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Selective increase of the potential anticarcinogen 4-methylsulphinylbutyl glucosinolate in broccoli

Kathy Faulkner[1,2], Richard Mithen[1] and Gary Williamson[2,3]

[1]John Innes Centre, Norwich Research Park, Colney Lane, Norwich NR4 7UH, UK and [2]Department of Biochemistry, Institute of Food Research, Norwich Research Park, Colney Lane, Norwich NR4 7UA, UK

[3]To whom correspondence should be addressed

The putative anticarcinogenic activity of Brassica vegetables has been associated with the presence of certain glucosinolates. 4-Methylsulphinylbutyl isothiocyanate (sulphoraphane), derived from the corresponding glucosinolate found in broccoli, has previously been identified as a potent inducer of the anticarcinogenic marker enzyme quinone reductase [NADP(H):quinone-acceptor oxidoreductase] in murine hepatoma Hepa 1c1c7 cells. We have therefore produced a broccoli hybrid with increased levels of this anticarcinogenic glucosinolate and tested the ability of extracts to induce quinone reductase. A 10-fold increase in the level of 4-methylsulphinylbutyl glucosinolate was obtained by crossing broccoli cultivars with selected wild taxa of the *Brassica oleracea* (chromosome number, $n = 9$) complex. Tissue from these hybrids exhibited a >100-fold increase in the ability to induce quinone reductase in Hepa 1c1c7 cells over broccoli cultivars, due to both an increase in 4-methylsulphinylbutyl glucosinolate content and increased percentage conversion to sulphoraphane.

Introduction

Several complementary pieces of evidence suggest that isothiocyanates derived from the hydrolysis of methylsulphinylalkyl glucosinolates found in the Cruciferae family may be important in the human diet in reducing the risk of cancer, for a number of reasons: (i) dietary provision of cruciferous vegetables protects rodents against chemically induced cancer (1); (ii) methylsulphinylalkyl isothiocyanates are known to be potent inducers of phase II detoxification enzymes in murine hepatoma Hepa 1c1c7 cells in culture (2,3), which are associated with reduced susceptibility of mammals and mammalian cell cultures to the toxic and neoplastic effects of carcinogens; and (iii) sulphoraphane (4-methylsulphinylbutyl isothiocyanate) blocks the formation of mammary tumours in Sprague–Dawley rats treated with 9,10-dimethyl-1,2-benzanthracene (4). In general, epidemiological studies show that people with high levels of vegetables in their diet are less susceptible to cancer (5), but these studies are not able to identify food components as potential anticarcinogens.

3-Methylsulphinylpropyl and 4-methylsulphinylbutyl glucosinolates are found in several cruciferous vegetables, but are most abundant in broccoli (syn. calabrese: *Brassica oleracea* L. *italica*), which lacks a functional allele at the *GSL-ALK* locus (Figure 1): the presence of a functional *GSL-ALK* allele would convert these glucosinolates to their alkenyl homologues, which are poor inducers of phase II enzymes (3). The levels of glucosinolates in commercially grown broccoli are relatively low compared with those found in salad crops such as rocket (*Eruca sativa*), which accumulates 4-methylthiobutyl glucosinolate, and watercress (*Rorippa nasturtium-aquaticum*), which accumulates phenylethyl glucosinolate (7). Enhancing the levels of 4-methylsulphinylbutyl glucosinolate in broccoli would be expected to enhance the potency of induction of phase II enzymes. It would be unlikely to lead to reduced palatability as methylsulphinylalkyl glucosinolates are non-volatile and have a relatively small contribution to flavour, in contrast with the majority of other isothiocyanates found in vegetables and salad crops (7).

Many wild members of the *Brassica oleracea* species complex (chromosome number, $n = 9$) have high levels of individual aliphatic glucosinolates (8,9). Studies on the genetics of glucosinolates in these taxa have, in addition to elucidating the genetic pathway for glucosinolate biosynthesis (Figure 1), suggested several taxa that would be valuable in broccoli breeding programs that are designed to specifically enhance 4-methylsulphinylbutyl glucosinolate and, by so doing, its anticarcinogenic potential. Foremost amongst these are members of the *B.villosa-rupestris* complex from Sicily, which possess a non-functional *GSL-ALK* allele, and may be the wild progenitors of cultivated broccoli.

Measurement of the induction of quinone reductase (QR*) in murine hepatoma Hepa 1c1c7 cells provides a rapid and reliable indicator of the ability of vegetable extracts to induce enzymes such as glutathione transferase and UDP-glucuronosyl transferase via the antioxidant responsive element in mammalian cells (10), and hence of putative anticarcinogenic activity via a blocking mechanism (1). This assay has been used to assess the potential of synthetic isothiocyanates (2,11), extracts from cruciferous vegetables (6) and myrosinase-treated glucosinolates (3). The glucosinolate/isothiocyanate content of the vegetable extracts has generally not been reported. In this paper, we have used this assay to investigate the relationship between the ability to induce QR activity and the glucosinolate content of three wild members of the *B.oleracea* complex, which have high levels of 3-methylthiopropyl, 3-methylsulphinylpropyl and 2-propenyl glucosinolates in commercial broccoli cultivars and hybrids between the wild accessions and a commercial double haploid broccoli breeding line, respectively.

Materials and methods

Source of material and sampling

A double haploid broccoli breeding line derived from the cultivar Green Duke (GD DH) (12), three commercial cultivars (Trixie, Green Comet and Marathon) and three wild *Brassica* species: *B.drepanensis* Caruel (syn. *B.villosa* Biv. subsp *drepanensis*), *B.villosa* Biv. and *B.atlantica* (Coss.) O.E.Schultz, were

***Abbreviations:** QR, quinone reductase [NADP(H):quinone-acceptor oxidoreductase], EC 1.6.99.2; GSL, glucosinolate; CD value, concentration of an inducer required to double the quinone reductase specific activity in murine hepatoma Hepa 1c1c7 cells; GD DH, Green Duke double haploid broccoli breeding line; SPME, solid phase matrix extraction; GC-MS, gas chromatography-mass spectroscopy; EMSA, electrophoretic mobility shift assays.

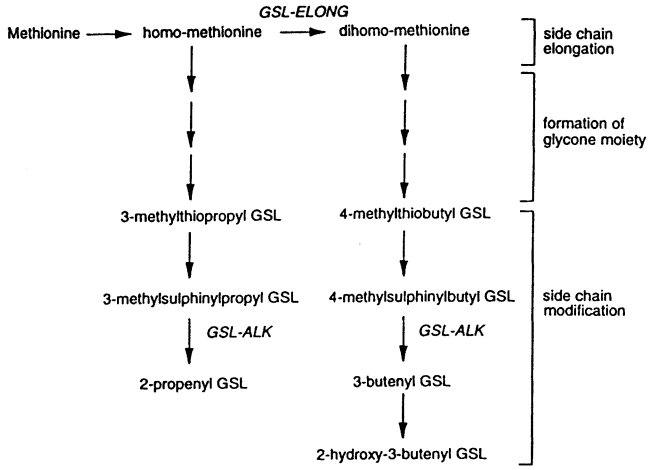

Fig. 1. Genetic model of aliphatic glucosinolate biosynthesis in *Brassica.*

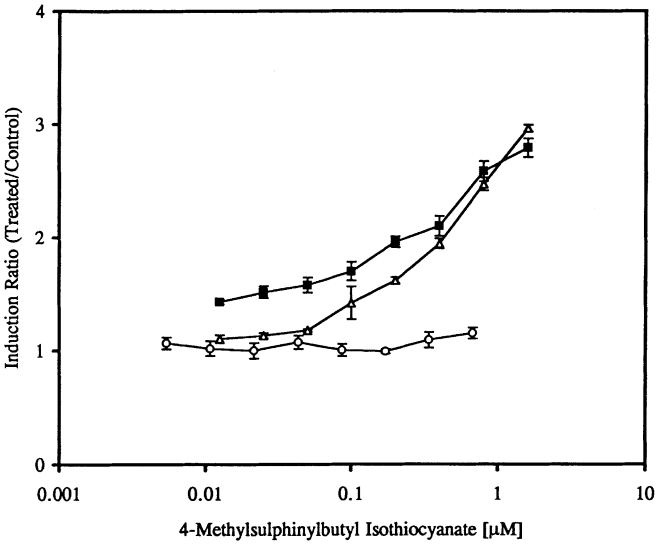

Fig. 2. Induction of QR activity in murine hepatoma Hepa 1c1c7 cells from the cultivar Marathon and synthetic sulphoraphane. Sulphoraphane (△); Marathon extract (0.125 mg/ml) with added sulphoraphane (■); Marathon extract (0.001 mg/ml to 0.125 mg/ml) (○). The estimated isothiocyanate concentration of the Marathon extract has been based on an assumption that 100% of the parent glucosinolate is converted to the isothiocyanate. Thus, Marathon extract (1 mg/ml) is equivalent to 5.4 μM 4-methylsulphinylbutyl isothiocyanate. Results for Marathon (0.125 mg/ml) with added sulphoraphane have been plotted with respect to the added synthetic sulphoraphane concentration only, as the Marathon extract alone has no significant effect on induction activity.

grown in a glasshouse under standard conditions as previously described (13). Each of the wild species was crossed to the GD DH breeding line, F_1 seeds were obtained and grown as previously described (13). Inflorescences were harvested from the cultivars after 8–12 weeks and from the hybrids after 12–16 weeks, immediately frozen in liquid nitrogen and freeze-dried. Synthetic sulphoraphane was kindly supplied by Professor P.Talalay, Johns Hopkins University School of Medicine, Baltimore, MD.

Glucosinolate analysis

Glucosinolates were extracted from the freeze-dried material, converted to desulphoglucosinolates and analysed by HPLC as previously described (13) using benzyl glucosinolate as an internal standard.

Induction of quinone reductase

Extracts from the freeze-dried material were assessed for their induction activity in murine hepatoma Hepa 1c1c7 cells. Approximately 0.1 g of milled freeze-dried material was moistened by addition of water (2 ml), homogenized and left at room temperature for 1 h with occasional mixing. Hot 70% (v/v) methanol (3 ml) was added and mixed thoroughly prior to incubation for 15 min at 70°C. The homogenates were cooled to room temperature and centrifuged for 5 min at 3000 *g*. Supernatants were removed and the volume decreased in a vacuum centrifuge to ~one-fifth of the initial volume. The resulting concentrates were filtered through sterile non-pyrogenic filters (0.22 μm) and stored at –70°C prior to testing. The concentrations of each extract were expressed as dry wt of original material to each ml of culture medium. Induction was measured according to published methods (3,14,15) with the following modifications. Each sample was analysed at eight concentrations using four replicates for each concentration and the data in all figures are presented with standard deviations. β-Napthoflavone was used as a positive control at a concentration of 0.2 μM. This typically produced a 3-fold induction (CD; 0.02 μM) and was comparable with previous determinations (15). Each cultivar/hybrid was extracted on three occasions and analysed separately.

Gas chromatography-mass spectroscopy (GC-MS) analysis

Non-volatile and volatile components of the hydrolytic breakdown products of the cultivars, wild species and the hybrids were analysed by GC-MS using a HP Chemstation GP800A equipped with a 30 m×0.25 mm HP1 cross-linked methylsilane column (Hewlett–Packard Co., Palo Alto, CA). Typically, the column was heated from 60°C to 250°C at 20°C/min and the mass spectra scanned from 35 to 250 m/z. Approximately 0.1 g of freeze-dried material was moistened with water, mixed thoroughly and incubated for 1 h with occasional mixing. Non-volatile hydrolytic products were extracted from the samples with methylene chloride and filtered prior to analysis. In order to analyse volatile products, freeze-dried material (0.1 g) was moistened with water (0.5 ml), the glass vial sealed immediately and volatile products collected from the vial head-space with a solid phase matrix extraction (SPME) probe (Supelco Inc., Bellefonte, PA).

Results and discussion

Glucosinolate analysis

The levels of 4-methylsulphinylbutyl glucosinolate in the broccoli cultivars were similar to those previously reported (16). Wild species had an ~10-fold greater level of total aliphatic glucosinolates than the cultivars. *B.villosa*, *B.drepanensis* and *B.atlantica* had predominantly 3-methylsulphinylpropyl, 3-methylthiopropyl and 2-propenyl glucosinolates, respectively (Table I). The differences in the glucosinolate profiles were attributed to differences in alleles at the *GSL-OXID* and *GSL-ALK* loci (9). Hybrids between broccoli and the wild species had similar total levels to those found in the florets of the wild species themselves. In hybrids with *B.drepanensis* and *B.villosa*, the major glucosinolates were 3-methylsulphinylpropyl and 4-methylsulphinylbutyl, which is similar to the profile found in broccoli, whereas in hybrids with *B.atlantica*, the major glucosinolates were 2-propenyl, 3-butenyl and 2-hydroxy-3-butenyl glucosinolate. The different glucosinolate profile in these hybrids resulted from the interaction of the genes in the respective parents.

Induction of quinone reductase

Synthetic sulphoraphane was used as a positive control to quantify induction of QR in Hepa 1c1c7 cells. It was a potent inducer and produced a three-fold induction at 1.6 μM (CD; 0.4 μM), compared with previous determinations (see Figure 2). No cytotoxicity was observed for any sample at any of the concentrations tested.

Extracts from all the cultivars were poor inducers. However, the induction was less than expected if 100% of the glucosinolates had been converted to isothiocyanates and not to other products such as thiocyanate or nitrile derivatives, which do not induce (7). For example, Marathon contained 5.4 μmol of 4-methylsulphinylbutyl glucosinolate/g dry wt (Table I). Therefore, an extract containing 75 μg/ml of Marathon would be expected to have a 4-methylsulphinylbutyl isothiocyanate concentration of 0.4 μM, which would result in a 2-fold

induction. However, no significant induction was observed at this concentration. Indeed, 2.5 mg/ml of Marathon extract was required for a 2-fold induction, which if 100% of the glucosinolate is converted to isothiocyanate, is equivalent to 13.5 μM 4-methylsulphinylbutyl isothiocyanate (unpublished data). Thus, either a small proportion of the glucosinolate had been converted to 4-methylsulphinylbutyl isothiocyanate, or the induction of QR activity was reduced by other components in the vegetable extract. To test for the presence of an inhibitor, Marathon extract (0.125 mg/ml) was spiked with synthetic sulphoraphane prior to application to the Hepa 1c1c7 cells. The induction observed was similar to that of pure sulphoraphane alone (Figure 2), which demonstrated that there had been no inhibitory effect by other components in the extracts. Therefore, it is possible that in Marathon (and also other cultivars) only a proportion of the glucosinolate had been converted to the isothiocyanate.

Extracts from both GD DH×*B.villosa* and GD DH×*B.drepanensis* were potent inducers of QR activity (Figure 4). Based on 100% conversion of the glucosinolates to isothiocyanates, the apparent concentration of an inducer required to double the quinone reductase-specific activity in murine hepatoma Hepa 1c1c7 cell (CD) values, of both extracts, was 0.3 μM (4-methylsulphinylbutyl glucosinolate equivalent), which is similar to that of pure sulphoraphane and to that reported in previous studies (10). Thus, while there was an ~10-fold increase in the levels of 4-methylsulphinylbutyl glucosinolate in GD DH×*B.villosa* and GD DH×*B.drepanensis* compared with Marathon and GD DH, there was a >100-fold difference in ability to induce QR activity (i.e. it requires at least 100 times the amount of Marathon tissue to induce equivalent QR activity compared with the GD DH hybrids). An explanation for this higher inducer activity could be that there is greater conversion of glucosinolates to isothiocyanates, instead of conversion to other hydrolytic products (thiocyanates and nitrile derivatives), because of the change in genetic background. For example, novel myrosinase alleles may have been introduced from the wild parent. Alternatively, there may be other compounds that induce QR activity, which are not present in *B.atlantica*.

In order to examine the composition and nature of the hydrolytic products, extracts were analysed by GC-MS (Figure 5). Hydration of freeze-dried leaves of *B.drepanensis* and *B.atlantica* led to the production of large amounts of volatile 3-methylthiopropyl and 2-propenyl isothiocyanates. 3-Methylsulphinylpropyl isothiocyanate was detected in methylene chloride extracts from *B.villosa*, which was con-

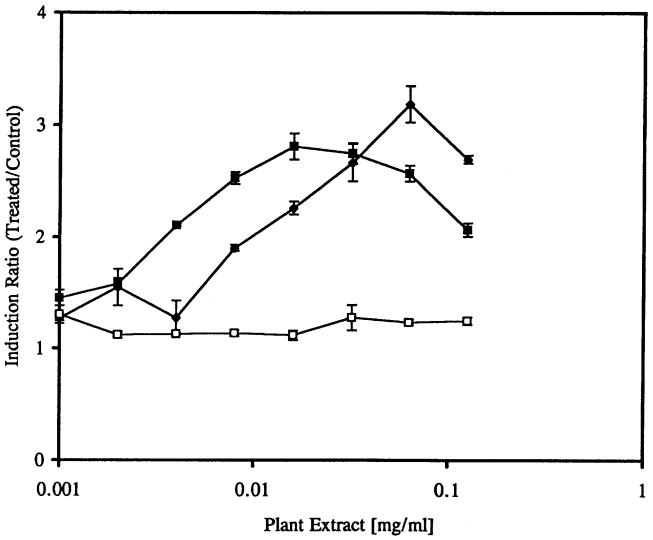

Fig. 3. Effect of extracts of *B.drepanensis* (■), *B.villosa* (◆), and *B.atlantica* (□) on induction of QR in murine hepatoma Hepa 1c1c7 cells.

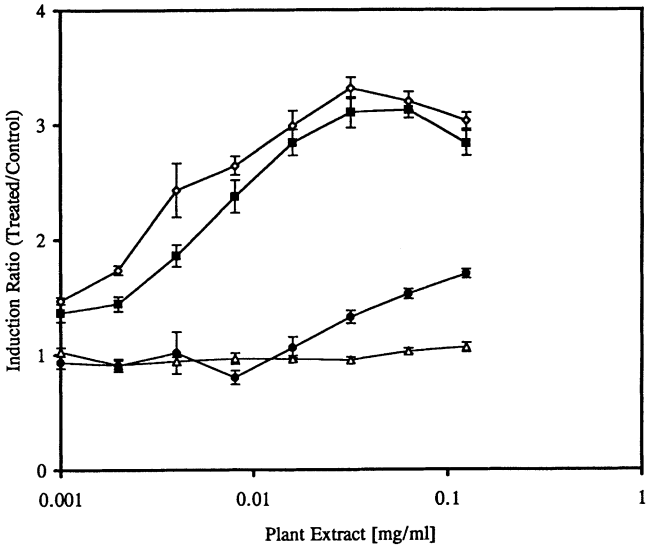

Fig. 4. The effect of extracts of the cultivar GD DH (●), GD DH×*B.drepanensis* (■), GD DH×*B.villosa* (◇), and GD DH×*B.atlantica* (Δ) on the induction of QR in murine hepatoma Hepa 1c1c7 cells.

Table I. Individual and total aliphatic glucosinolate content (μmoles/g dry wt ± 1 SE) of broccoli cultivars, wild *Brassica* species and hybrids produced from crosses between GD DH and the wild *Brassica* species

	MSP	MSB	PROP	BUT	MTP	OH-BUT	Total
Green Comet	0.1 ± 0.1	0.8 ± 0.5	0.2 ± 0.1	0.0	2.7 ± 0.5	0.5 ± 0.3	4.3 ± 0.5
GD DH	0.2 ± 0.2	4.6 ± 1.1	0.0	0.0	2.3 ± 0.6	0.0	7.1 ± 1.1
Marathon	1.0 ± 0.3	5.4 ± 1.1	0.2 ± 0.1	0.0	4.1 ± 0.7	0.0	10.7 ± 1.8
Trixie	0.4 ± 0.2	11.1 ± 2.1	0.2 ± 0.1	0.0	4.9 ± 1.2	0.0	16.6 ± 2.6
B.atlantica	0.9 ± 0.7	0.0	92.8 ± 25.4	0.5 ± 0.3	1.1 ± 1.0	0.0	95.3 ± 26.6
B.drepanensis	11.0 ± 1.7	0.0	0.0	0.0	51.6 ± 9.3	0.0	62.6 ± 10.9
B.villosa	119 ± 18	1.4 ± 0.2	0.0	0.1 ± 0.1	3.4 ± 0.9	0.1 ± 0.1	124 ± 19
GD × *B.atlantica*	2.2 ± 0.8	5.3 ± 1.4	76.9 ± 20.8	23.6 ± 6.3	2.0 ± 0.7	43.7 ± 6.7	154 ± 30
GD×*B.drepanensis*	26.2 ± 2.9	76.5 ± 8.9	0.0	0.0	1.9 ± 0.4	0.0	105 ± 12
GD×*B.villosa*	26.4 ± 2.7	81.8 ± 5.0	0.0	0.0	1.0 ± 0.3	0.0	109 ± 7

MSP, 3-methylsulphinylpropyl; MSB, 4-methylsulphinylbutyl; PROP, 2-propenyl; BUT, 3-butenyl; MTP, 3-methylthiopropyl; OH-BUT, 2-hydroxy-3-butenyl, GD, GD DH.

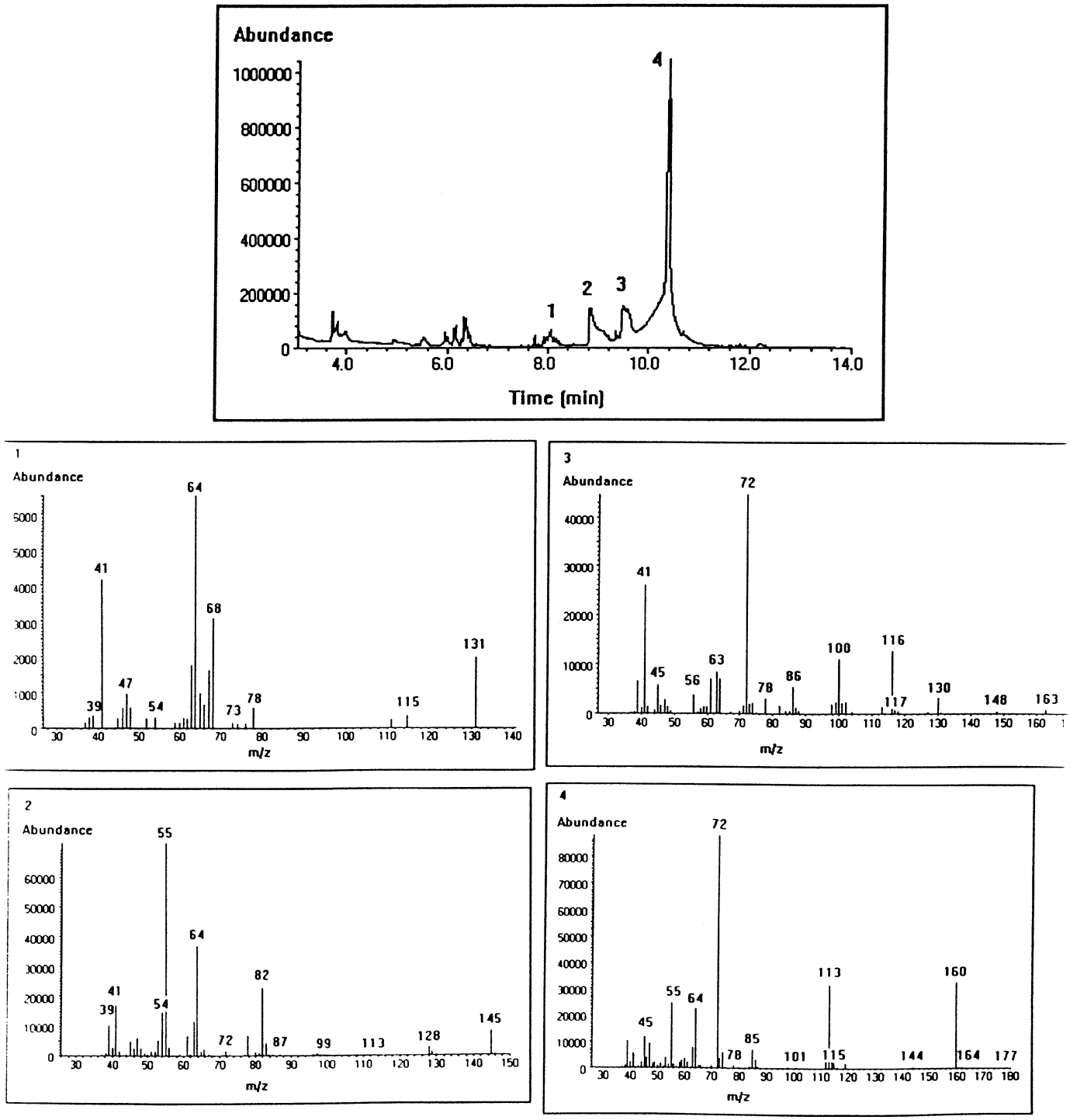

Fig. 5. Gas chromatograph profile of an extract from GD DH×*B.drepanensis*. Mass spectrometry confirmed the peaks as (**1**), 3-methylsulphinylpropyl nitrile; (**2**), 4-methylsulphinylbutyl nitrile; (**3**), 3-methylsulphinylpropyl isothiocyanate; and (**4**), 4-methylsulphinylbutyl isothiocyanate.

sistent with the glucosinolate profiles. In extracts of GD DH×*B.villosa* and GD DH×*B.drepanensis*, the dominant isothiocyanate was 4-methylsulphinylbutyl isothiocyanate, as was expected. Relatively low levels of 3-methylsulphinylpropyl isothiocyanate and nitrile derivatives were also detected (Figure 5). In contrast, only trace amounts of 4-methylsulphinylbutyl isothiocyanate were detected in the cultivars. This indicated that the 100-fold difference in ability to induce QR activity between the two hybrids and the cultivars was caused by an increase in 4-methylsulphinylbutyl glucosinolate and to a greater conversion to isothiocyanate.

There have been a large number of studies that have used animal models to show that glucosinolate breakdown products, such as isothiocyanates, inhibit chemically-induced carcinogenesis by up to 100%. Furthermore, studies in humans have shown that consumption of *Brassica* vegetables can reduce the amount of DNA damage and increase the level of detoxifying enzymes (17,18). The most potent glucosinolate breakdown

products for induction of detoxification enzymes are the methylsulphinylalkyl isothiocyanates, of which the most reported is sulphoraphane (4-methylsulphinylbutyl isothiocyanate) (2). The results also highlight that the anticarcinogenic properties of broccoli depend both on the concentration of 4-methylsulphinylbutyl glucosinolate and on the conversion of this compound into the isothiocyanate on autolysis. The latter is affected by temperature, pH, endogenous levels of myrosinase and on the concentration of reactants (7).

Hybrids between commercial broccoli cultivars and the two wild species *B.villosa* and *B.drepanensis* are fully fertile, and back-cross populations have been developed. Thus, it is feasible to develop broccoli lines with enhanced levels of 4-methylsulphinylbutyl glucosinolate and its associated anticarcinogenic activity. The efficiency of development of these lines is considerably enhanced by the availability of molecular markers to select for both glucosinolate content and the desired genetic background. These lines may be of value for experimental purposes in dietary intervention studies and for commercialization.

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