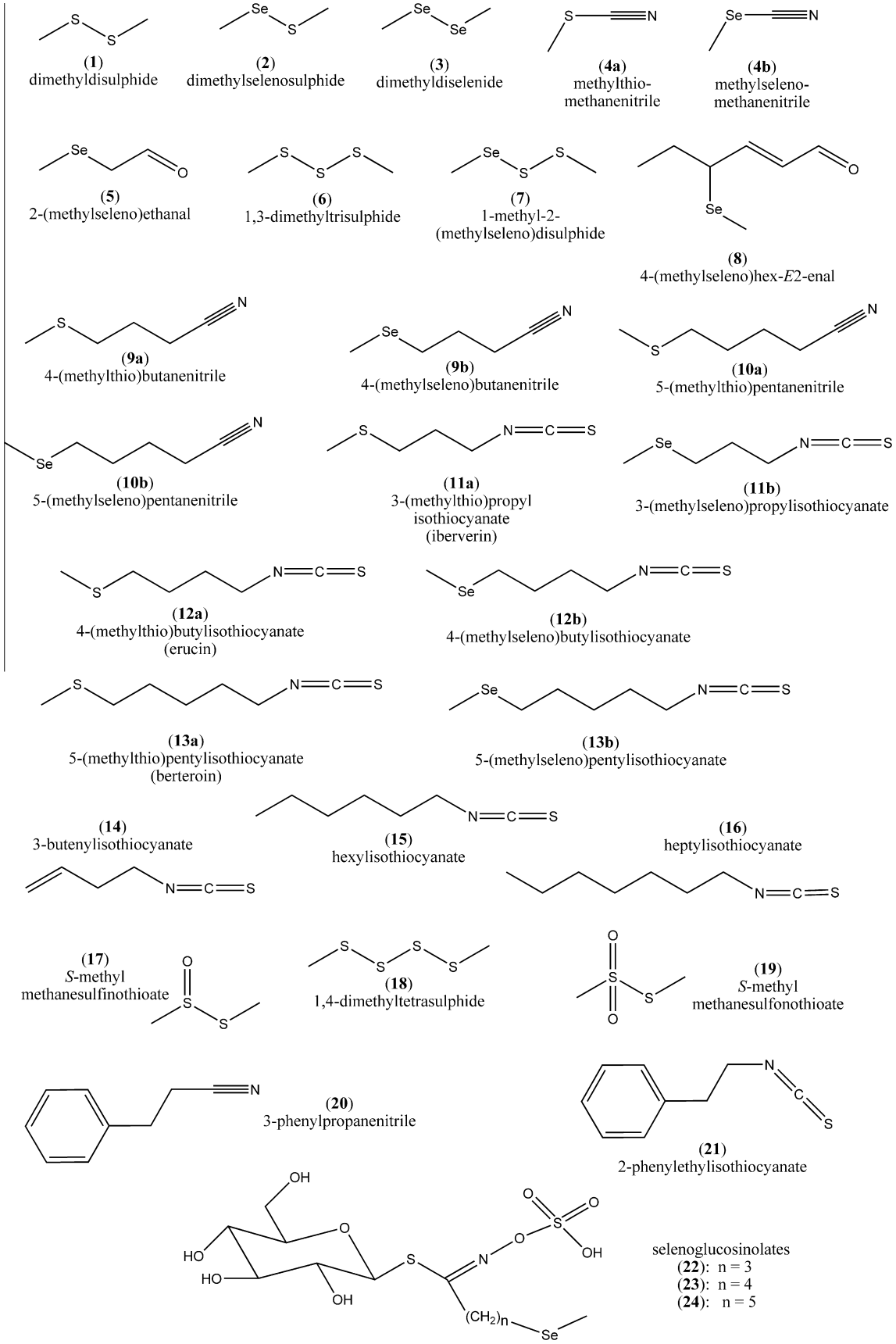

Fig. 1. Structures of selenium and sulphur compounds identified during this and previous studies.

(Methylseleno)pentanenitrile (**10b**) had an M^+ of *m/z* 177.0055 (calcd. for $C_6H_{11}NSe$, 177.0057) and a slightly different fragmentation pattern (Fig. 2), viz. *m/z* 162 (M^+-CH_3), *m/z* 135 ($M^+-C_2H_4N$), *m/z* 109 ($M^+-C_3H_6CN$ to give $CH_3SeCH_2^+$), *m/z* 95 (CH_3Se^+), and *m/z*

Table 1
Semi-quantitative GC–MS analysis (μg kg^{-1} FW) of selenium-containing volatiles and, where found, of their sulphur analogues in extracts of broccoli and cauliflower floret tissue and forage rape root tissue from control plants and from plants fertilised with 5 mM sodium selenate (Na_2SeO_4). Concentrations are the average (±SEM) of three samples, each sample comprising tissue combined from two separate plants. Chemical structures are given in Fig. 1.

Compound	RT (min)	RI (Wax)[a]	Broccoli cv. 'Triathlon'		Cauliflower cv. 'Liberty'		Forage rape cv. 'Maxima'	
			Control	Na_2SeO_4	Control	Na_2SeO_4	Control	Na_2SeO_4
CH_3SSCH_3 (**1**)[b]	4.71	1060	2700 (130)	2200 (510)	2700 (680)	1460 (350)	260 (140)	80 (20)
CH_3SeSCH_3 (**2**)[c]	7.2	1135	–[k]	310 (73)	–	164 (7)	–	104 (38)
$CH_3Se_2CH_3$ (**3**)[b]	9.85	1210	–	13 (2)	–	10 (4)	–	16 (4)
CH_3SCN (**4a**)[d]	10.93	1240	290 (14)	280 (44)	270 (130)	174 (38)	15 (4)	13 (2)
$CH_3S_3CH_3$ (**6**)[d]	14.91	1339	5800 (1230)	3500 (790)	1600 (250)	1150 (420)	280 (100)	98 (34)
CH_3SeCN (**4b**)[e]	18.39	1424	–	–	–	34 (34)	–	–
$CH_3S_2SeCH_3$ (**7**)[f]	18.99	1459	–	26 (9)	–	23 (11)	–	7 (0.3)
$CH_3SC_3H_6CN$ (**9a**)[g]	28.18	1742	80 (16)	320 (45)	2070 (340)	1490 (590)	23 (6)	70 (34)
$C_3H_6(CH_3Se)C_3H_3O$ (**8**)[h]	29.59	1815	–	5.7 (0.4)	–	11 (5)	–	–
$CH_3SeC_3H_6CN$ (**9b**)[g]	29.86	1831	–	1350 (160)	–	400 (120)	0.9 (0.1)	26 (13)
$CH_3SC_4H_8CN$ (**10a**)[d]	30.81	1888	1800 (420)	1600 (230)	160 (74)	90 (50)	460 (110)	620 (125)
$CH_3SC_3H_6NCS$ (**11a**)[d]	31.62	1939	9 (1)	19 (6)	170 (130)	390 (240)	43 (16)	63 (10)
$CH_3SeC_4H_8CN$ (**10b**)[h]	32.34	1983	–	1050 (100)	–	4 (1)	0.5 (0.1)	260 (140)
$CH_3SeC_3H_6NCS$ (**11b**)[h]	33.04	2032	–	260 (33)	–	110 (73)	–	46 (13)
$CH_3SC_4H_8NCS$ (**12a**)[i,j]	33.82	2086	320 (70)	340 (17)	45 (10)	40 (20)	5700 (1980)	6900 (810)
$CH_3SeC_4H_8NCS$ (**12b**)[h]	35.17	2181	–	1000 (44)	–	4 (2)	–	2040 (460)
$CH_3SC_5H_{10}NCS$ (**13a**)[i]	35.6	2209	–	–	–	–	9100 (970)	14800 (800)
$CH_3SeC_5H_{10}NCS$ (**13b**)[g]	36.81	2301	–	–	–	–	–	320 (83)

[a] Retention Index on DB-Wax GC column with respect to a series of straight-chain hydrocarbons.
[b] Identified against authentic compounds.
[c] Identified previously (Matich et al., 2009).
[d] Mass spectrum library matching (NIST, 2002).
[e] Yang et al. (2004).
[f] Meija and Caruso (2004), Swearingen et al. (2006) and Shah et al. (2007).
[g] Tentatively identified by MS analysis.
[h] Identified by synthesis of authentic compounds.
[i] Kjaer et al. (1963).
[j] Vercammen et al. (2001).
[k] Not detected.

82 ($C_4H_8CN^+$). These fragmentations are analogous to those observed for the sulphur analogues of these compounds (Spencer and Daxenbichler, 1980). We confirmed the identification of **9b** and **10b** by chemical synthesis. These two organoselenides and their sulphur analogues (**9a** and **10a**) were present in the extracts of all three *Brassica* spp. Compounds **9a** and **10a** have been reported in steam distillates of cabbage, broccoli and cauliflower (Buttery et al., 1976; Valette et al., 2003). Small amounts of organoselenides **9b** and **10b** were also identified in the forage rape control samples (no selenium fertiliser used) confirming the natural occurrence of these compounds (Table 1). The concentration of organoselenide **9b** in the selenium-fertilised forage rape samples was ca. 30-fold higher than in the control samples, and for **10b** it was ca. 500-fold higher. In total, the concentration of organoselenides (nitriles plus isothiocyanates) in the selenised forage rape roots was ca. 2000-fold higher than in the control samples.

The anti-cancer properties of *Brassica* nitriles have not received as much attention as the isothiocyanates and it has been suggested this is because few bioactive nitriles have been reported (Keck and Finley, 2004). In some studies nitriles have a similar anti-cancer activity to the isothiocyanates, but generally they are less active (Nastruzzi et al., 2000; Keck and Finley, 2004). Another biological activity reported for the *Brassica* nitriles is as a plant chemical defence against specialist herbivores. Predation is discouraged by a combination of reduced attraction of the specialist herbivore to the plant and the attraction of parasites that feed on the herbivore (Mumm et al., 2008).

2.1.4. Selenium-containing isothiocyanates

The mass spectra of compounds **11b**, **12b** and **13b** led us to suggest they were homologues (Fig. 4). With M^+ differing by 14 amu, viz. *m/z* 195, 209, and 223, respectively, they all displayed M^+-15 (*m/z* 180, 194, and 208, respectively), and M^+-74 (*m/z* 121, 135, and 149, respectively). All compounds produced *m/z* 109 ($CH_3Se^+CH_2$), *m/z* 93 (Se^+CH), and *m/z* 72 ($CH_2{=}N^+CS$) which is a well known isothiocyanate fragment (Kjaer et al., 1963). We surmise that M^+-74 represents the sum of the loss of HNCS and CH_3, and that these compounds are methylselenoalkylisothiocyanates. The fragment *m/z* 101 in compound **11b** (vs. *m/z* 114 in **13b** and *m/z* 128 in **13b**) was not totally consistent with the series, but a discontinuity in the fragmentation patterns for the sulphur analogues of these compounds is also observed (Kjaer et al., 1963). This fragment corresponds to a cyclisation and loss of $CH_2Se^{\cdot}$ to produce the 4,5-dihydro-3H-pyrrole-2-thiol ion (Fig. 4, $C_4H_7N^+S$). In contrast, *m/z* 114 and 128 are probably $C_4H_8NCS^+$ and $C_5H_{10}NCS^+$, respectively.

We confirmed the identity of 4-(methylseleno)butylisothiocyanate (**12b**) by synthesis. Compounds **11b** and **13b** were therefore assigned as 3-(methylseleno)propylisothiocyanate and 5-(methylseleno)pentylisothiocyanate, respectively, based upon their GC retention times and the similarity of their fragmentation patterns to that of **12b** (Fig. 4) and that of their sulphur analogues (Kjaer et al., 1963). 3-(Methylseleno)propylisothiocyanate (**11b**), 4-(methylseleno)butylisothiocyanate (**12b**) and their sulphur analogues **11a** and **12a** were identified in all three *Brassica* extracts, whilst 5-(methylseleno)pentylisothiocyanate (**13b**) and its sulphur analogue **13a** were found only in the extracts of forage rape.

Methylselenoalkylisothiocyanates **11b**–**13b** were previously unknown in nature and their biological activity is yet to be determined. However their sulphur analogues, and other isothiocyanates, exhibit anti-cancer activities (Zhang et al., 1992; Hwang and Lee, 2006; Melchini and Traka, 2010). Erucin (**12a**), was originally identified at high levels in the salad vegetable "Rocket" (*Eruca sativa*) and its mode of action as an anti-cancer phytochemical investigated (Wang et al., 2005; Melchini and Traka, 2010). Iberverin (**11a**) activates phase II detoxification enzymes, which are

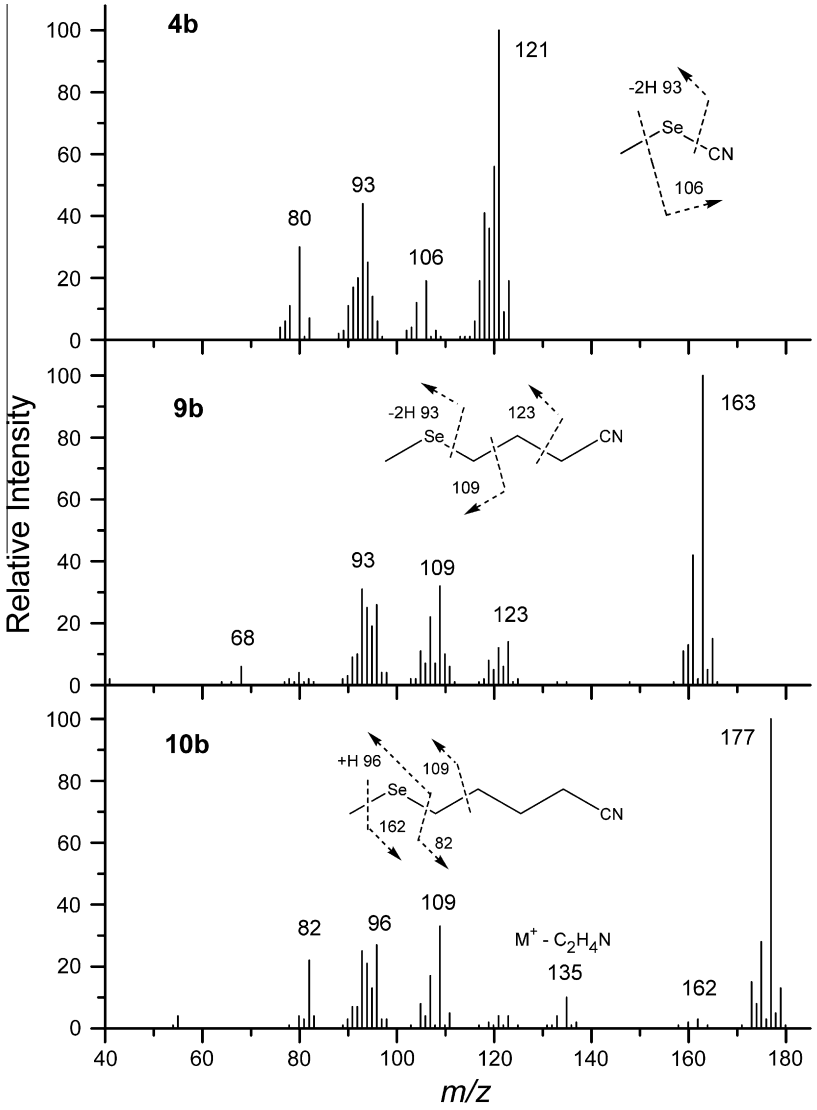

Fig. 2. Mass spectra of methylselenomethanenitrile (CH_3SeCN, **4b**), 4-(methylseleno)butanenitrile ($CH_3SeC_3H_6CN$, **9b**), and 5-(methylseleno)pentanenitrile ($CH_3SeC_4H_8CN$, **10b**).

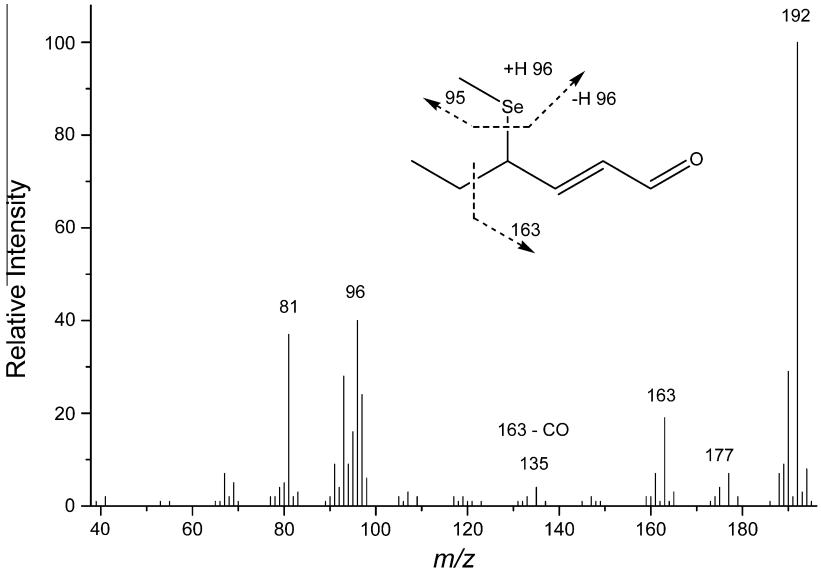

Fig. 3. Mass spectrum of 4-(methylseleno)hex-*E*2-enal (**8**).

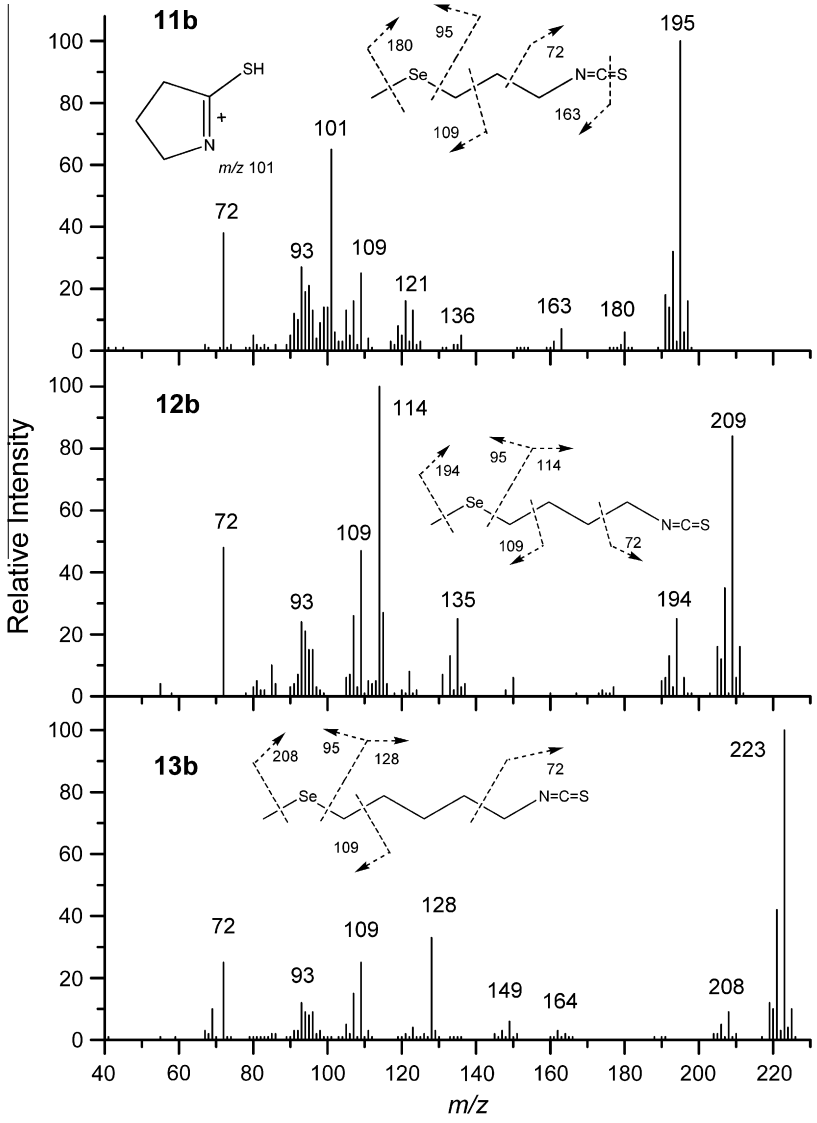

Fig. 4. Mass spectra of 3-(methylseleno)propylisothiocyanate ($CH_3SeC_3H_6NCS$, **11b**), 4-(methylseleno)butylisothiocyanate ($CH_3SeC_4H_8NCS$, **12b**), and 5-(methylseleno)pentylisothiocyanate ($CH_3SeC_5H_{10}NCS$, **13b**).

known to protect against chemical carcinogenesis (Munday and Munday, 2004). As a chemical class, isothiocyanates seem to be generally active against cancers (Russo et al., 2010). Therefore, compounds **11b–13b** may also show such activity, and given the suggestion that some selenium compounds are more potent anticancer compounds than their sulphur analogues (Sharma et al., 2009; Emmert et al., 2010), the discovery of these compounds provides new opportunities for understanding and improving the cancer-preventative properties of *Brassica*.

2.1.5. Concentrations of the organoselenides and their sulphur analogues

Table 1 gives semi-quantitative measurements of the tissue concentrations of the selenium compounds, and their sulphur analogues, identified in the solvent extracts of *Brassica* fertilised with sodium selenate. Table 2 lists the sulphur compounds for which the corresponding selenium analogues were not identified. These tables do not represent an exhaustive identification of all sulphur compounds present in the solvent extracts, just those easily identifiable and, in Table 1, of biosynthetic relevance to the selenium compounds discovered. The compounds of greatest interest were the isothiocyanates and the nitriles, because they are selenium analogues of compounds that are produced by the *Brassica* sulphur assimilation pathway.

The selenium-containing nitriles **9b** and **10b**, were at their highest concentrations in the broccoli samples at around 1 mg kg^{-1} FW (Table 1), with concentrations at similar orders of magnitude to those of their sulphur analogues (**9a** and **10a**), and with **9b** (1.35 mg kg^{-1} FW) 4-fold more concentrated than its sulphur analogue **9a** (0.32 mg kg^{-1} FW). In broccoli both selenium-containing isothiocyanates (**11b** and **12b**) were also at higher concentrations than their sulphur analogues (**11a** and **12a**). Concentrations of these compounds in cauliflower were lower than in the broccoli and the selenium compounds were at lower concentrations than their sulphur counterparts (Table 1). Cauliflower florets were however, the only plant tissue to contain methylselenomethanenitrile (**4b**), even though all three *Brassica* spp. contained its sulphur analogue (**4a**) (Table 1).

The selenium compound at highest concentration in forage rape roots was 4-(methylseleno)butylisothiocyanate (**12b**) at 2 mg kg^{-1}, which was 30% of the concentration of its sulphur analogue (**12a**). The forage rape roots had the highest concentrations of sulphur aglycones, in the form of the isothiocyanates **12a** and **13a** (~7 and ~15 mg kg^{-1} FW, respectively, Table 1). However, the concen-

Table 2
Semi-quantitative analysis of sulphur-containing volatiles, measured by GC–MS (μg kg^{-1} FW), in extracts of broccoli and cauliflower floret tissue and forage rape root tissue from control plants and plants fertilised with 5 mM sodium selenate (Na_2SeO_4), for which selenium analogues were not identified. Compound identification is by mass spectrum library matching (NIST, 2002). Concentrations are the average (±SEM) of three samples, each sample comprising tissue combined from two separate plants. Chemical structures are given in Fig. 1.

Compound	RT (min)	RI (Wax)[a]	Broccoli cv. 'Triathlon'		Cauliflower cv. 'Liberty'		Forage rape cv. 'Maxima'	
			Control	Na_2SeO_4	Control	Na_2SeO_4	Control	Na_2SeO_4
$CH_2{=}CHC_2H_4NCS$ (**14**)	18.27	1425	4 (1)	31 (4)	2.1 (0.5)	1 (0.1)	360 (40)	230 (46)
$C_6H_{13}NCS$ (**15**)	23.38	1555	23 (4)	16 (3)	–	–	25 (4)	40 (7)
$C_7H_{15}NCS$ (**16**)	25.06	1609	–[b]	–	–	–	65 (18)	110 (14)
$CH_3S(O)SCH_3$ (**17**)	25.25	1610	7100 (1570)	5700 (890)	5900 (1500)	4200 (140)	123 (36)	72 (16)
$CH_3S_4CH_3$ (**18**)	27.11	1687	430 (210)	200 (35)	180 (60)	70 (23)	13 (1)	12 (4)
$CH_3S(O_2)SCH_3$ (**19**)	31.54	1932	40 (25)	75 (34)	360 (170)	280 (230)	105 (35)	76 (52)
PhC_2H_4CN (**20**)	32.50	1994	220 (10)	84 (10)	60 (20)	15 (7)	1600 (190)	2100 (550)
PhC_2H_4NCS (**21**)	35.05	2175	560 (21)	340 (26)	100 (24)	60 (25)	80,000 (11,800)	83,000 (7660)

[a] Retention Index on DB-Wax GC column with respect to a series of straight-chain hydrocarbons.
[b] Not detected.

trations of their corresponding seleno-aglycones (**12b** and **13b**) were not similarly high, and were at around the same concentrations as those of the selenium-containing nitriles and isothiocyanates in the broccoli samples. Generally, the forage rape samples had higher concentrations of isothiocyanates than of nitriles, while in the broccoli and cauliflower the reverse was observed. The concentration of 5-(methylseleno)pentylisothiocyanate (**13b**) in forage rape root was around 16% of the concentration of 4-(methylseleno)butylisothiocyanate (**12b**), even though there was 2-fold more of 5-(methylthio)pentylisothiocyanate (**13a**) present than there was of 4-(methylthio)butylisothiocyanate (**12a**). Apparently, the plant was more likely to produce the selenium analogue of **12a** than **13a**, even though these compounds differ in structure by only one methylene group.

The summed concentrations of the selenoglucosinolate aglycones **9b**–**13b** in the forage rape roots was around 2.7 mg kg^{-1} (12% of the total concentration of the sulphur analogues **9a**–**13a**). For broccoli, the seleno-aglycones **9b**–**12b** totalled 3.7 mg kg^{-1} (160% of **9a**–**12a**), and for the cauliflower the seleno-aglycones **4b**, **9b**–**12b** totalled 0.6 mg kg^{-1} (25% of **4a** and **9a**–**12a**). Thus, there was a greater percentage incorporation of selenium into the broccoli and cauliflower glucosinolate aglycones than there was into those of forage rape. The sum of all selenium compounds in the broccoli (4 mg kg^{-1}), cauliflower (0.76 mg kg^{-1}), and forage rape (2.8 mg kg^{-1}) samples constituted 21%, 7.5% and 2.5%, respectively of the combined total of sulphur and selenium compounds identified (Tables 1 and 2). In part, the forage rape samples had the lowest percentage incorporation of selenium into the sulphur assimilation pathway because the major forage rape root aglycone is 2-phenylethylisothiocyanate (**21**) (McCully et al., 2008). This compound constituted 75% of the combined total of the sulphur and selenium compounds present in the forage rape roots but no selenium analogue of this compound was present.

Most of the compounds in Table 2 do not contain the methylthio moiety, and therefore seleno-amino acid-derived analogues of these compounds (**14**, **15**, **16**, **20**, and **21**) do not appear to exist. This is consistent with the exclusive incorporation of selenium into the selenoglucosinolates via selenomethionine. The selenium analogues of **17** and **19** have not been reported in the literature, possibly because at room temperature they are unstable and undergo the selenoxide β-elimination reaction (March, 1985). The 2-phenylethylisothiocyanate (**21**), found in the forage rape root extract (Table 2), was at high concentrations of around 83 mg kg^{-1} (FW). Concentrations of grams per kg of **21** have previously been reported in forage rape roots (McCully et al., 2008) and in Brassicaceae such as watercress (Newman et al., 1992). If there was incorporation of selenium into the isothiocyanate (R-NCS) group to produce isoselenocyanates (R-NCSe), then it would most likely to be observed for **21**, which is the highest concentration isothiocyanate we identified in these samples. In the present study we were able to identify seleno-aglycones at concentrations as low as ca. 5 μg kg^{-1} which is less than 0.01% of the concentration of the 2-phenylethylisothiocyanate in the selenium-treated forage rape sample (Table 2). Despite this, we did not identify any isoselenocyanates in our *Brassica* extracts. However, it cannot be conclusively determined that the plants in this study did not produce these compounds, because we examined only one tissue type from the plants and some isoselenocyanates are unstable under light and in aqueous environments (Sonoda et al., 1972; Kjaer and Skrydstrup, 1987).

2.2. LC–MS analysis of selenoglucosinolates

Having identified their hydrolysis products, we next sought to identify the parent selenoglucosinolates directly using high resolution LC–MS. The LC–MS chromatograms of the ethanolic extracts of ground, frozen broccoli and forage rape tissues contained chromatographic peaks whose high resolution pseudomolecular ions and isotope patterns matched those reported previously for glucosinolates in these *Brassica* spp. (Tian et al., 2005; Bialecki et al., 2010; Cataldi et al., 2010). These nonselenium-containing glucosinolates constituted the majority of the compounds in the ethanolic extracts.

Small amounts of three selenium-containing glucosinolates (**22**–**24** in Fig. 1) were identified in the ethanolic extracts of broccoli and forage rape tissues. We have named these compounds glucoselenoiberverin (**22**), glucoselenoerucin (**23**), and glucoselenoberteroin (**24**): they are the selenium analogues of the well known glucosinolates glucoiberverin, glucoerucin, and glucoberteroin, respectively (Nugon-Baudon and Rabot, 1994). All three selenoglucosinolates were clearly identified in the forage rape sample, and glucoselenoiberverin and glucoselenoerucin were also identified in the broccoli sample (Table 3).

2.2.1. LC–MS identification of the selenoglucosinolates

Glucoselenoerucin (**23**) (RT = 7.0 min, Fig. 5) was the most abundant of the three selenoglucosinolates and there was sufficient compound present to observe a good selenium isotope pattern and obtain high-resolution mass spectral data. Several diagnostic daughter ions were also measured from fragmentation of the pseudomolecular ion *m/z* 467.9899 ($[M-H]^-$, −0.7 mDa, calcd. for $C_{12}H_{22}NO_9S_2Se$, 467.9906). Most daughter ions were diagnostic for glucose (Fig. 6), viz. *m/z* 275 ($C_6H_{11}O_8S_2^-$) from rearrangement of $[M-H]^-$ and neutral loss of $CH_3SeC_4H_8N{=}C{=}O$ (*m/z* 193); *m/z* 259 ($C_6H_{11}O_9S^-$) from rearrangement and neutral loss of $CH_3SeC_4H_8N{=}C{=}S$ (209); and the thioglucose group ($C_6H_{11}O_5S^-$)

Table 3
Percentage composition (LC–MS peak area of *m/z* [M−H]$^-$) of glucosinolates for which selenium analogues were detected in ethanolic extracts, and ratio of incorporation of selenium and sulphur (Se:S) into the methylseleno/methylthio group of these compounds.

Compound	Broccoli florets cv. 'Triathlon'	Forage rape roots cv. 'Maxima'
Glucoiberverin	7	0.3
Glucoselenoiberverin (**22**)	4.2 (38:62)	0.03 (9:81)
Glucoerucin	76	26
Glucoselenoerucin (**23**)	11.3 (13:87)	1.2 (4:96)
Glucoberteroin	1.4	72
Glucoselenoberteroin (**24**)	nd (0:100)	0.4 (0.6:99.4)

at *m/z* 195. The bisulphate ion was also identified at *m/z* 97 (HSO_4^-). These fragmentation ions agree with those reported (Cataldi et al., 2010; Clarke, 2010) for the sulphur glucosinolates and were used to identify the sulphur analogue of **23** (4-(methylthio)butylglucosinolate, glucoerucin), which eluted 0.8 min (RT = 6.2 min) before the selenium compound with [M−H]$^-$ *m/z* 420.0460 (calcd. for $C_{12}H_{22}NO_9S_3$, 420.0462) and a distinctive S_3 isotope pattern.

The above glucoselenoerucin ions indicate the presence of two sulphur atoms in the glucosinolate, with one in the sulphate group and the second as the bridge between glucose and the rest of the molecule. Therefore, the selenium atom is probably located in the amino acid-derived side chain. In the sulphur analogue (erucin) we observed the neutral loss M−H−Glc−SO_3 to produce *m/z* 178.0372 (+1.1 mDa, calcd. for $C_6H_{12}NOS_2$, 178.0361) with the structure $CH_3SC_4H_8C(S^-)$=NOH (Clarke, 2010). In selenium compound **23** we also observed this ion, but containing selenium with *m/z* 225.9813 (+0.8 mDa, calcd. for $C_6H_{12}NOSSe$, 225.9805) and, by analogy, with the structure $CH_3SeC_4H_8C(S^-)$=NOH (Fig. 6). This confirmed that the selenium atom was in the side chain of the glucosinolate molecule and accords with our GC–MS identification of a number of methylselenoalkly isothiocyanates and nitriles in the solvent extracts.

Glucoselenoberteroin (**24**) (RT = 8.9 min) was identified from its pseudomolecular ion at *m/z* 482.0067 ([M−H]$^-$, −0.4 mDa, calcd. for $C_{13}H_{24}NO_9S_2Se$, 482.0063) with a distinctive selenium isotope pattern. The sulphur analogue eluted 1 min earlier (RT = 7.92 min) with pseudomolecular ion *m/z* 434.0618 ([M−H]$^-$, +0.5 mDa, calcd. for $C_{13}H_{24}NO_9S_3$, 434.0613) and a distinctive S_3 isotope pattern. Identification of glucoselenoiberverin **22** (RT = 5.5 min) was less definitive. This minor compound gave *m/z* 453.9732 ([M−H]$^-$, −1.7 mDa, calcd. for $C_{11}H_{20}NO_9S_2Se$, 453.9745), but neither its selenium isotope pattern nor its daughter ions could be measured. The sulphur analogue, 3-(methylthio)propylglucosinolate, was identified eluting 0.65 min before **22** (RT = 4.85 min), and was identified from its pseudomolecular ion at *m/z* 406.0272 ([M−H]$^-$; −3.4 mDa; calcd. for $C_{11}H_{20}NO_9S_3$, 406.0306) and its distinct S_3 isotope pattern.

2.2.2. Previous reports of selenoglucosinolates

There appear to be only three previous reports of selenoglucosinolates; an organic synthesis of benzyl- and methyl-selenoglucosinolate (Kjaer and Skrydstrup, 1987), an unspecified incorporation of selenium into 2-phenylethylglucosinolate in root cultures of Prince's plume (Wielanek et al., 2005) and one of trace levels of 3-butenylselenoglucosinolate in watercress fertilised with selenium fertilisers (Bertelsen et al., 1988). For the two studies in which the position of selenium incorporation was reported, it was identified as D-selenoglucose. This contrasts with compounds **4b**, **9b**, **10b**–**13b**, **22**–**24** identified here where the selenium was located in the amino acid-derived side chain as a methylseleno group.

2.2.3. Relative concentrations of the selenoglucosinolates

Integration of the [M−H]$^-$ peaks (Fig. 5), for the three selenoglucosinolates and their three sulphur analogues in the ethanolic extract of the forage rape root sample, indicated that the sulphur-glucosinolates were the major analogues present (Table 3), as were their hydrolysis products, the methylthio nitriles and isothiocyanates by GC–MS (Table 1). The major selenoglucosinolate was glucoselenoerucin (**23**) at around 4.5% of the concentration of glucoerucin. These relative amounts were reflected in the aglycone hydrolysis products in which 4-(methylseleno)isothiocyanate (**12b**) was at the highest concentration and was at around 30% of the concentration of 4-(methylthio)isothiocyanate (**12a**, erucin) (Table 1). The selenoglucosinolates in the forage rape roots constituted approximately 1.6% of the six glucosinolates in Table 3, and

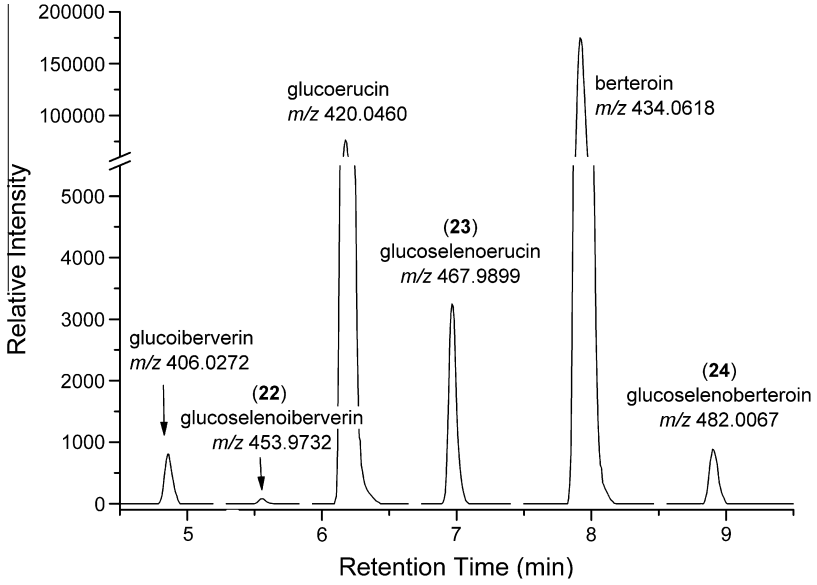

Fig. 5. Reconstructed ion chromatogram (RIC) of the pseudomolecular ions [M−H]$^-$ of methylseleno and corresponding methylthio glucosinolates identified in an ethanolic extract of 'Maxima' forage rape root.

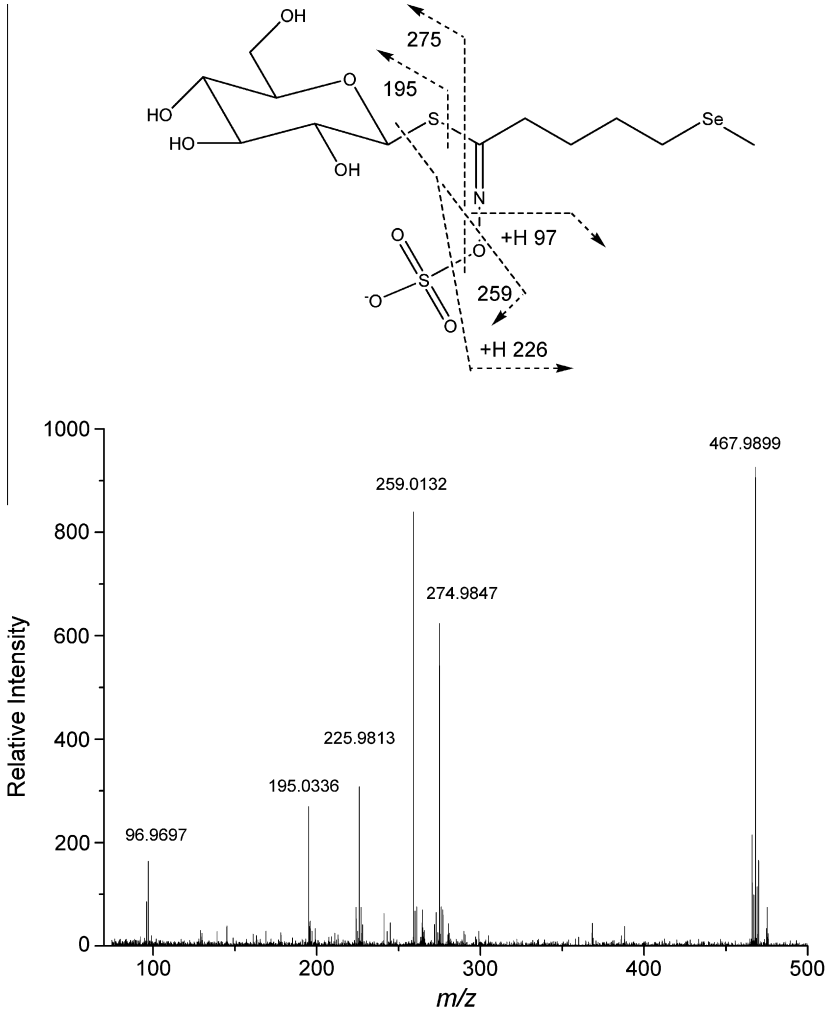

Fig. 6. Fragmentation of the pseudomolecular ion ($[M-H]^-$, *m/z* 468) of 4-(methylseleno)butylglucosinolate (glucoselenoerucin, **23**) identified in an ethanolic extract of 'Maxima' forage rape root.

9% of their aglycones were organoselenides (Table 1). Incorporation of selenium into these broccoli glucosinolates, and their aglycones, was considerably higher. The broccoli glucosinolates shown in Table 3, were 15.5% organoselenides, and their aglycones (Table 1) were 78% organoselenides. We did not measure the glucosinolates in the cauliflower samples, but approximately 21% of their aglycones contained selenium.

Incorporation of selenium into the broccoli and forage rape glucosinolates was higher for the short-chain aglycone compounds (Table 3). In the forage rape sample the Se:S ratio decreased with increasing chain length from 9:81 to 0.6:99.4, and for the broccoli sample the ratios decreased from 60:40 to 0:100. The Se:S ratio of the isothiocyanate aglycones showed the same behaviour. The broccoli Se:S ratio decreased from 93:7 to 75:25, for cauliflower from 23:77 to 8:92, and for forage rape the ratios were 42:58, 23:77, and 2:98.

2.3. The biosynthetic origins of the organoselenides

In *Brassica*, methylthioalkyl nitriles and isothiocyanates are myrosinase hydrolysis products of methylthioalkyl glucosinolates, with the sulphur being derived from methionine (Halkier and Gershenzon, 2006; Kissen and Bones, 2009). Thus in the present work the methylselenoalkyls would be hydrolysis products of methylselenoalkyl glucosinolates, with the selenium being sourced from selenomethionine. The sulphur atom in the isothiocyanate moiety of glucosinolates is derived from glutathione (Sonderby et al., 2010), and so by analogy isoselenocyanates would be expected to be derived from selenoglutathiones. However, no isoselenocyanates (R-NCSe) were found in the present study. Isoselenocyanates are not well known in nature with only one report in which trace amounts of 3-butenylselenoglucosinolate were reported in Brassicaceae (*S. pinnata*) treated with high concentrations of selenium fertiliser (Bertelsen et al., 1988). Bertelsen et al. proposed that the poor incorporation of selenium into the glucosinolates indicated the ability of the plants to detect the chemical non-equivalency of sulphur and selenium.

Methylthio and methylseleno alkyl nitriles and isothiocyanates were identified in all extracts, but the nitriles occurred at higher concentrations in the broccoli and cauliflower while the isothiocyanates were the major species in the forage rape roots. The nitrile:isothiocyanate ratios in the three different plants were 70:30, 80:20, and 3:97, respectively. Thus while all plants showed myrosinase activity, the forage rape roots produced the largest quantities of isothiocyanates in agreement with previous reports that the major glucosinolate hydrolysis products in *B. napus* roots are isothiocyanates (Brown and Morra, 1996; McCully et al., 2008).

Previous reports of glucosinolate hydrolysis products report either low molecular weight nitriles (Vidal-Aragón et al., 2009; Krumbein et al., 2010), or significant amounts of both nitriles

and isothiocyanates (Buttery et al., 1976; Di Cesare et al., 2001, 2002; Valette et al., 2003). The studies that identified the low molecular weight nitriles involved headspace analyses, whereas the other studies involved thermal extraction methods such as hydrodistillation. Damage to broccoli tissue at room temperature produces nitriles as the primary hydrolysis products, as a result of intercession by epithiospecifier proteins (Matusheski et al., 2004, 2006). However, heating the broccoli inactivates the epithiospecifier proteins and allows production of isothiocyanates. In the present study, solvent extraction of all tissue samples was performed at low temperatures, which is consistent with the identification of nitriles as the main glucosinolate hydrolysis products in broccoli and cauliflower.

3. Concluding remarks

Brassica treated with selenate fertiliser incorporate selenium via the sulphur assimilation pathway to produce selenoglucosinolates, selenium analogues of well known *Brassica* compounds. Selenium in the selenoglucosinolates was present as a methylselenoalkyl group that was probably derived from selenomethionine. There was no evidence for incorporation of selenium into either the sulphate or the thioglucose moieties of the glucosinolate.

There was a significant incorporation of selenium into roots of forage rape, with selenoglucosinolates present at up to 10% of the concentration of their sulphur analogues. The concentrations of the selenium-containing aglycones of the three glucosinolates were as high as 70% of the concentrations of their sulphur analogues. The broccoli and cauliflower samples showed a considerably higher relative incorporation of selenium. The broccoli selenoglucosinolates reached 60% of the concentrations of their sulphur analogues, and the selenium aglycone concentrations were up 1300% of their sulphur analogues. The cauliflower aglycones also had high concentrations of selenium, with an Se:S ratio as high as 0.3:1. Incorporation of selenium into the glucosinolates and their hydrolysates appeared to be dependent upon the length of the side chain derived from selenomethionine. However, overall these results indicate the potential for the production of significant concentrations of organoselenium compounds by *Brassica* species.

4. Experimental

4.1. Plant, growth and harvesting

One cultivar each of broccoli (*B. oleracea* L. var. *italica* cv. 'Triathlon'), cauliflower (*B. oleracea* L. var. *botrytis* cv. 'Liberty'), and forage rape (*B. napus* cv. 'Maxima') were grown in 8 l bags of potting mix at Plant & Food Research, Palmerston North. Plants were watered once daily via watering spikes for 1 min. Selenium feeding was commenced for broccoli and cauliflower following the emergence of immature floret material from the meristem and for forage rape once the plants had a well established stem (ca. 16 cm from soil to top of meristem) and approximately 10 fully expanded leaves. Plants were fed via the soil with 20 ml of 5.0 mM sodium selenate twice weekly, for four weeks, after watering. We estimate this selenium application to be equivalent to ca. 15 kg ha^{-1} which is very high compared with the 2–20 g ha^{-1} reported for agricultural and forage crops (Pulsipher et al., 2004; Broadley et al., 2006; Eich-Greatorex et al., 2007). Previous workers reported that broccoli produced at this higher rate of selenium application contained at least 500-fold more selenium than control samples (Finley et al., 2000) but suffered no adverse effects upon its growth. Control plants received no added selenium. Following the selenium feeding, mature floret tissue was harvested from the broccoli and cauliflower plants, and the lower stem/tap root (3–4 cm long) was harvested from the forage rape plants. For the forage rape tissue the outer fibrous ring was removed and the inner pith sampled. All material was immediately frozen in liquid nitrogen before storage at −80 °C until extraction.

4.1.1. Extraction of volatile compounds for GC–MS analysis

Triplicate extractions were performed, each replicate comprising tissue from two separate plants. For each replicate 10–15 g FW was accurately weighed and ground, with dry ice, to a powder in a coffee grinder. The sample was combined with 2 vol. (w/v) of pre-chilled 50:50 pentane/diethyl ether on ice in a Schott bottle, sealed and left at 1 °C for a week with daily mixing before the solid material was frozen at −20 °C and the solvent poured off. The solvent extracts were dried ($MgSO_4$), and their volumes reduced to ca. 1.5 ml under a gentle stream of nitrogen.

4.1.2. Extraction of glucosinolates for LC–MS analysis

Frozen floret tissue (1 g) of broccoli, fertilised with 5 mM Na_2SeO_4, was ground in liquid nitrogen in a mortar and pestle and poured into 80% ethanol (10 ml) preheated and then held at 80 °C for 5 min in order to inactivate myrosinase (Tian et al., 2005). Samples were cooled at 5 °C for 30 min, filtered, and the filtrate stored at −20 °C. Frozen forage rape root tissue (1 g), partially ground with sand in liquid nitrogen, was quenched into 80% ethanol (20 ml) held at 80 °C for 5 min, and combined with a further 20 ml of 80% ethanol used to rinse the mortar.

4.2. GC–MS analysis of volatile compounds

Separations were performed on an Agilent 6890N GC coupled to a Waters GCT time of flight (ToF) mass spectrometer with an EI energy of 70 eV and a scan time of 0.4 s. One microlitre split (5:0.9) injections were made at 220 °C onto a 20 m × 0.18 mm i.d. × 0.18 μm film thickness DB-Wax (J & W, Scientific) capillary column with a He flow of 0.9 ml min^{-1}. The oven temperature programme was 1 min at 35 °C, 3 °C min^{-1} to 100 °C, 7 °C min^{-1} to 240 °C, hold 5 min. External standards used for semi-quantification (using total ion current peak areas) were $CH_3SeSeCH_3$ (**3**) for compounds **1**–**3**, **6**–**8** and **17**–**19**, and $CH_3SeC_3H_6CN$ (**9b**) for the nitriles and isothiocyanates, at concentrations of 0.0005, 0.00005 and 0.000005 μl ml^{-1} in order to cover the range of concentrations of compounds measured in the solvent extracts. The external standards and the plant extracts were spiked with internal standards (undecane, tridecane, and tetradecane) at concentrations covering three orders of magnitude. The hydrocarbon standards enabled correction for the plant extract sample volumes and for GC–MS system responses. Retention indices of compounds were determined against a standard containing straight-chain hydrocarbons ($C_{10}H_{22}$–$C_{26}H_{54}$).

4.3. LC–MS analysis of glucosinolates

LC–MS analysis enabled identification of three selenoglucosinolates: 3-(methylseleno)propylglucosinolate (**22**, glucoselenoiberverin), 4-(methylseleno)butylglucosinolate (**23**, glucoselenoerucin), and 5-(methylseleno)pentylglucosinolate (**24**, glucoselenoberteroin), and their known sulphur analogues. Separations were performed on a Dionex Ultimate® 3000 Rapid Separation LC with a micrOTOF-Q II mass spectrometer (Bruker Daltonics, Germany) fitted with an electrospray source operating in negative mode. Sample injections (2 μl), were made onto a Zorbax™ SB-C18 2.1 × 100 mm, 1.8 μm (Agilent) analytical column maintained at 50 °C and with a flow of 400 μl min^{-1}. Solvent A was 10:90 H_2O:MeOH, and Solvent B was 0.5:99.5 HCO_2H:H_2O. The solvent ramp was 1:99 (Solvent A:Solvent B) from injection to 0.5 min, followed by a linear gradient to 30:70 (0.5–8 min), to 75:25 (8–13 min), to 100:0 (13–15 min), hold at 100:0 (15–17 min). The

micrOTOF-Q II source parameters were: temp. 200 °C; drying N_2 flow 8 l min^{-1}; nebuliser N_2 4 bar (400 kPa), endplate offset −500 V, capillary voltage +3500 V; mass range 100–1500 Da at 2 scans s^{-1}. Post-acquisition internal mass calibration used sodium formate clusters with the sodium formate delivered by a syringe pump at the start of each analysis. Mass spectral data were processed using Compass DataAnalysis (Bruker Daltonics).

4.4. Chemical synthesis and mass spectral data

4-(Methylseleno)hex-*E*2-enal (**8**, $C_3H_6(CH_3Se)C_3H_3O$) synthesis: $CH_3Se_2CH_3$ (100 mg, 0.53 mmol, Aldrich) was added to a solution of $NaBH_4$ (60 mg, 1.59 mmol) in absolute EtOH (5 ml) under N_2. After 5 min of vigorous reaction the colour of the solution changed from yellow to white. Bromoacetaldehyde dimethylacetal (0.127 g, 0.75 mmol, Aldrich) was added and stirred for 21 h. Water (10 ml) was added and the organic phase extracted with Et_2O (4 × 25 ml) and dried ($MgSO_4$). The solvent was removed and the crude product taken up in tetrahydrofuran (3 ml). Water (200 µl), HCl (25 µl conc.) and hex-*E*2-enal (15 µl, Aldrich) were added and the solution stirred for 20 h. $NaHCO_3$ (3 ml, sat. aq.) was added and the organic phase extracted with dichloromethane (4 × 25 ml). Attempts to purify the desired product by silica flash chromatography were unsuccessful. GC–MS analysis showed the major product was 2,2-bis(methylseleno)acetaldehyde with **8** also present. EI-ToF-MS (Fig. 3), *m*/*z* (rel. int.): 192 $[M]^+$ (100), 190 ($[M]^+$, Se^{78}) (24), 177 $[M-CH_3]^+$ (8), 163 $[C_5H_7SeO]^+$ (16), 135 (4), 97 (20), 96 $[M-SeCH_4]^+$ (38), 95 $[CH_3Se]^+$ (17), 94 (10), 93 (25), 81 $[C_5H_5O]^+$ (31), 69 (5), 67 (8).

4-(Methylseleno)butanenitrile (**9b**, $CH_3SeC_3H_6CN$) synthesis: $CH_3Se_2CH_3$ (100 mg, 0.53 mmol, Aldrich) was added to a solution of $NaBH_4$ (60 mg, 1.59 mmol) in 5 ml of absolute EtOH under N_2. After 5 min of vigorous reaction the colour of the solution changed from yellow to white. 5-Bromobutanenitrile (0.157 g, 1.06 mmol, Acros) was then added and stirred for 2.5 h. Et_2O (10 ml) was added, the reaction mixture was washed with 5 ml each of water and brine, extracted with 3 × 25 ml of Et_2O, and dried ($MgSO_4$). The solvent was removed and the crude product purified by flash chromatography on silica (petroleum ether:Et_2O, 90:10 and 85:15) to give **9b** as a yellow oil (87 mg, 50%). EI-ToF-MS (Fig. 2), *m*/*z* (rel. int.): 163 $[M]^+$ (100), 161 (42), 123 (14), 121 (12), 109 $[CH_3SeCH_2]^+$ (32), 107 (22), 96 $[CH_3SeH]^+$ (26), 95 $[CH_3Se]^+$ (19), 94 $[CH_2Se]^+$ (25), 93 $[CHSe]^+$ (31), 68 (6). ^1H NMR (500 MHz, $CDCl_3$): δ2.01 (s, 3H, CH_3Se), 2.01 (q, 2H, J = 7.1 Hz, $CH_2\boldsymbol{CH_2}CH_2$), 2.53 (t, 2H, J = 7.1 Hz, CH_2CN), 2.66 (t, 2H, J = 7.0 Hz, CH_2Se).

5-(Methylseleno)pentanenitrile (**10b**, $CH_3SeC_4H_8CN$) synthesis: $CH_3Se_2CH_3$ (100 mg, 0.53 mmol, Aldrich) was added to a solution of $NaBH_4$ (60 mg, 1.59 mmol) in 5 ml of absolute EtOH under N_2. After 5 min of vigorous reaction the colour of the solution changed from yellow to white. 5-Bromopentanenitrile (0.17 g, 1.06 mmol, Acros) was then added and stirred for 2.5 h. Et_2O (10 ml) was added, the reaction mixture was washed with 5 ml each of water and brine, extracted with 2 × 25 ml of Et_2O, and dried ($MgSO_4$). The solvent was removed to give **10b** as a yellow oil (107 mg, 57% yield). EI-ToF-MS (Fig. 2), *m*/*z* (rel. int.): 177 $[M]^+$ (100), 175 (28), 173 (15), 135 (10), 109 $[CH_3SeCH_2]^+$ (33), 107 (17), 96 $[CH_3SeH]^+$ (27), 94 $[CH_2Se]^+$ (21), 93 $[CHSe]^+$ (25), 82 (22), 55 (4). ^1H NMR (500 MHz, $CDCl_3$): δ1.75–1.88 (m, 4H, CH_2CH_2), 2.00 (s, 3H, CH_3Se), 2.38 (t, 2H, J = 6.8 Hz, CH_2CN), 2.57 (t, 2H, J = 6.9 Hz, CH_2Se).

3-(Methylseleno)propylisothiocyanate (**11b**, $CH_3SeC_3H_6NCS$) EI-ToF-MS (Fig. 4), *m*/*z* (rel. int., fragment): 197 ($[M]^+$, Se^{82}) (16), 195 ($[M]^+$, Se^{80}) (100), 193 ($[M]^+$, Se^{78}) (32), 191 ($[M]^+$, Se^{76}) (18), 180 $[M-CH_3]^+$ (6), 163 $[M-S]^+$ (7), 136 $[M-HNCS]^+$ (5), 121 $[CH_3SeC_2H_2]^+$ (16), 109 $[CH_3Se^{80}CH_2]^+$ (25), 107 $[CH_3Se^{78}CH_2]^+$ (16), 101 $[M-SeCH_2]^+$ (65), 100 $[M-SeCH_3]^+$ (14), 95 $[SeCH_3]^+$ (21), 94 (19), 93 (27), 72 $[CH_2NCS]^+$ (38).

4-(Methylseleno)butylisothiocyanate (**12b**, $CH_3SeC_4H_8NCS$) synthesis: 4-(methylseleno)butanenitrile was synthesised, as described above, from 500 mg (2.6 mmol) of $CH_3Se_2CH_3$ and 0.79 g (5.33 mmol) of 4-bromobutanenitrile. The product was dissolved in dry Et_2O (25 ml) and added drop-wise to a slurry of $LiAlH_4$ (200 mg, 5.3 mmol) in dry Et_2O (25 ml) under N_2 over 5 min followed by 15 min of stirring. Wet Et_2O (25 ml) was added, 5 ml of NH_4Cl (sat. aq.), and 1 ml of conc. ammonia to solubilise the amine in the organic phase which was dried ($MgSO_4$) and removed to give 4-(methylseleno)butamine. EI-MS, *m*/*z* (rel. int.): 167 $[M]^+$ (24), 152 $[M-CH_3]^+$ (7), 135 $[M-CH_3-NH_3]^+$ (13), 122 $[M-CH_3-CH_2NH_3]^+$ (4), 109 $[C_2H_5Se]^+$ (9), 93 $[CHSe]^+$ (9), 72 $[C_4H_8NH_2]^+$ (100), 55 (33), 43 (62) (Budzikiewicz and Pesch, 1974). Thiophosgene ($CSCl_2$, 750 µl, Janssen) in dry dichloromethane (25 ml) was added drop wise to the above amine in dry dichloromethane (25 ml) containing diethylamine (0.5 ml) under N_2. After stirring for 23 h, 2.5 ml of $NaHCO_3$ (sat. aq.) was added with vigorous stirring, the mixture was filtered through a plug of silica and dried ($MgSO_4$). The solvent was removed, the product taken up in dry Et_2O, filtered and the solvent removed to give **12b** as a yellow oil. EI-ToF-MS (Fig. 4), *m*/*z* (rel. int.): 209 $[M]^+$ (84), 194 $[M-CH_3]^+$, 135 $[CH_3SeC_2H_4]^+$ (25), 114 $[M^+-SeCH_3]^+$ (57), 109 $[CH_3SeCH_2]^+$ (19), 93 $[SeCH]^+$ (11), 85 $[C_2H_3NCS]^+$ (19), 72 $[CH_2NCS]^+$ (95), 55 (100), 41 (25). ^1H NMR (500 MHz, $CDCl_3$): δ1.81 (m, 4H, CH_2CH_2), 2.01 (s, 3H, CH_3Se), 2.57 (t, 2H, J = 6.48 Hz, $SeCH_2$), 3.56 (t, 2H, J = 6.18 Hz, CH_2N).

5-(Methylseleno)pentylisothiocyanate (**13b**, $CH_3SeC_5H_{10}NCS$) EI-ToF-MS (Fig. 4), *m*/*z* (rel. int., fragment): 223 ($[M]^+$, Se^{80}) (100), 221 ($[M]^+$, Se^{78}) (42), 208 $[M-CH_3]^+$ (9), 164 $[M-HNCS]^+$ (2), 149 $[SeC_5H_9]^+$ (6), 128 $[M-CH_3Se]^+$ (33), 109 $[CH_3Se^{80}CH_2]^+$ (25), 107 $[CH_3Se^{78}CH_2]^+$ (15), 93 $[SeCH]^+$ (12), 72 $[CH_2NCS]^+$ (25), 69 $[C_5H_9]^+$ (10).

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