Identification of Bis(4-isothiocyanatobutyl) Disulfide and Its Precursor from Rocket Salad (*Eruca sativa*)

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An unknown compound, observed as a prominent peak in extracts of Rocket salad (*Eruca sativa*) that were analyzed for pesticide residues by gas chromatography with the electron capture detection (GC-ECD), was isolated by high-performance liquid chromatography (HPLC). The compound was identified by gas chromatography/mass spectrometry (GC/MS), Fourier transform-infrared spectroscopy (FTIR), and nuclear magnetic resonance spectroscopy (NMR) as bis(4-isothiocyanatobutyl) disulfide. For the final structural confirmation, the compound was synthesized. Its formation by the oxidation of 4-(mercapto)butyl isothiocyanate, a glucosinolate breakdown product, was elucidated.

Keywords: *Rocket salad; Eruca sativa; bis(4-isothiocyanatobutyl) disulfide; 4-(mercapto)butyl isothiocyanate; glucosinolates*

INTRODUCTION

Rocket salad (*Eruca sativa*) from the family of cruciferous crops is used especially as a salad or a food ingredient and has a characteristic horseradish-like odor and biting taste. In Asia, the plant serves as an important source of oil seeds.

During routine tests for dithiocarbamate fungicides in various vegetables by a carbon disulfide (CS_2) evolution method with spectrophotometric determination (Keppel, 1969, Keppel, 1971), positive results at levels from 0.5 to 5 mg CS_2/kg have been frequently found in Rocket salad samples, although an application of dithiocarbamates is unusual for that crop. Positive results were also found in plants cultivated from seeds in our laboratory without any pesticide treatment. The presence of CS_2 after hydrolysis of the Rocket salad in a hot $HCl/SnCl_2$ mixture was confirmed by a gas chromatography (GC) headspace technique with a flame photometric detector in the sulfur-selective mode (FPD-S; Friedrichs *et al.*, 1995). Therefore, a sulfur-containing plant constituent or constituents decomposing to CS_2 were considered as a possible reason for the false positive results.

In addition, samples of Rocket salad analyzed by the AOAC official pesticide multiresidue method (AOAC, 1995) showed a prominent GC peak on various capillary columns with selective GC detectors, such as the electron-capture detector (ECD), the thermionic nitrogen-phosphorus detector (NPD), and the FPD-S. No matching compound was found in our retention times data base. Using GC with the flame ionization detector (FID), the estimated content of the unknown compound (R1) ranged from 10 to 600 mg/kg.

The objective of the present study was to isolate R1, the unknown compound, by high-performance liquid chromatography (HPLC), and to identify it by GC with specific detectors, gas chromatography/mass spectrometry (GC/MS), Fourier transform-infrared spectroscopy (FTIR), and nuclear magnetic resonance spectroscopy (NMR).

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MATERIALS AND METHODS

Reagents. Chemicals and reagents were purchased from E. Merck SA (Switzerland) and Fluka Chemie AG (Switzerland). Solvents were of residue quality or HPLC-grade, and all other reagents were of analytical grade.

Plant Material. Samples of *E. sativa* were obtained from producers in Ticino (southern part of Switzerland) and Italy. Additionally, plants were cultivated from seeds in the laboratory.

Sample Preparation. The extraction of R1 was carried out by the modified AOAC multiresidue method for organochlorinated and organophosphorus pesticides (AOAC, 1995). A 30-g sample was blended with 45 mL of acetonitrile and 2 mL of water (acetonitrile:water ratio, 1.5:1, including water from the sample). The extract was filtered through a paper filter, 25 mL of the filtrate (representing 10 g of the sample) were diluted with 200 mL of 2% sodium sulfate solution in water, and residues were re-extracted into 25 mL of a hexane:dichloromethane mixture (2:1) by liquid−liquid partitioning. The organic phase was separated, dried with 5 g of anhydrous sodium sulfate, and evaporated nearly to dryness under reduced pressure on a rotary evaporator at 40 °C. The residue was dissolved in 10 mL of hexane, and a 2-mL aliquot (corresponding to 2 g of sample) was taken for the cleanup on 2 g of partially deactivated Florisil in a 10-mm i.d. chromatographic column. The unknown compound was eluted in the 3rd fraction with 20 mL of 30% diethyl ether in hexane (1st fraction, 15 mL of hexane; 2nd fraction, 12 mL of 4% ether in hexane). The 3rd fraction was evaporated to 2 mL under a gentle stream of nitrogen gas at 40 °C, and the volume was adjusted to 5 mL with hexane. The collected fractions were stored at 4 °C.

Isolation of the Unknown Compound by HPLC. First, 25 μL of the sample prepared by the AOAC method (3rd fraction from Florisil column cleanup) was injected repeatedly onto a silica gel HPLC column (E. Merck Lichrosorb Si 60, 5 μm particle size, 250 mm length, 4 mm i.d.). The mobile phase was cyclohexane:ethanol (95:5) at a flow rate of 1 mL/min, and a UV detector (Perkin Elmer LC 95) at 205 nm was used for monitoring. R1 was collected from 3.5 to 4.0 mL, and the identity of the fraction was confirmed by GC/MS. The corresponding fractions from consecutive injections were collected in the same small glass tube (2 mL), and every fraction was evaporated to dryness under a gentle stream of nitrogen at room temperature before the next fraction was collected. The resulting yellowish oil (10 mg) was stored at 4 °C for further testing.

Gas Chromatography. GC was carried out on two different systems. The first system was a Varian 3600 GC with

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an ECD, a NPD, or a FPD-S (394 nm filter), an on-column injector, a Restek Rtx-1 fused silica (FS) capillary column (30 m length; 0.53-mm i.d.; 0.5-μm film thickness), and a Restek Rtx-200 FS column (30 m; 0.53-mm i.d.; 0.5 μm). The operating conditions were as follows: carrier gas, He at 50 kPa; injector temperature program, from 90 to 270 °C at 150 °C/min; oven temperature program, from 90 to 270 °C at 8 °C/min, and 2.5 min at 270 °C; detector temperature, 300 °C; and injection volume, 1 μL. Data were acquired and evaluated on a Varian Star chromatography data system (version 4.0).

The second GC system was Hewlett Packard (HP) 5890 Series II GC equipped with an ECD and a FID, and a J&W DB-1 FS capillary column (30 m; 0.32 mm i.d.; 0.1 μm). The operating conditions were as follows: carrier gas, He at 85 kPa; column temperature program, from 90 to 270 °C at 8 °C/min, then hold at 270 °C for 7.5 min; on-column injector tracking the column oven temperature; and detector at 300 °C. Data acquisition was accomplished with a HP ChemStation (version 3.34).

CG/MS. A Varian 3600 GC directly interfaced to a Varian Saturn 4D ion-trap mass spectrometer was used. The system was operated alternatively in the electron impact (EI) mode at 70 eV and in the chemical ionization (CI) mode with *i*-butane or acetonitrile as reagents. The GC was equipped with an on-column injector and a J&W DB-1 FS capillary column (30 m; 0.25 mm i.d.; 0.1 μm). The operating conditions were as follows: carrier gas, He at 85 kPa; column temperature program, from 90 to 270 °C at 8 °C/min, then hold at 270 °C for 6.5 min; injector temperature program, from 90 to 270 °C at 150 °C/min; transfer line, at 260 °C; and injection volume, 1 μL. The MS operating conditions were as follows: ion trap temperature, 220 °C; scan range, 40−450 amu; scanning speed, 1 scan/s. Data acquisition and evaluation were performed with Varian Saturn GC/MS software (version 5.2).

GC/High-Resolution Mass Spectroscopy (HRMS). High-resolution EI mass spectra were obtained on a Finnigan MAT 95 instrument directly coupled to a Varian 3400 GC. For the GC separation, a Restek Rtx-5 FS capillary column (25 m; 0.25-mm i.d.; 0.25-μm film thickness) was used. The GC operating conditions were as follows: injection volume, 1 μL; splitless injector, 280 °C; carrier gas, hydrogen at 70 kPa; temperature program, from 60 to 270 °C at 5 °C/min; and transfer line, 270 °C. The MS operating conditions were as follows: ion source, 200 °C; ionization energy, 70 eV; resolution, 6500; scan range, 60−350 amu; scanning speed, 1 scan/0.6 s; and perfluorokerosene as reference. A DEC 3100 data system with Finnigan ICIS software (version 9.3) was used for data acquisition and evaluation.

FTIR. The IR spectra were recorded on a Perkin Elmer 1650 FTIR spectrometer using a liquid film of the compound on a KBr disk. The instrument was calibrated with polystyrene at 1601 cm^{-1}.

NMR. R1 isolated by HPLC (10 mg) was dissolved in $CDCl_3$ and transferred into a 5-mm o.d. NMR tube. NMR spectra (^1H; ^{13}C; ^{13}C-DEPT; C-H correlation experiments) were obtained on a Bruker AM 400 spectrometer operating at 400 and 100.6 MHz, respectively. Tetramethysilane (TMS) was used as an internal standard.

RESULTS AND DISCUSSION

R1 was observed as a sharp symmetrical peak on a fully deactivated GC system; on GC systems with some active sites, a distinct peak tailing was observed. The relative retention time in linear temperature program (RRTP) to the pesticide chlorpyrifos (CAS RN 2921-88-2) on a nonpolar polysiloxane capillary column (DB-1) was 1.53, as shown in Figure 1. On a more polar trifluoropropylmethyl polysiloxane column (Rtx-200), the RRTP was 1.60. A strong GC response was obtained on the ECD, NPD, and FPD-S detectors, but no response was observed on the FPD-P detector. Therefore, the presence of sulfur, nitrogen, and eventually halogen as heteroatoms in the molecule of R1 was assumed. A

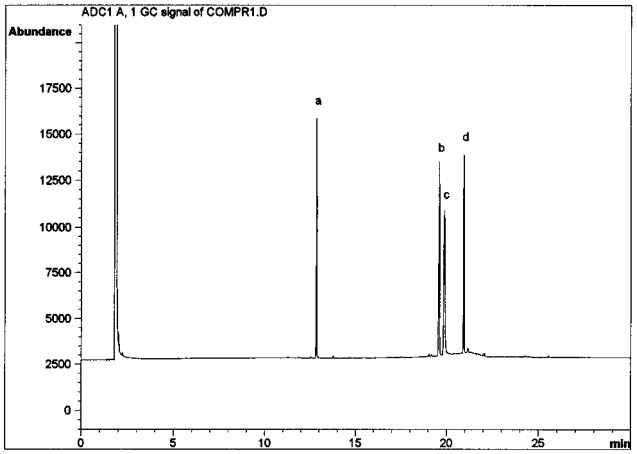

Figure 1. GC-FID chromatogram of (a) 25 ng of chlorpyrifos, (b) 25 ng of the compound R1, (c) 100 ng of DPDT, and (d) 10 ng of di-*n*-octyl phthalate on the DB-1 capillary column (GC system 2).

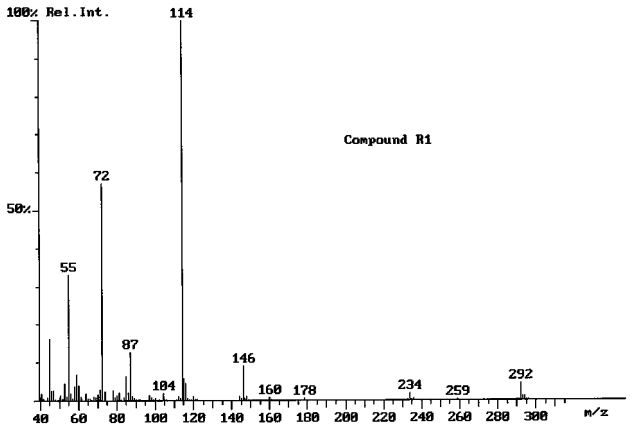

Figure 2. EI mass spectrum of compound R1 [bis(4-isothiocyanatobutyl) disulfide].

FPD-S response at various concentrations was compared with responses of known substances containing different number of sulfur atoms. Taking a nonlinear response of the FPD-S into consideration, four sulfur atoms in the molecule of R1 were calculated in comparison with the response of the pesticide ethion ($C_9H_{22}O_4P_2S_4$, CAS RN 563-12-2). An estimated molecular weight of 292 for R1 from the GC/MS analysis was used in the calculation. Approximate concentrations of R1 in the various plant samples were determined with the FID using chlorpyrifos as an external standard. Concentrations of R1 in the range from 10 to 600 mg/kg (average 230 mg/kg for five different samples) were found. R1 was also present in purified extracts from the seeds and in the sprouts of *E. sativa*.

The full-scan EI spectrum of R1 (Figure 2) contains several important ions, especially at *m/z (%)* 292 (4.3), 146 (9.6), 114 (100.0), 87 (12.8), 72 (54.2), 60 (3.9), 59 (6.7), and 55 (31.3). The ion at *m/z* 292 was assigned to the molecular ion $[M]^+$. The CI experiments with *i*-butane and acetonitrile (Figure 3) showed a formation of the protonated molecular ion $[M+1]^+$ at *m/z* 293. According to the nitrogen rule, an even nominal molecular weight and the presence of nitrogen in the compound indicated an even number of nitrogen atoms. No fragment clusters of characteristic isotopic patterns for chlorine and bromine were found.

In the NIST/EPA/NIH mass spectral database, a similar mass spectrum assigned to dipyrrolidylthiuram disulfide (DPDT, $C_{10}H_{16}N_2S_4$, CAS RN 496-08-2) was

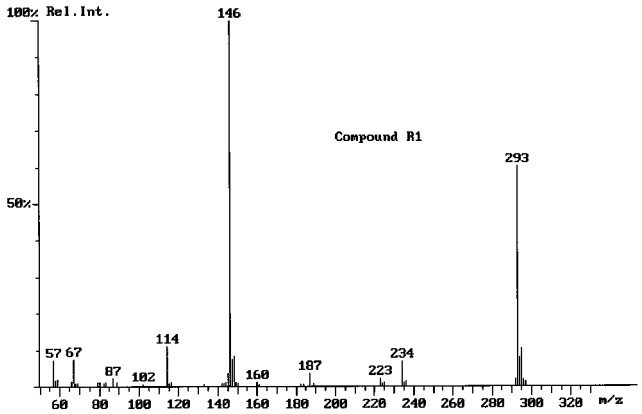

Figure 3. CI mass spectrum of compound R1, with acetonitrile as reagent.

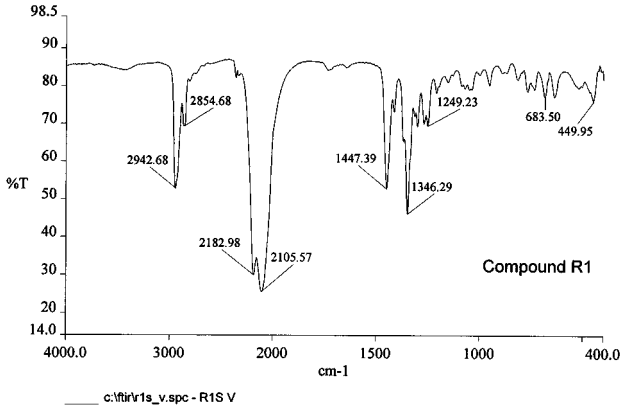

Figure 4. FTIR spectrum of compound R1.

found. The compound was introduced as a fungicide named Lutiram by BASF in Germany (Schlör, 1970; Melnikov, 1971). No reference substance was available because the production had been discontinued. Therefore, the compound was prepared by the oxidation of pyrrolidyl dithiocarbamate by iodine in an ethanolic solution. Pyrrolidyl dithiocarbamate was obtained from pyrrolidine and CS_2 (Hoffmeister and Tarbell, 1965). The RRTP of DPDT on a nonpolar column was 1.55; that is, the compound was baseline separated from R1 (Figure 1). The mass spectra of R1 and DPDT exhibit important differences: *m/z* 87 is present only in R1, *m/z* 260 $[M\text{-}S]^+$ only in DPDT, and the relative abundance of matching fragments in both compounds differ strongly. However, both substances have distinct similarity: a symmetrical disulfide structure with $[M]^+$ at *m/z* 292, a fission product at *m/z* 146 $[M/2]^+$, a base peak at *m/z* 114 $[M/2\text{-}S]^+$, and a peak at *m/z* 72, which was described as $[CH_2NCS]^+$ in the mass spectrum of DPDT (Madsen *et al.*, 1967). The same fragment is characteristic for alkyl isothiocyanates (Kjaer *et al.*, 1963).

An accurate mass determination was performed by GC/HRMS for key ions at *m/z* 72, 87, 114, and 292. The following elemental compositions were determined: *m/z* 72, C_2H_2NS, (difference between theoretical and experimental value, −0.1 mmu); *m/z* 87, C_4H_7S, (0.8 mmu); *m/z* 114, C_5H_8NS, (2.0 mmu), and *m/z* 292, $C_{10}H_{16}N_2S_4$, (−1.0 mmu). From the elemental composition of the molecular ion, four double bonds in the compound were calculated (Clerc *et al.*, 1981).

An FTIR spectrum of R1 isolated by HPLC is shown in Figure 4. A strong absorption doublet observed at 2106 and 2183 cm^{-1} (asymmetric stretching of −N═C═S) and an absorption band at 1346 cm^{-1} are characteristic for alkyl isothiocyanates (Svatek *et al.*, 1959).

Table 1. ^{13}C NMR and ^1H Chemical Shifts (δ_C and δ_H) of Compound R1 (ppm Relative to TMS)

δ_C	group	assignment	δ_H
130.4	C	C^5	
44.7	CH_2	C^4	3.57 (t)
37.8	CH_2	C^1	2.73 (t)
28.6	CH_2	C^2 or C^3	1.83 (m)
26.0	CH_2	C^2 or C^3	1.83 (m)

In the proton decoupled ^{13}C NMR spectrum, four chemical shifts (δ_C) with similar intensities at 26.0, 28.6, 37.8, and 44.7 ppm were found; a weaker broad signal appeared at 130.4 ppm. The DEPT experiment together with the C-H correlation experiment indicated that the four lower peaks are CH_2 groups. No hydrogen is attached to carbon at 130.4 ppm; the value is within the δ_C range (125−140 ppm) for isothiocyanates (Breitmaier, 1993).

The ^1H NMR spectrum contained one multiplet at 1.83 ppm, and two triplets at 2.73 and 3.57 ppm; the integration ratio was 2:1:1. The C−H correlation showed that the multiplet at 1.83 ppm inhered two equal signals connected to ^{13}C shifts at 26.0 and 28.6 ppm. The ^1H NMR data confirmed a presence of the $-CH_2-CH_2-CH_2-CH_2-$ chain.

Considering all experimental data from GC/MS, GC/HRMS, and NMR, the unknown compound R1 was assigned the molecular structure of bis(4-isothiocyanatobutyl) disulfide $[S{=}C^5{=}N{-}C^4H_2{-}C^3H_2{-}C^2H_2{-}C^1H_2{-}S{-})_2]$.

The measured ^{13}C NMR shifts (δ_C) 130.4 and 44.7 ppm are consistent with the tabulated shifts for *n*-butyl isothiocyanate (131 ppm for SCN− and 45.0 ppm for α-C). The experimental δ_C of C connected to S (37.8 ppm) is in a good agreement with the calculated value 38.2 ppm, which is based on tabulated additive increments (Clerc *et al.*, 1981). The shifts 26.0 and 28.6 ppm correspond to values of CH_2 groups in the middle of the chain.

The experimental ^1H shift of 3.57 ppm for hydrogens on C adjacent to the SCN− group agrees with the tabulated ^1H NMR shifts (δ_H) for ethyl isothiocyanate (3.64 ppm). The tabulated δ_H values for *n*-propyl alkyl disulfide (i.e., 2.63 ppm for hydrogens on α-C, and 1.7 ppm for hydrogens on β-C) are in accordance with the found shifts. All δ_C and δ_H values for the mentioned compounds were taken from the tables compiled by Pretsch *et al.* (1986). The NMR data assignments to the proposed structure are summarized in Table 1.

Searching the chemical literature revealed that the substance was synthesized among various sulfur-containing compounds by Vinkler *et al.* (1968) and registered under CAS RN 18729-71-0; the CA name is isothiocyanic acid:dithiobis(tetramethylene) ester. The authors described a strong antimicrobial activity of the compound. The multistep procedure started with the reaction of *N*-(4-bromobutyl) phthalimide (prepared from potassium phthalimide and 1,4-dibromobutane) with sodium thiosulfate to form the sodium salt of δ-phthalimidobutane thiosulfuric acid (Bunte salt). The product was refluxed with sodium 4-chlorothiophenolate in ethanol to give δ-phthalimidobutyl 4-chlorophenyl disulfide. By heating with hydrazine hydrate, two compounds were obtained: 4-chlorothiophenol and 4-amino-*n*-butylthiol. The latter was formed by the exchange reaction between the phthalimide and hydrazine (Ing-

Manske procedure). Both thiols were oxidized by the air oxygen into the corresponding aromatic and aliphatic disulfides. The latter, bis(4-aminobutyl) disulfide was separated and converted with thiofosgene to bis(4-isothiocyanatobutyl) disulfide.

Following the described method, 1.6 g of bis(4-isothiocyanatobutyl) disulfide were prepared. The properties of the yellowish oily compound were examined by GC, GC/MS, and NMR. All obtained data were fully consistent with the data of the compound R1 isolated from the plant. The compound is readily soluble in many organic solvents (hexane, dichloromethane, acetone), only slightly soluble in methanol, and insoluble in water. Solutions in hexane were very stable; that is, no changes of the concentration for >1 year were observed. The compound is nearly odorless.

Under the conditions used for the decomposition of dithiocarbamate fungicides (Keppel, 1969), the formation of CS_2 from the compound was experimentally examined; an average yield of CS_2 was only 0.1%. Due to high concentrations of R1 in *E. sativa*, this could possibly explains false positive results by the determination of dithiocarbamates. Otherwise, the formation of CS_2 from an impurity in the synthesized compound cannot be excluded, and other sulfur-containing compounds from the plant may also decompose to CS_2.

Isothiocyanates (ITC), also known as mustard oils, are formed from cruciferous crops by autolytic hydrolysis of glucosinolates by the action of the enzyme thioglucoside glucohydrolase (myrosinase). Over 90 different glucosinolates have been described. Depending on the structure of the glucosinolate side chain, different ITCs are produced. 4-(Methythio)butyl isothiocyanate [CAS RN 4430-36-8; CA name, isothiocyanic acid: 4-(methylthio)butyl ester] was described as a principal hydrolysis product of a glucosinolate with the trivial name glucoerucin from *E. sativa* (Fenwick *et al.*, 1983; Cole, 1976). Under certain conditions, a formation of 4-(methylthio)butyl thiocyanate from *E. sativa* was reported (Schlüter and Gmelin, 1972).

To elucidate the formation of bis(4-isothiocyanatobutyl) disulfide, the following experiments were carried out. First, a 30 g sample of *E. sativa* was blended with 30 mL of water and incubated at 30 °C for 30 min. The sample was diluted with 170 mL of water and submitted to steam distillation in the presence of hexane in a Clevenger apparatus. An aliquot of the hexane layer was analyzed by GC/MS. As a main volatile compound, 4-(methythio)butyl ITC (MTBI) was found, and the disulfide R1 was present only as a trace (<1% of MTBI).

In the second experiment, 30-g samples were homogenized with 30 mL of water and autolyzed at 30 °C for 30 min. The mixture was directly extracted by dichloromethane. The crude extract was filtered and examined without delay by GC/MS. The extract contained a broad strongly tailing peak 1, a sharp symmetrical peak 2 (MTBI), a tailing peak 3, and the disulfide R1 (peak 4), as shown in Figure 5.

The published MTBI spectrum (Kjaer, 1963) matched the spectrum of peak 2. The MS spectrum of peak 3 was identical with the spectrum of 4-(methylsulfinyl)-butyl ITC (CAS RN 4478-93-7; common name, sulforaphane) presented by Spencer and Daxenbichler (1980). After oxidation with H_2O_2, the compound was no longer present in the extract. Sulforaphane is an important glucosinolate breakdown product from cabbage, cauliflower, Brussel sprouts, and broccoli (Fenwick *et al.*, 1983; Kore *et al.*, 1993).

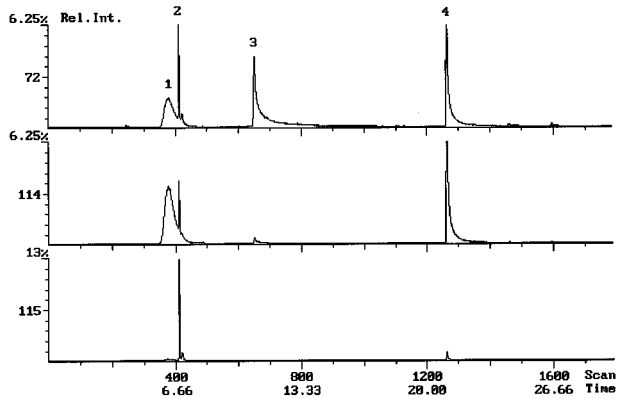

Figure 5. GC/MS chromatogram of a dichloromethane extract of *E. sativa*. The upper trace at *m/z* 72, in the center *m/z* 114, and at the bottom *m/z* 115. Peak 1, 4-(mercapto)butyl ITC; peak 2, MTBI; peak 3, 4-(methysulfinyl)butyl ITC; peak 3, R1, bis(4-isothiocyanatobutyl) disulfide.

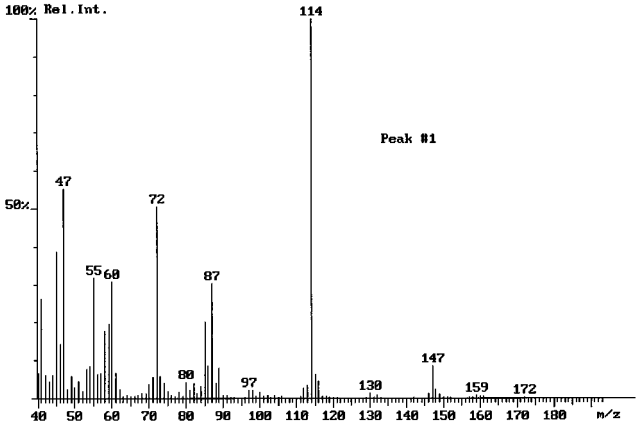

Figure 6. EI mass spectrum of 4-(mercapto)butyl ITC.

The EI mass spectrum of peak 1 (Figure 6) exhibited prominent ions at *m/z* (%) 147 (8.4), 114 (100.0), 87 (30.3), 72 (50.7), 60 (30.7), 59 (19.7), and 55 (31.9). The fragmentation pattern below *m/z* 147 is very similar to that of the disulfide R1; the ion at *m/z* 147 was assigned to the molecular ion. The CI experiment with acetonitrile confirmed the formation of the protonated $[M+1]^+$ at *m/z* 148. By a mild oxidation of the extract with hydrogen peroxide (by 10 min shaking with 10% solution in water), the compound was completely converted to the disulfide R1. Peak 1 was consequently identified as 4-(mercapto)butyl isothiocyanate ($H-S-CH_2-CH_2-CH_2-CH_2-N=C=S$). The compound was not found in the CAS data base.

The concentration of the ITCs in the dichloromethane extract was determined by GC-FID. Calculated on the basis of the fresh plant material, 170 mg/kg of the disulfide R1 was found, and the concentration of the precursor was nearly the same. The concentration ratio of MTBI to R1 was ~0.5, and the concentration ratio of 4-(methylsulfinyl)butyl ITC to R1 was ~2.

These experiments established that besides MTBI and 4-(methylsulfinyl)butyl ITC, 4-(mercapto)butyl isothiocyanate is a principal product of an enzymatic hydrolysis of glucosinolates from *E. sativa*. The compound is difficult to detect because of its polar, unstable character. It is easily oxidized, even by the air oxygen, to the corresponding low-volatile disulfide.

ACKNOWLEDGMENT

We thank Hans Schaufelberger for the acquisition of the FTIR spectra and Julius Glaser (The Royal Institute of Technology, Stockholm, Sweden) for the recording of the NMR spectra.

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Received for review May 22, 1996. Accepted October 3, 1996.[⊗]

JF960361R

[⊗] Abstract published in *Advance ACS Abstracts,* November 15, 1996.