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Isolation and Identification of *trans*-4-(Methylthio)-3-butenyl Glucosinolate from Radish Roots (*Raphanus sativus* L.)

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A procedure for preparative isolation of *trans*-4-(methylthio)-3-butenyl glucosinolate (4MTB-GLS) as a potassium salt from radish roots is described. The main steps of this procedure are (i) lyophilization of suitable ripe radish roots; (ii) extraction using 70% methanol with 50 mM imidazole–formate, pH 4.15; (iii) ion-exchange chromatography on DEAE-Sephadex A-25; (iv) concentration and desalting; and (v) precipitation of 4MTB-GLS in ethanol at −20 °C. The 4MTB-GLS was identified on the basis of its physicochemical properties, UV, IR, ^{1}H and ^{13}C NMR, and mass spectra.

Glucosinolates (GLSs) are an important and unique class of secondary plant compounds found in the seeds, roots, stems, and leaves of plants belonging to at least 11 families of dicotyledonous angiosperms, among which the Cruciferae and its genus *Raphanus* (Kjaer, 1960; Tookey et al., 1980; Larsen, 1981; Daxenbichler et al., 1991). In addition, each genotype generally contains one predominant GLS (Daxenbichler et al., 1991). This finding is important for both the taxonomy and genetics of these plant families and is also important for successfully isolating these compounds, especially with high yield and purity. GLSs include approximately 100 identified naturally occurring thioglucosides (Kjaer and Skrydstrup, 1987) with a common structure (Figure 1) characterized by side chains (R) with varying aliphatic, aromatic, and heteroaromatic carbon skeletons, all presumably derived from amino acids by a chain-lengthening process and hydroxylation or oxidation (Larsen, 1981).

In the intact cell, the GLSs are separated from β-thioglucosidase (EC 3.2.3.1), an enzyme generally known as myrosinase, which catalyzes the hydrolysis of these compounds when the plant cell structure is damaged. The enzymatic hydrolysis of GLSs yields D-glucose, sulfate, and a series of compounds including isothiocyanates, thiocyanates, and nitriles, depending on both the substrate and the reaction conditions, especially the pH (Figure 1).

Numerous studies have demonstrated that some of these breakdown products, in addition to being responsible for the characteristic pungent flavor, have various nutritive and antinutritional properties (Fenwick et al., 1983), inhibit neoplastic effects of some carcinogens (McDanell et al., 1988), and show antifungal and antibacterial activities (Chew, 1988).

Radish (*Raphanus sativus* L.) contains three main GLSs: 4-(methylsulfinyl)butyl glucosinolate (glucoraphanin) and 4-(methylsulfinyl)but-3-enyl glucosinolate (glucoraphenin), which prevail in the seed (Daxenbichler et al., 1991), and *trans*-4-(methylthio)-3-butenyl glucosinolate (4MTB-GLS), which is the most important in the root (Carlson et al., 1985) (Figure 1).

The literature on cruciferous material containing 4MTB-GLS, and/or its corresponding isothiocyanate, reports that this plant material possesses some characteristic and interesting properties, presumably attributable to 4MTB-GLS, such as (i) the pungent flavor typical of radish (Ishii, 1991); (ii) the capacity, as some other GLSs, to keep nematodes in soil under control (Caubel and Chaubert, 1985; Tacconi et al., 1989; Brown et al., 1991); and (iii) a certain biological activity against some liver diseases (Schmid and Karrer, 1948).

The presence of 4MTB-GLS in radish roots was indirectly established by Friis and Kjaer (1966) by isolating and characterizing the *trans*-4-(methylthio)-3-butenyl isothiocyanate obtained by myrosinase-catalyzed hydrolysis.

Several unsuccessful attempts were made by the same authors to isolate a significant amount of this GLS intact. They suggested that their failure was due to the particularly fast enzymatic hydrolysis of 4MTB-GLS. This supposition was supported by the detection of considerable increases in free glucose during the first phase of the purification process (Friis and Kjaer, 1966).

In recent years, several efficient methods of preparative isolation and purification of some intact GLSs from plant material have been described (Bjerg and Sörensen, 1987; Thies, 1988) but not 4MTB-GLS. The present paper describes an isolation procedure for 4MTB-GLS from radish roots and reports its spectroscopic characterization.

MATERIALS AND METHODS

Plant Material. Radish seeds (*Raphanus sativus* L. cv. Pegletta) were obtained from P. H. Petersen Saatzücht Lundsgaard (Hannover, RFG). Plants were grown in Bologna during the spring of 1990. Radish roots were collected when the plants were at the first stage of seed ripening. At this growth stage the amount of 4MTB-GLS per unit of root was optimal (Lazzeri et al., manuscript in preparation). After washing in tap water, the whole intact roots were frozen and lyophilized using an Edwards freeze-dryer Model Minifast MF DO.1. The dried roots were ground in a Retsch grinder Model ZM1 equipped with a 0.75-mm sieve. The resulting dry white powder was stored in a sealed vessel at room temperature.

Chemicals. The sinigrin and the standard glucose solution were supplied by Sigma. TMS and D_2O were purchased from Fluka. The other reagents were of analytical grade.

Extraction of Glucosinolates. A typical procedure for the extraction of 4MTB-GLS was as follows: 50 g of freeze-dried material was added (1:20 w/v) to a boiling solution (72–74 °C) of 50 mM imidazole–formate, pH 4.15, and methanol (3:7). The mixture was homogenized in an Ultraturrax Model TP 18/2N for 20 min at medium speed, cooled, and left in a ice bath until the suspension decanted. The solid residue was extracted again and filtered. The combined methanolic extracts (1240 mL) were centrifuged at 17700*g* for 30 min using a Beckman Model J-21

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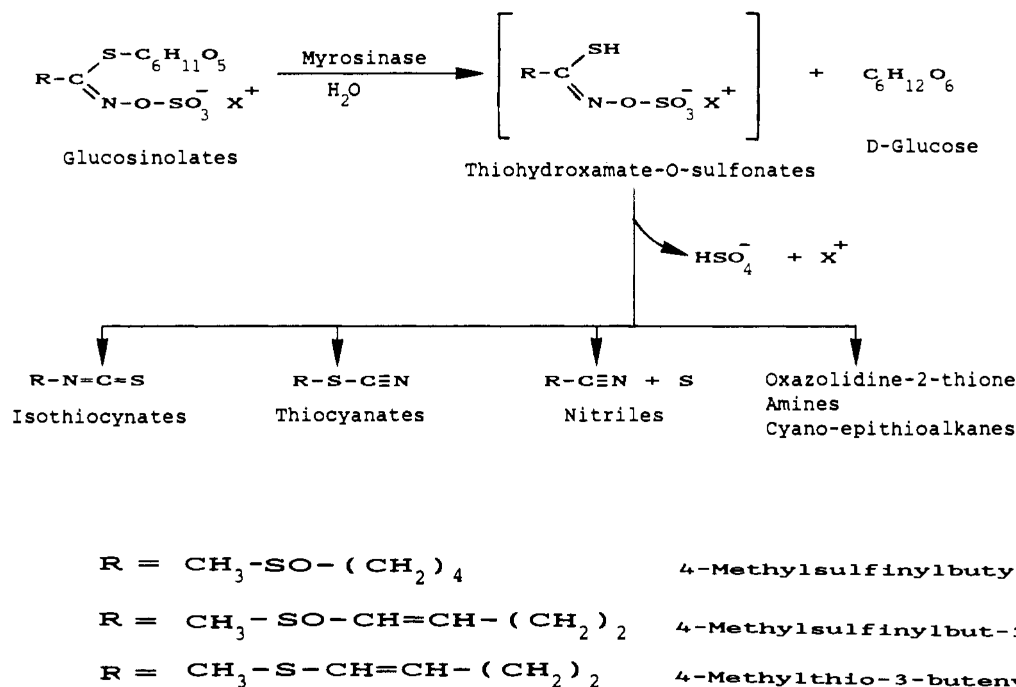

Figure 1. Scheme of the myrosinase-catalyzed hydrolysis of GLSs.

centrifuge. The supernatant was concentrated to about 40% of the initial volume (i.e. when the major part of methanol was removed) in a Büchi Model RE 121 rotary evaporator at 40 °C under vacuum. The concentrated extract was diluted to the initial volume with 50 mM imidazole–formate, pH 4.15. Finally, the extract was deproteinized using a solution of lead and barium acetate (each 0.5 M) (Thies, 1988) in the ratio of 1 mL of solution to 50 mL of extract. After precipitation of the proteins, the extract was centrifuged for 15 min at 17700*g*.

Chromatography. After any necessary adjustment in the initial pH, the entire extract was loaded on a DEAE-Sephadex A-25 anion-exchange column (2.6 × 15 cm) (Pharmacia, Uppsala, Sweden) conditioned with 50 mM imidazole–formate, pH 4.15. After loading, the column was washed successively with 400 mL of distilled water, 100 mL of formic acid–2-propanol–water (3:2:5) and another 500 mL of distilled water. Finally, the 4MTB-GLS was eluted with 260 mL of 0.5 M K_2SO_4 containing 5% 2-propanol.

Separation of K_2SO_4. The eluate was dried using the rotary evaporator by adding ca. 30 mL of absolute ethanol in small amounts at irregular intervals during the last phase of the evaporation process to remove the residual water. Boiling methanol was added to the solid up to about the initial volume and the mixture left at room temperature to allow the K_2SO_4 to settle.

Isolation of 4MTB-GLS. After filtration to remove the remaining particles of K_2SO_4, the methanolic extract (ca. 240 mL) was concentrated up to ca. 40 mL. The warm solution (40–45 °C) was then poured slowly in small amounts into an Erlenmeyer flask containing 200 mL of absolute ethanol previously cooled to −20 °C. A white powder was obtained. The ethanolic mixture was stored at −20 °C overnight to complete 4MTB-GLS precipitation. After centrifugation, the precipitate was dried in a desiccator with P_2O_5 under vacuum.

Spectroscopy. Infrared spectra were obtained using a Perkin-Elmer FT-IR Model 1860X spectrophotometer following the diffusive reflectance technique with the sample mixed in KBr. ^1H and ^{13}C NMR spectra were obtained using a Bruker AC 300 MHz apparatus. The sample was dissolved in D_2O. The chemical shifts were determined with reference to a water signal at δ 4.70 in relation to that of TMS. Mass spectra were obtained with a Finnigam Mat-8222 apparatus using the DCI (ammonia) technique with direct sample introduction. The source temperature was 160 °C; source pressure, 66 Pa; resolution, 2000. Optical rotation was measured in a 10-cm cell in a Perkin-Elmer Model 241 automatic polarimeter. The ultraviolet spectrum of desulfo-GLS was obtained by HPLC analysis with a diode array detection of the pure elution peak, while that for the whole GLC in water solution was performed with a Cary Model 219 recording spectrophotometer.

HPLC Analyses. The purity of 4MTB-GLS was tested using glucotropaeolin as the internal standard by analyzing an aqueous solution of 0.65 mg/mL with a Hewlett-Packard Model 1090 L HPLC equipped with a diode array detector, monitoring the absorbance of the eluates at 229 nm. Glucotropaeolin was isolated from seeds of *Lepidium sativum* via the method described by Thies (1988). The purity of glucotropaeolin was tested by polarography determining the glucose released (Iori et al., 1983), by HPLC using sinigrin as internal standard, and by NMR and mass spectroscopy. The HPLC analyses were carried out on desulfo-GLS using the reverse phase technique as described by Büchner (1987).

RESULTS AND DISCUSSION

Isolation Procedure. Although the procedure used for isolating 4MTB-GLS was essentially based on the method described by Thies (1988) for isolating and crystallizing sinigrin and glucotropaeolin, our slight modifications were of crucial importance for obtaining highly pure 4MTB-GLS powder in acceptable yield.

Although we made several attempts with the above procedure (which is probably the best in the literature for a large number of GLSs), we were not successful in the case of 4MTB-GLS. In addition, to the best of our knowledge, this GLS has never been isolated before in pure form due to its high hygroscopicity and, in particular, because it is much more prone to rapid degradation by myrosinase than other GLSs.

One therefore has to take in account that myrosinase is a glycoprotein which is generally extremely stable toward both heat and organic solvents. For instance, the myrosinase isolated from *Sinapis alba*, even when solubilized in a isooctane–reverse micelles system, shows its maximum activity with sinigrin at about 60 °C (Hochkoeppler and Palmieri, 1992).

Although in the first step of extraction we lost nearly 40% of the 4MT-GLS (presumably due the combined action of myrosinase and heat), we are convinced that our success was due mainly to the adopted isolation procedure

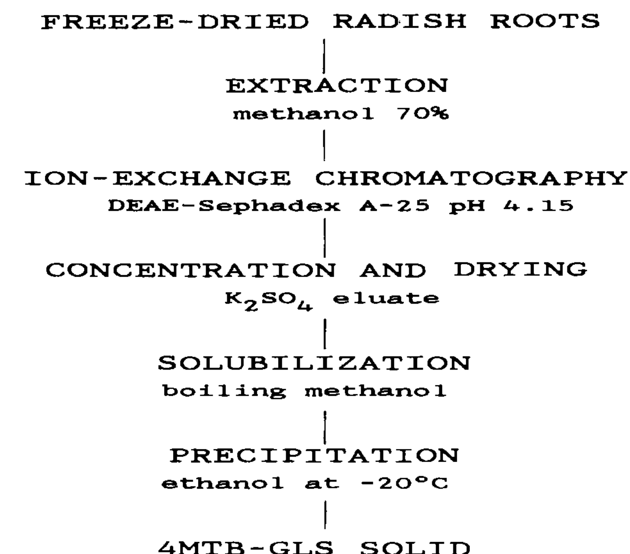

Figure 2. Flow sheet of 4MTB-GLS isolation.

reported in Figure 2, viz. (i) the use of suitable ripe radish roots in which 4MTB-GLS is essentially the only GLS present; (ii) the lyophilization of the intact roots before extraction; (iii) the addition of small amounts of ethanol during the concentration of the K_2SO_4 eluate to avoid the formation of a sticky paste, from which it is very difficult to isolate pure 4MTB-GLS; and finally, (iv) the precipitation of 4MTB-GLS in cool ethanol.

The variations introduced in our method permits one to obtain yields ranging from 9.8 to 24.7%. Nevertheless, we believe that the most important modification is in the use of lyophilization, which allows one to obtain a fine dry powder as the starting raw material. This expedient appears to be crucial for producing a faster deactivation of myrosinase in the first phase of 4MTB-GLS extraction.

Finally, the precipitation of 4MTB-GLS in cool ethanol, while it is a common technique found in many other isolation procedures and, in this case, based on simple empirical solubility trials, is essential for improving the final physical state and the purity of 4MTB-GLS. In fact, by concentrating the methanolic solution one obtains a pale dense yellowish-green product, which still contains some apparent impurities, presumably phenolic in nature. This step could also be useful to improve the isolation of other highly hygroscopic GLSs, exploiting the different solubilities when passing from warm methanol to −20 °C absolute ethanol. Using this expedient one obtains GLSs as a fine white powder, which then can be easily dehydrated in a desiccator with P_2O_5.

In fact, the HPLC profile of 4MTB-GLS isolated without any further purification (Figure 3) shows one main peak with a retention time greater than glucotropaeolin (internal standard) and a very small amount of glucoraphenin (retention time ca. 5 min). The purity of 4MTB-GLS calculated by both HPLC and glucose-release polarographic analyses appears to be above 97%.

Structure and Composition. Studies of ^{1}H and ^{13}C NMR (Tables I and II) suggest the structure reported in Figure 4. The signals of the ^{13}C NMR spectrum were consistent with the presence of one methyl, three methylenes, two vinyls, five CH carbons, and one quaternary carbon group. Experiments carried out in DEPT 135 confirmed these data. In the ^{1}H NMR spectrum (Figure 4), the signal at δ 2.12 (3 H, s) is attributable to a methyl group attached to a sulfur atom, the signal at δ 2.42 (2 H, q, J = 7.5 Hz) to the methylene protons attached to a double bond, and the signal at δ 2.70 (2 H, t, J = 7.5 Hz) to the methylene protons next to the quaternary carbon. Two signals are present in the vinylic proton region. The signal at δ 5.42 (1 H, dt, J = 15.0, 7.5 Hz) can be assigned to vinyl proton 3 (see Figure 4) and the signal at δ 6.10 (1 H, d, J = 15.0 Hz) to vinyl proton 4 attached to the methylthio-bearing carbon atom.

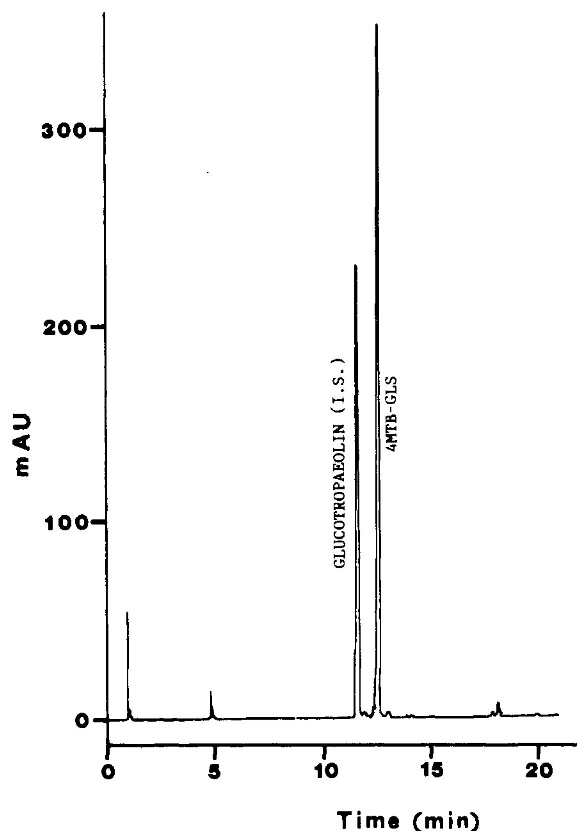

Figure 3. HPLC profile of desulfo-4MTB-GLS.

Table I. ^{1}H NMR Data for Glucosinolate (300 MHz in D_2O)

H1′	4.91	d	J = 9.5 Hz
H2′, H3′, H4′, H5′	3.30 − 3.55	m	
H6′	3.60	dd	J = 12.5, 5.5 Hz
	3.80	dd	J = 12.5, 2.0 Hz
H1	2.70	t	J = 7.5 Hz
H2	2.42	q	J = 7.5 Hz
H3	5.42	dt	J = 15, 7.5 Hz
H4	6.10	d	J = 15 Hz
H5	2.12	s	

Table II. ^{13}C NMR Data for Glucosinolate (300 MHz in D_2O)

C1′	82.54	C1	32.81
C2′	72.68	C2	30.84
C3′	77.78	C3	125.33, 125.92
C4′	69.85	C4	
C5′	80.81	C5	14.45
C6′	61.32	C6	164.15

The double bond configuration was chosen as 3*E* from the ^{1}H NMR coupling constant ($J_{H3/H4}$ = 15.0 Hz). The complex group of signals between δ 3.30 and 3.85 and the signal at δ 4.91 is characteristic of the glucose moiety. The multiplet at δ 3.30–3.55 (4 H) can be attributed to protons 2′, 3′, 4′, and 5′ of the glucose. The signals at δ 3.60 (1 H, dd, J = 12.5, 5.5 Hz) and δ 3.80 (1 H, dd, J = 12.5, 2.0 Hz) can be attributed to the methylene group of glucose. Finally, the signal at δ 4.91 (1 H, d, J = 9.5 Hz) is that of the anomeric proton (Olsen and Sörensen, 1980, 1981).

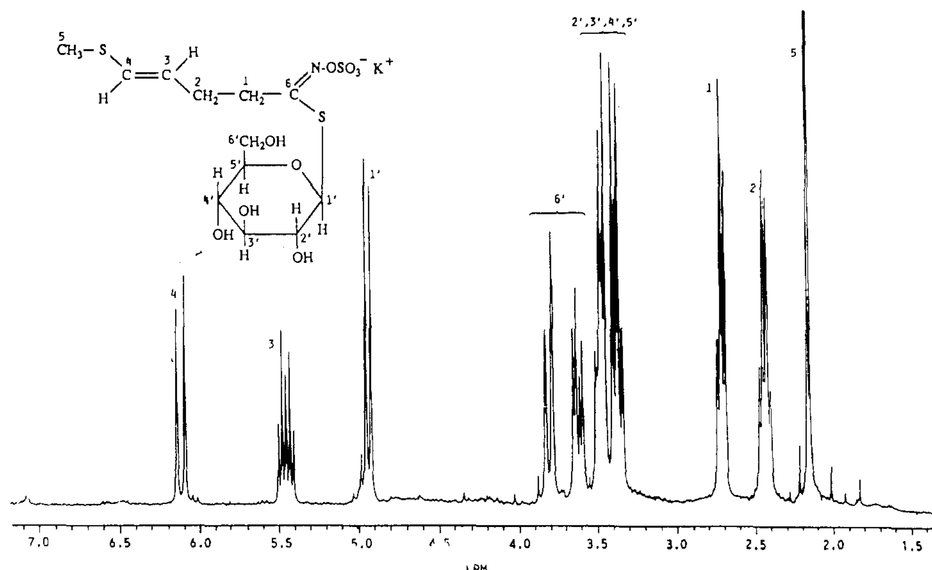

Figure 4. ^{1}H NMR spectrum of 4MTB-GLS.

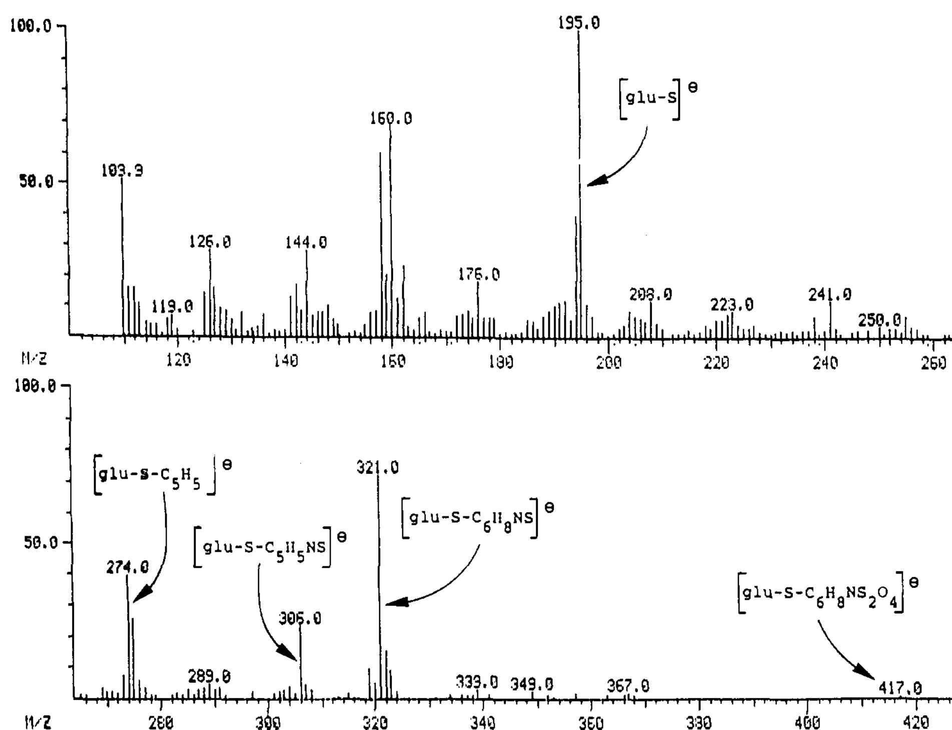

Figure 5. Mass spectrum of 4MTB-GLS.

Spatial configurations of H1′–H2′ were determined to be diaxial from the ^{1}H NMR coupling constant ($J_{H1'/H2'}$ = 9.5 Hz). Decoupling experiments of ^{1}H NMR disclosed that the signal at δ 4.91 is in relation to the multiplet at δ 3.30–3.55.

Mass spectral fragmentation of 4MTB-GLS (Figure 5) is in agreement with the structure of the compound. The peak m/z 417 is $[M - K - H]^-$ ion; m/z 321 represents the ion $[Glu - S - C_6H_8NS]^-$ formed by the loss of a sulfate group; the m/z 306 and 274 peaks are, respectively, the demethylated and the dethyomethylated anions reported above; finally, the m/z 195 peak can be attributed to the $[Glu - S]^-$ (Figure 5).

The IR spectrum seems to confirm the structure of Figure 4. In fact, IR ν_{max} (KBr, cm^{-1}): 3360 (OH), 1590 (C=C, C=N), 1350 (CH_3S), 1390, 1270, 1120, 1070 (SO_4).

Similarly to some other GLSs, the UV spectrum of desulfo-4MTB-GLS shows a UV spectrum with a maximum absorption around 225 nm, while that of the intact 4MTB-GLS has a broader absorption maximum, even though its NMR and mass spectra indicate a nearly homogeneous compound. Nevertheless, the curve of the second derivative of the 4MTB-GLS spectrum in the UV region shows several peaks, three of which, at 226.8, 231.2, and 235.0 nm, are well defined. The molar extinction coefficient at 226.8 nm and the polarization index, both determined in water, are 9220 ± 70 M^{-1} cm^{-1} and $[\alpha]^{20}_D$ = −23°, c = 1.0, respectively.

Conclusions. The majority of papers that have appeared in the last few decades regarding GLSs and their aglucon derivatives in cruciferous materials generally emphasize the drawbacks of this class of compounds such as their toxicity and endemic hypothyroidism in humans and animals. Much less has been written about the advantages that might emerge from their biological activities and new technological uses. In particular, an

important aspect of direct practical interest for 4MTB-GLS is the presumed biological activity of its corresponding isothiocyanate to control the attack and multiplication of nematodes (*H. schachtii*) in the soil. Several experiments carried out in our laboratory seem to confirm this activity in vitro (results to be published).

This paper presents some simple modifications of a well-known procedure, which permit the isolation of appreciable amounts of 4MTB-GLS from radish roots in nearly homogeneous form. In fact, the proposed procedure allows one to obtain ca. 100 mg of this GLS in one batch, an amount that in our case was sufficient to determine its main molecular properties and structure (Figure 1).

In conclusion, we wish to emphasize that 4MTB-GLS, usually described as a highly labile compound, especially in the presence of myrosinase, and, therefore, difficult to isolate and characterize directly, can be obtained in a high level of purity and appreciable yield using this simple procedure.

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LITERATURE CITED

Bjerg, B.; Sörensen, H. Isolation of intact glucosinolates by column chromatography and determination of their purity. In *Glucosinolates in Rapeseeds: Analytical Aspects*; Wathelet, J. P., Ed.; M. Nijhoff Publishers: Dordrecht, 1987; pp 59–75.

Brown, P. D.; Morra, M. J.; McCaffrey, J. P.; Auld, D. L.; Williams, L., III. Allelochemicals produced during glucosinolate degradation in soil. *J. Chem. Ecol.* **1991**, *17* (10), 2021–2034.

Büchner, R. Comparison of procedures for optimum extraction. Purification and analysis of desulfoindolyl glucosinolates. In *Glucosinolates in Rapeseed: Analytical Aspects*; Wathelet, J. P., Ed.; M. Nijhoff Publishers: Dordrecht, 1987; pp 76–79.

Carlson, D. G.; Daxenbichler, M. E.; VanEtten, C. H.; Hill, C. B.; Williams, P. H. Glucosinolates in radish cultivars. *J. Am. Soc. Hortic. Sci.* **1985**, *110* (5), 634–638.

Caubel, G.; Chaubet, B. *Agronomie* **1985**, *5*, 463–466.

Chew, F. S. Biological effects of glucosinolates. In *Biologically Active Natural Products for Potential Use in Agriculture*; Cutler, H. G., Ed.; American Chemical Society: Washington, DC, 1988; pp 155–181.

Daxenbichler, M. E.; Spencer, G. F.; Carlson, D. G.; Rose, G. B.; Brinker, A. M.; Powell, R. G. Glucosinolate composition of seeds from 297 species of wild plants. *Phytochemistry* **1991**, *30*, 2623–2638.

Fenwick, G. R.; Heaney, R. K.; Mullin, W. J. Glucosinolates and their breakdown products in food and food plants. *CRC Crit. Rev. Food Sci.* **1983**, *18*, 123–201.

Friis, P.; Kjaer, A. 4-Methylthio-3-butenyl isothiocyanate, the pungent principle of radish root. *Acta Chem. Scand.* **1966**, *20*, 698–705.

Hochkoeppler, A.; Palmieri, S. Kinetic properties of myrosinase in hydrated reverse micelles. *Biotechnol. Prog.* **1992**, *8*, 91–96.

Iori, R.; Leoni, O.; Palmieri, S. A polarographic method for the simultaneous determination of total glucosinolates and free glucose of cruciferous material. *Anal. Biochem.* **1983**, *134*, 195–198.

Ishii, G. Glucosinolate in Japanese radish, *Raphanus sativus* L. *JARQ* **1991**, *24*, 273–279.

Kjaer, A. Naturally derived isothiocyanates (mustard oil) and their parent glucosides. *Fortschr. Chem. Org. Naturst.* **1960**, *18*, 122–176.

Kjaer, A.; Skrydstrup, T. Selenoglucosinolates: synthesis and enzymatic hydrolysis. *Acta Chem. Scand.* **1987**, *B41*, 29–33.

Larsen, P. O. Glucosinolates. In *The Biochemistry of Plants*; Conn, E. E., Stumpf, P. K., Eds.; Academic Press: New York, 1981; Vol. 7, pp 501–525.

McDanell, R.; McLean, A. E. M.; Hanley, A. B.; Heaney, R. K.; Fenwick, G. R. Chemical and biological properties of indole glucosinolates (glucobrassicin): A review. *Food Chem. Toxicol.* **1988**, *26*, 59–70.

Olsen, O.; Sörensen, H. Glucosinolate and amines in *Reseda media*. *Phytochemistry* **1980**, *19*, 1783–1787.

Olsen, O.; Sörensen, H. Recent advances in the analysis of glucosinolates. *J. Am. Oil Chem. Soc.* **1981**, *58*, 857–865.

Schmid, H.; Karrer, P. *Helv. Chim. Acta* **1948**, *31*, 1017–1028.

Tacconi, R.; Mambelli, S.; Menichetti, P.; Pola, R. *Nematol. Medit.* **1989**, *17*, 21–25.

Thies, W. Isolation of sinigrin and glucotropaeolin from cruciferous seeds. *Fat Sci. Technol.* **1988**, *8*, 311–314.

Tookey, H. L.; VanEtten, C. H.; Daxenbichler, M. E. Glucosinolates. In *Toxic Constituents of Plant Foodstuffs*, 2nd ed.; Liener, I. E., Ed.; Academic Press: New York, 1980; pp 103–142.

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