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4-HYDROXY-3-METHOXYBENZYLGLUCOSINOLATE, A NEW GLUCOSINOLATE IN SEEDS OF *BRASSICA ELONGATA*

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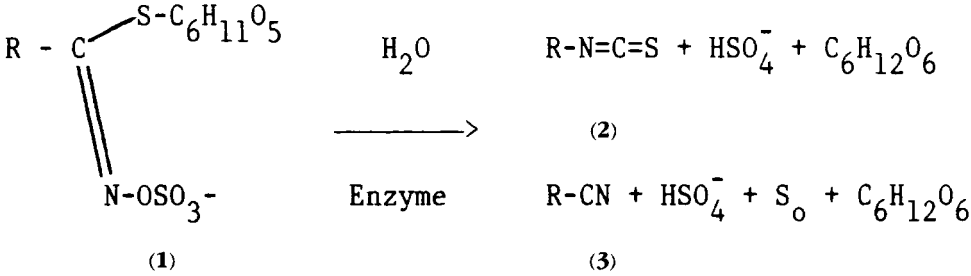
ABSTRACT.—A new compound, 4-hydroxy-3-methoxybenzylglucosinolate, is reported in the seed of *Brassica elongata* Ehrh. Other glucosinolates include 3,4-dimethoxybenzyl- and *p*-hydroxybenzyl-glucosinolates.

Seed of *Brassica elongata* Ehrh. was reported previously to contain glucosinolates, but the specific kinds were not identified (1). We report here the presence in this seed of a novel glucosinolate, 4-hydroxy-3-methoxybenzylglucosinolate (4-hydroxy-3-methoxybenzyl-GS), in addition to *p*-hydroxybenzyl-GS and 3,4-dimethoxybenzyl-GS, which had been found previously in other crucifer plants (2,3). Glucosinolates (**1**) are readily hydrolyzed by endogenous enzyme to release the aglucon as isothiocyanate (**2**) or as a nitrile (**3**) (4). These aglucon products have been employed extensively both to identify the parent glucosinolates and to provide quantitative measurements of them (4-8).



RESULTS AND DISCUSSION

Preliminary gc/ms data indicated the presence of a new substituted benzyl isothiocyanate from seed of *B. elongata* in addition to the known *p*-hydroxybenzyl (3) and 3,4-dimethoxybenzyl isothiocyanates (-NCS) (4) (confirmed by spectral library correlations). Isolation of the new isothiocyanate and the corresponding nitrile with subsequent gc/ms (including TMS derivative), thiourea derivatization (uv/tlc analysis) and nmr data confirmed the aglucon structure of the parent 4-hydroxy-3-methoxybenzyl-GS.

The glc measurements of the liberated 3,4-dimethoxybenzyl-NCS and 4-hydroxy-3-methoxybenzyl-NCS indicated the presence of their respective glucosinolates in defatted seed meal in a ratio of 3:2 and accounted for about 2/3 of the 6% total glucosinolate in the seed, based on data from enzymatically released glucose. Most of the remainder glucosinolate-glucose measurement could be assigned to *p*-hydroxybenzyl-GS from thiocyanate ion determinations.

The ms data for the new isothiocyanate yielded major ions of $M^+ = m/z$ 195 and a base peak of m/z 137 (M^+-NCS). Spectral analysis of the TMS derivative produced major ions of $M^+ = m/z$ 267, base peak m/z 209 (M^+-NCS), m/z 179 (**4**), and m/z 73

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(TMS), all corresponding to the addition of one TMS moiety due to the presence of single hydroxyl function.

Spectral of the nitrile resulted in two major ions, base peak $M^+ = m/z$ 163 and m/z 148 (M^+-CH_3). The presence of only two major ions M^+ and M^+-CH_3 without a loss of the nitrile function corresponds to spectra obtained for 3,4-dimethoxybenzyl nitrile (5). Derivatization with TMS resulted in the predicted spectra of $M^+ = m/z$ 235, m/z 220 (M^+-CH_3), base peak m/z 205 (**5**) and m/z 73 (TMS). Formation of structure **4** was reported for TMS derivatized carbidopa metabolites (9) and lends support to a 3,4-substituted benzyl structure.

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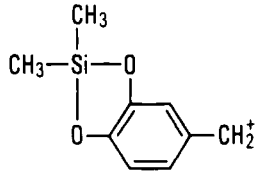

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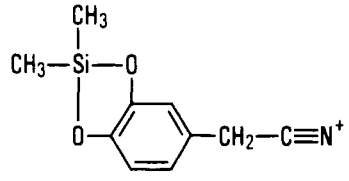

Mass Spectral Ion Structures

A thiourea derivative was formed to further confirm the presence of the isothiocyanate function. The uv absorption at γ max 244 nm showed a marked increase after derivatization. Thiourea formation was shown to be quantitative by the change in Rf of a single spot on tlc after derivatization.

The nmr data of both the nitrile and isothiocyanate substantiated the hydroxy, methoxy structure. Spectra for the isothiocyanate produced a singlet at δ 1.25 (CH_2), a singlet δ 3.90 (O-CH_3), a singlet δ 4.60 (OH), and protons at δ 6.83, 6.88, 7.26 (aromatic). Spectra for the nitrile produced a singlet δ 1.25 (CH_2), singlet δ 3.90 (O-CH_3), and protons at δ 6.83, 7.26 (aromatic). In an attempt to confirm the relative positions of the hydroxyl and methoxyl functions, two known benzyl alcohol isomer standards were analyzed by nmr: the 3-hydroxy-4-methoxy isomer, producing a singlet at δ 1.58 (CH_2), singlet δ 3.89 (O-CH_3), broad singlet δ 4.59 (CH_2-OH), singlet δ 5.63 (Ar-OH), and protons δ 6.83, 6.95, 7.26 (aromatic); and the 4-hydroxy-3-methoxy isomer producing a singlet δ 1.60 (CH_2), singlet δ 3.90 (O-CH_3), two peak δ 4.56-4.62 (CH_2-OH), singlet δ 5.63 (Ar-OH), and protons δ 6.86, 6.92, 7.26 (aromatic). The aromatic proton at δ 7.26 present in all spectra indicates a position removed enough to be unaffected by the hydroxyl and methoxyl positioning, thus supporting a 3,4 substituted structure. The difference in chemical shift between the remaining two protons was as follows: the new isothiocyanate 0.05 ppm, the 3-hydroxy-4-methoxy alcohol isomer 0.12 ppm, and the 4-hydroxy-3-methoxy alcohol isomer 0.06 ppm. The similar chemical shift between the isothiocyanate and the latter isomer supports the 4-hydroxy-3-methoxybenzyl-NCS structure.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—The hplc was performed using a Waters 6000A pump with a U6K injector and a tandem detection system of a Waters 401 refractometer and a Shoeffel 701 uv detector set at 240 nm. A Waters μ-Bondapak octadecasilane column was used with an isocratic solvent system of acetonitrile-water (1:1) and a flow rate of 2 ml/min. The tlc was carried out on silica gel with ether-hexane (1:1); glc conditions were as described by Daxenbichler and VanEtten (8). Mass spectrometry analyses were performed on tandem gc/ms using conditions described by Spencer and Daxenbichler (5). Thiourea absorption measurements were made with a Beckman DK6 uv visible spectrophotometer scanning from 320-220 nm. ^{1}H-nmr was carried out in $CDCl_3$ solvent with a Brücker WH-90 90 MHz Spectrometer.

GLUCOSINOLATE EXTRACTION.—Seed of *B. elongata* was collected and identified by Prof. Osman Tosun, Ankara University, Turkey. The seed was ground in a mortar and pestle and extracted with pen-

tane-hexane (four times) in a small glass percolator. After extraction, the defatted meal was allowed to air-dry at ambient temperature. Deactivation of the endogenous enzyme system and subsequent extraction of the intact glucosinolate was accomplished by addition of the meal to boiling methanol. Boiling was maintained for 5 min, followed by filtration and three additional extractions with boiling methanol-water (70:30). The combined extracts were concentrated under vacuum and made to a known volume with distilled water.

ISOTHIOCYANATE ISOLATION.—An aliquot of glucosinolate extract equivalent to 2 g meal was transferred to a Teflon-capped vial along with 4 ml of 0.2 M phosphate buffer containing 120 mg thioglucosidase (glucosinolate glucohydrolase, E.C. 3.2.3.1) prepared from *Sinapis alba* seed as previously described (6). Dichloromethane (25 ml) was added to the vial and the hydrolysis products were extracted during 16 h shaking at room temperature. This procedure favors the hydrolysis of glucosinolates to isothiocyanates (7). The vial contents were transferred quantitatively to a separatory funnel, and the CH_2Cl_2 phase was collected. The aqueous phase was reextracted twice with 40 ml CH_2Cl_2, and the combined extracts were dried over Na_2SO_4, filtered and concentrated (*in vacuo*) to 0.5-ml volume. This concentrate was placed on a 1×25 cm column packed with 12 g silica gel, washed with 40 ml of hexane, and then eluted with 20-ml portions of ethylether-hexane starting with ethylether-hexane (1:10) and increasing by increments of 10% ether until the final portion was pure ethyl ether. The eluants were collected as 10-ml fractions and tlc/gc was used to detect components. Fractions 6 and 7 (hexane-ether) were found to contain a mixture of all three components of interest, thus requiring further separation by hplc. All three components were completely resolved; their retention times were as follows: *p*-hydroxybenzyl-NCS (11 min), 4-hydroxy-3-methoxybenzyl-NCS (14 min), and 3,4-dimethoxybenzyl-NCS (21 min). The fractions collected were each extracted four times with double volumes of CH_2Cl_2; the extracts then were dried over Na_2SO_4, filtered, and concentrated to 1-ml volume for analysis by gc/ms. An aliquot of each was taken and treated in pyridine with a mixture of hexamethylsilazane and trimethylchlorosilane (2:1) to form the TMS derivative. A second aliquot was treated with ammoniacal ethanol to form a thiourea as further evidence of a NCS function (tlc analysis). Preparation for nmr analysis was accomplished by removing the CH_2Cl_2 under a stream of nitrogen and redissolving in $CDCl_3$.

NITRILE ISOLATION.—An aliquot of glucosinolate extract equivalent to 12 g of meal was cooled in an ice bath along with a second vial containing 6 g defatted *Crambe abyssinica* meal; these conditions favor the formation of nitriles from glucosinolates (4,9). The extract was then transferred and mixed thoroughly with the crambe meal, allowed to stand 5 min in the ice bath, followed by 15 min at ambient temperatures. The mixture was extracted with 40 ml of CH_2Cl_2, after which the CH_2Cl_2 was decanted, dried over Na_2SO_4, filtered, and concentrated to 0.5-ml volumes.

Initial separation was again achieved on a silica gel column, as previously described, with the exception of using 40-ml volumes of each increment. Components were detected as follows: isothiocyanates fractions 5-9, benzylic nitriles 11-13, and *Crambe* episulfide nitriles 15-20.

Isothiocyanate separation was carried out as previously described. Separation of the nitriles was accomplished using the same hplc system as described, but with isocratic elution with acetonitrile-water (1:2). The order of elution was the same as with the isothiocyanate separation (retention times 16, 18, 24 min), but with incomplete separation of the *p*-hydroxybenzyl cyanide from the hydroxy-methoxybenzyl cyanide. This fraction was rechromatographed with an isocratic system of methanol-water (4:1) at a 2 ml/min flow rate. The separation produced three fractions with retention times as follows: *p*-hydroxybenzyl cyanide, 28 min; trace amount of apparent isomer of hydroxy-methoxybenzyl cyanide, 37 min; and 4-hydroxy-3-methoxybenzyl cyanide, 46 min. Extraction and preparation of each fraction for analysis by gc/ms (including TMS) and nmr was as previously described.

Note added in proof: The results reported here disagree with the glucosinolate composition for *B. elongata* seed included in a recent report [P.J. Horn and J.G. Vaughan, *Phytochemistry* **22**, 465 (1983)] that came to our attention after this manuscript was submitted for publication. Vaughan *et al.* graciously provided us a sample of their botanically well-defined seed from *B. elongata*.

The composition we report here was verified, although there were some quantitative differences in the two accessions.

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