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Isolation and Structure Elucidation of a New Thermal Breakdown Product of Glucobrassicin, the Parent Indole Glucosinolate

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The thermal breakdown of glucobrassicin, the major natural indole glucosinolate present in cruciferous vegetables, has been studied. This study has been conducted using pure synthetic glucobrassicin instead of raw vegetable material to eliminate possible other sources (i.e., enzymatic or chemical) of degradation. After 1 h in aqueous solution at 100 °C, 10% of the original glucobrassicin is degraded and yields a unique degradation compound. This compound is described for the first time and has been identified as 2-(3′-indolylmethyl)glucobrassicin on the basis of data obtained by ^{1}H nuclear magnetic resonance spectroscopy as well as tandem mass spectrometric experiments carried out from positive and negative electrospray produced ions.

KEYWORDS: Glucosinolates; glucobrassicin; indole derivatives; indole condensation product; anticarcinogenic substances; thermal breakdown, structure elucidation

INTRODUCTION

Glucosinolates constitute a wide class of natural compounds predominantly from the Cruciferae family and are mainly responsible for the flavor and biological activity of these vegetables. Glucosinolates and their breakdown products have long been known for their goitrogenic and antinutritional activity and have given rise to intense research work for their cancer-preventing activities (*1*). They all possess a common chemical structure consisting of a 1-thio-β-D-glucopyranose unit and an O-sulfated thiohydroxymate moiety and vary only in the nature of their side chain, which can be aliphatic, aromatic, or heterocyclic.

Glucobrassicin (**1**, **Figure 1a**) represents the most widespread indole glucosinolate and is present in particular in cruciferous vegetables of the *Brassica* genus (*2*, *3*). This indolylmethyl glucosinolate is involved via its breakdown products, such as indole-3-carbinol, 3,3′-diindolylmethane, and subsequent oligomerization products, in the anticarcinogenic properties of cruciferous vegetables, and these condensation products are known to induce detoxification enzymes such as cytochrome P-450 or glutathione *S*-transferases (GST) (*4*−*7*).

Most of the biological effects as well as the flavors appreciated by consumers are not associated with the glucosinolates per se but with their degradation products, and the active indole breakdown products issuing from **1** may be the result of various degradation processes, including enzymatic, chemical, or thermal degradation (*2*, *3*, *8*−*13*).

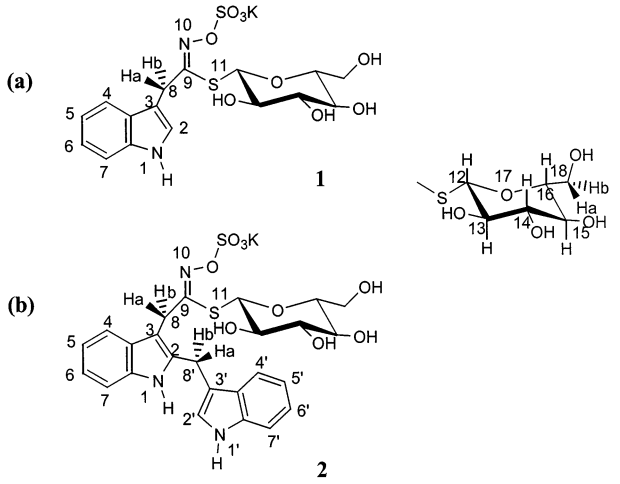

Figure 1. Chemical structures of (a) glucobrassicin (**1**) and (b) 2-(3′-indolylmethyl)glucobrassicin (**2**).

Data concerning the enzymatic and chemical degradation of indole glucosinolates have been reviewed by McDanell et al. in 1988 (*3*). The main studies on the thermal degradation of **1** and other indole glucosinolates have been carried out by Slominski and Campbell (*8*, *9*) in the examination of the indole glucosinolate degradation during heat processing of *Brassica*

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vegetables. In these studies, indole-3-carbinol and indole-3-acetonitrile were identified as the main decomposition products of **1**.

In a recent study on the thermal breakdown of **1** using for the first time the radiolabeled indole glucosinolate prepared in our laboratory (*14*), we have reported that after exposure to heat treatment, glucobrassicin was weakly degraded (10% within 1 h) and gave rise to a new minor indole condensation product corresponding to a (3′-indolylmethyl)glucobrassicin (**2**, **Figure 1b**), whereas neither indole-3-carbinol nor indole-3-acetonitrile was obtained (*13*). The occurrence of such a degradation product had never been reported for the thermal degradation of an intact indole glucosinolate. However, at this time, only a partial structural characterization of this compound appearing after 1 h at 100 °C could be carried out by LC-MS (*13*), and the position of the indolylmethyl group on the glucobrassicin remained to be specified. We now report the isolation and structure elucidation of **2** on the basis of MS and ^{1}H NMR.

MATERIALS AND METHODS

Chemicals. Synthetic glucobrassicin with a chemical purity (determined by HPLC) of >97% was prepared as described in our previous work (*13*). HPLC grade acetonitrile was purchased from Scharlau (Barcelona, Spain).

Thermal Breakdown. Glucobrassicin (15 μg) was dissolved in 1 mL of ultrapure water from a Milli-Q system (Millipore, St Quentin Yvelines, France), introduced into a closed vial, and kept at 100 °C for 1 h. The thermal breakdown assays were conducted in quadruplicate. For the preparation of **2**, 10 mg of glucobrassicin was prepared in 2 mL of ultrapure water, introduced into a closed vial, and kept at 100 °C for 1 h.

HPLC Analyses. Separation of **1** and **2** was performed using a Jasco pump HPLC series PU-980 (Merck, Nogent-sur-Marne, France) equipped with a Rheodyne 7725i injection valve fitted with a 100 μL sample loop, a Jasco series L6-980-02 gradient former (Merck), a 250 mm × 4.6 mm i.d., 5 μm, Ultrabase C18 column (S.F.C.C., Eragny, France), and a 18 mm × 4.6 mm i.d., 10 μm, Ultrabase C18 guard column (Shandon-L.S.I., Cergy-Pontoise, France). The HPLC mobile phases consisted of acetonitrile/water/acetic acid mixtures, 10/90/0.5 (v/v/v) for A and 70/30/0.5 (v/v/v) for B, respectively. Elution was achieved using a linear gradient from 0 to 46% of B in 15 min followed by isocratic conditions at 46% of B during 10 min. The flow rate was 1 mL/min, at a temperature of 30 °C controlled by a Stabitherm column oven (Prolabo, Fontenay-sous-Bois, France). Ultraviolet (UV) detection was carried out at 280 nm with a Jasco detector series UV-975 (Merck). Two hundred microliter aliquots were sampled, evaporated to dryness under a nitrogen stream, and reconstituted in 100 μL of mobile phase A before injection. HPLC retention times for **1** and **2** were 7.9 and 15.5 min, respectively, using the above gradient elution.

The preparative fractionation for the isolation of **2** was performed with the same HPLC system (injection valve fitted with a 200 μL sample loop). Two hundred microliter reaction mixture aliquots were directly injected. **2** was manually collected at the column outlet. The mobile phase was evaporated to dryness, and the sample was stored at −20 °C until analysis.

MS Analyses. Mass spectrometric experiments were carried out on a Finnigan LCQ quadrupole ion trap mass spectrometer equipped with a Finnigan electrospray ionization source operating under either positive or negative ionization conditions. Sample solutions (typically 5−10 ng/μL in 50:50 MeOH/H_2O) were infused at 3 μL/min into the electrospray interface. Typical potentials applied to the ESI source were as follows: needle, 5000 V; heated transfer capillary, 5−20 V. All other operating parameters were manually tuned to optimize the signal for the ion of interest. Hydrogen/deuterium exchange experiments were performed using MeOD/D_2O as the solvent. Structural information was obtained using sequential MS^n experiments carried out into the trap. All spectra were acquired under normal scan (unit resolution) and automatic gain control conditions, using helium as the collision gas for MS^n experiments.

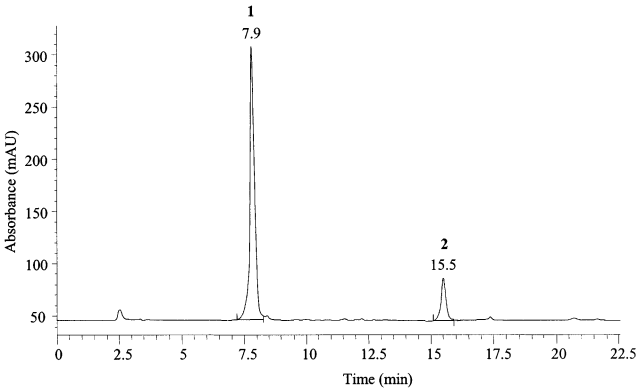

Figure 2. HPLC chromatogram at 235 nm of the thermal breakdown of glucobrassicin after 1 h of incubation at 100 °C.

High-resolution mass spectrometry on **2** was achieved on an API-Q-Star-Pulsar-*i* quadrupole time-of-flight hybrid instrument from Applied Biosystems MDS Sciex (Les Ulis, France), using negative electrospray as the ionization technique (needle voltage, −4500 V; declustering potential, −60 V). Exact mass measurements were made on the $[M - H]^-$ ion of **2** at a resolution of 5000, using taurocholic acid ($C_{26}H_{45}NO_7S$, M_w 514.2839) as the internal reference standard.

NMR Analyses. The NMR analyses were recorded on a Bruker AMX-400 spectrometer working at 400.13 MHz in CD_3OD. The central solvent signal of CD_3OD was used as internal reference (^{1}H, δ 3.31 relative to TMS).

RESULTS AND DISCUSSION

The HPLC chromatogram resulting from the thermal breakdown of **1** after 1 h of incubation at 100 °C is presented in **Figure 2**. This chromatogram is quite similar to that obtained in our previous study, when we were working with the [5-^{3}H]-glucobrassicin, although retention times differed slightly due to chromatographic conditions (phosphate buffer replaced by acetic acid) (*13*). In this work, it was clearly demonstrated that no loss of tritium occurred during incubation and that the total radioactivity was distributed between unchanged [^{3}H]glucobrassicin and the radiolabeled breakdown product **2**, the relative proportions being 90/10 after 1 h of incubation (*13*).

These results revealed that under drastic heat treatment conditions, glucobrassicin underwent degradation into a unique breakdown product that has never been described before. This main degradation compound, eluting at 15.5 min (**Figure 2**), was manually collected at the column exit and then submitted to both tandem MS and NMR analyses.

The analyses by MS were carried out using both positive and negative electrospray ionization. In the latter case, the $[M - H]^-$ ion was observed at *m*/*z* 576, which was in agreement with a molecule of glucobrassicin substituted by an indolylmethyl group. The CID spectrum (**Figure 3**) obtained after isolation and decomposition of the *m*/*z* 576 ion into the ion trap device exhibited several diagnostic daughter ions. As shown in the decomposition pathway proposed in **Figure 4**, several competitive decomposition processes took place from the $[M - H]^-$ species, leading in particular to the *m*/*z* 496, 380, 291, and 259 main characteristic fragment ions. The formation of the *m*/*z* 496 and 380 ions can be attributed to the loss of sulfate and the elimination of a neutral thioglucose moiety, respectively. Conversely, the *m*/*z* 291 and 259 fragment ions result from cleavages occurring at both carbon−sulfur bonds of the thioglucose moiety with transfer of the sulfate group, as presented in **Figure 4**. The formation of the *m*/*z* 334 ion should involve the elimination of glucose as a consecutive process occurring

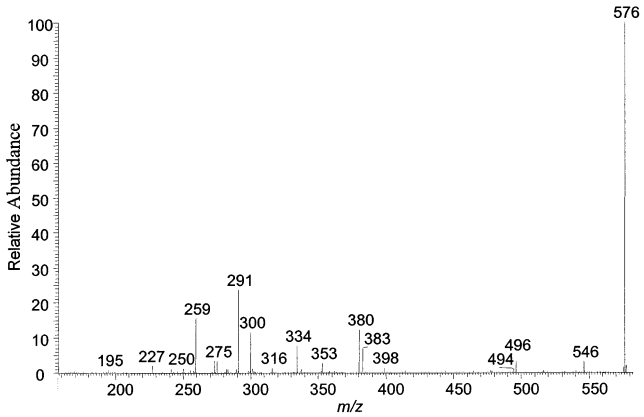

Figure 3. Tandem mass spectrum of ESI-produced $[M - H]^-$ (*m/z* 576) ion from **2**.

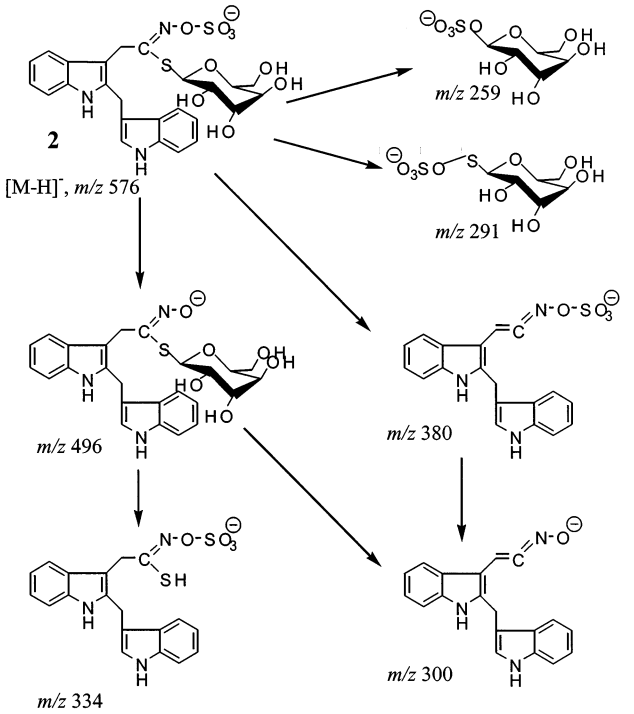

Figure 4. Proposed structures for the fragment ions generated in the MS/MS analysis of the ESI-produced $[M - H]^-$ (*m/z* 576) ion from **2**.

from the *m/z* 496 fragment ion (i.e. 334 = 496 − 162), whereas the structure proposed for the *m/z* 300 ion is consistent with a consecutive decomposition occurring either from the *m/z* 496 ion (elimination of thioglucose) or from the *m/z* 380 ion (loss of sulfate).

The structure and decomposition scheme proposed for **2** were further supported by data obtained from hydrogen−deuterium exchange experiments carried out using am MeOD/D_2O mixture as the electrospray solution. The *m/z* ratios of the various ion species of interest generated in both tandem mass spectrometric experiments under negative electrospray ionization are reported in **Table 1**. The quasi-molecular $[M - H]^-$ ion (*m/z* 576) was shifted to a $[M_{d7} - D]^-$ species at *m/z* 582, meaning that the molecule contained seven exchangeable hydrogens. This allowed the possible substitution of the indolic nitrogen atom by the additional indolyl methyl group to be ruled out, which would have produced a six exchangeable hydrogen atom species. As indicated in **Table 1**, all fragment ion *m/z* ratios were shifted in agreement with the number of exchangeable hydrogens of the structures proposed in **Figure 4**.

Table 1. *m/z* Ratios of Molecular and Fragment Ions Obtained from the ESI-MS Analysis of 2-(3′-Indolylmethyl)glucobrassicin in Nondeuterated and Deuterated Media

	solvent: MeOH/H_2O	solvent: MeOD/D_2O
quasi-molecular species	$[M - H]^-$; *m/z* 576	$[M_{d7} - D]^-$; *m/z* 582
main fragment ions (*m/z*)	496	502
	383	385
	380	382
	334	337
	300	302
	291	295
	259	263

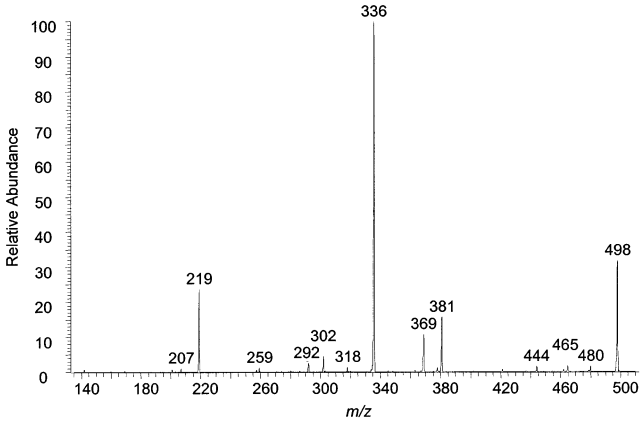

Figure 5. MS^3 spectrum of *m/z* 498 ion obtained from the decomposition of the ESI-produced MH^+ (*m/z* 578) ion from **2**.

As expected, under positive ionization conditions, the MH^+ ion was observed at *m/z* 578 as determined by LC-ESI/MS in a previous study (*13*). In this work, the MH^+ ion has been submitted to collisional activation into the trap. The resulting MS/MS spectrum exhibited the *m/z* 498 ion as the only daughter ion (data not shown), corresponding to the loss of the sulfate moiety of the molecule. Submitted to a sequential MS^3 experiment, the *m/z* 498 fragment ion mainly decomposed into the *m/z* 381, 369, 336, and 219 ions, as indicated in **Figure 5**. These ions were consistent with the postulated structure. The *m/z* 336 ion can be interpreted as the result of the loss of glucose from the *m/z* 498 ion. The elimination of an indole ring from the *m/z* 498 ion can lead to the *m/z* 381 fragment ion, whereas the *m/z* 219 ion can be generated when this elimination occurs consecutively from the *m/z* 336 ion. Finally, the loss of the indolylmethyl group accompanied by a hydrogen transfer from the leaving group can explain the formation of the *m/z* 369 daughter ion, which corresponds to a protonated form of nonsulfated glucobrassicin. Further information could be obtained by subsequent isolation and decomposition of the *m/z* 336 fragment ion present in the CID spectrum discussed above. Indeed, the *m/z* 336 ion decomposed into the *m/z* 259, 219, and 130 main fragment ions, which was in agreement with previous data obtained by LC-ESI/MS using in-source CID conditions (*13*). In particular, the occurrence of the *m/z* 259 ion, corresponding to the 2-(3′-indolylmethyl)-3-methylindole cation, provided further evidence for the structure established for **2**.

Figure 6a shows the aromatic part of the ^1H NMR spectrum measured at 400 MHz for glucobrassicin. The chemical shifts as well as the signal multiplicity observed are representative of an indole nucleus in which H-4 and H-7 protons (**Figure 1**) appear as doublets and H-5 and H-6 protons as multiplets, whereas the noncoupled H-2 proton is characterized by a singlet

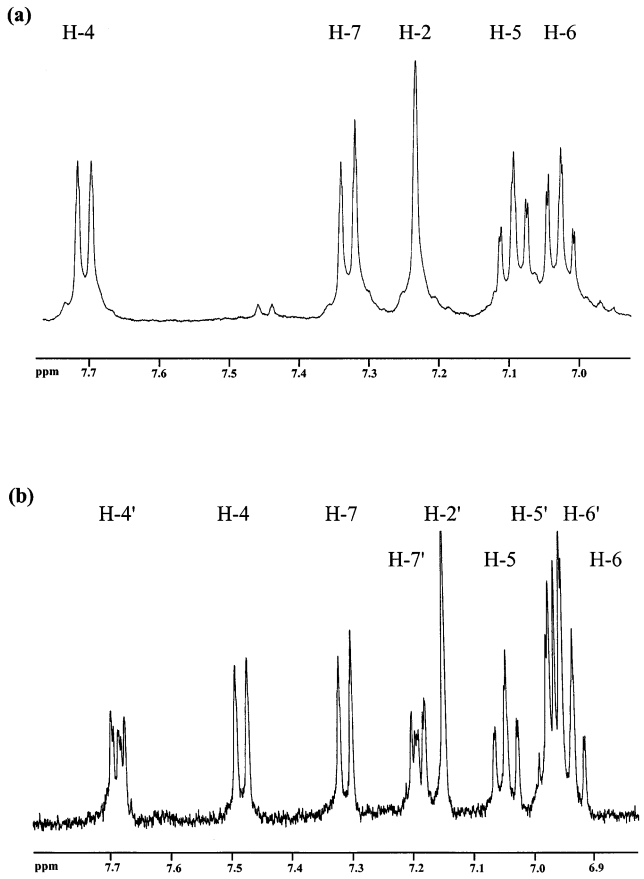

Figure 6. Part of the ^{1}H NMR spectra corresponding to the indole region of (a) glucobrassicin (**1**) and (b) 2-(3′-indolylmethyl)glucobrassicin (**2**).

Table 2. ^{1}HNMR Assignments of the Compound 2-(3′-Indolylmethyl)glucobrassicin in CD_3OD

atom	δ	J (Hz)	atom	δ	J (Hz)
H-2′	7.14	s	H-4	7.48	d (7.9)
H-4′	7.68	m	H-5	7.04	dt (7.9, 0.9)
H-5′	6.96	m	H-6	6.93	dt (6.8, 1.0)
H-6′	6.96	m	H-7	7.31	d (8.1)
H-7′	7.19	m			
H-8′α	4.33	s			
H-8′β	4.33	s			
H-8α	4.16[a]	d (16.2)			
H-8β	4.31[a]	d (16.2)			
H-12	4.82	d (nd[b])			
H-13	3.02	t (8.8)			
H-14	3.10	t (9.6)			
H-15	3.21	t (9.4)			
H-16	2.93	m			
H-18α	3.51[c]	dd (12.3, 5.6)			
H-18β	3.65[c]	dd (12.2, 2.1)			

[a] May be reversed. [b] nd, not determined. [c] May be reversed.

at 7.23 ppm. These data were in good agreement with previously published data (*15*, *16*). The spectrum of **2** is reported in **Figure 6b**. The aromatic portion of this spectrum showed the presence of two indole units (nine aromatic protons between 6.9 and 7.8 ppm). A representative singlet at 7.14 ppm for H-2′ indicates that position 3′ (**Figure 1**) is substituted, and multiplets are observed at 7.19 and 7.68 ppm for H-7′ and H-4′ protons, respectively (see **Figure 6b** and **Table 2**), whereas H-5′ and H-6′ protons appear as an unresolved multiplet at 6.96 ppm. In contrast, the 2,3-disubstituted indole moiety showed well-resolved aromatic protons from H-4 to H-7. We also observed two equivalent methylene protons for H-8′ (α and β) at 4.33 ppm (**Table 2**), whereas the multiplet pattern of H-8 showed two doublets ($J \sim 16$ Hz) corresponding to the diastereotopic H-8α and H-8β protons (4.16 and 4.31 ppm; see **Table 2**). Finally, analysis of the pyranose moiety showed a characteristic coupling of the H-18α and H-18β protons between 3.4 and 3.7 ppm, as well as characteristic triplet patterns for H-13, H-14, and H-15 (**Table 2**). On the basis of all of these structural data, the identity of **2** could be established as 2-(3′-indolylmethyl)-3-indolylmethyl glucosinolate or 2-(3′-indolylmethyl)glucobrassicin (**Figure 1b**).

This structure was further confirmed by high-resolution mass spectrometry measurements which gave an exact mass experimental value of 576.1083 for the $[M - H]^-$ ion, in agreement within 4.6 ppm with the calculated value for this ion (calculated value for $C_{25}H_{26}N_3O_9S_2$: 576.1110).

In the absence of data on the reactivity of the indole nucleus of intact glucobrassicin, some considerations may be developed from studies carried out on its degradation indole derivatives. Several indole-3-carbinol condensation products have already been described, consisting of indole-3-carbinol substituted by an indolylmethyl group at position 1 or 2 (*17*, *18*). Amat-Guerri et al. (*17*) showed that the condensation at position 2 can take place in the reaction between some 3-(hydroxymethyl)indoles and 3-substituted indoles. De Kruif (*19*) studied the potency of indole-3-carbinol to form condensation products other than 3,3′-diindolylmethane under acidic aqueous conditions. HPLC-MS and ^{1}H and ^{13}C NMR analyses showed the presence of a trimer, exhibiting an indole group 2,3-disubstituted with two indolyl methyl moieties [2,3-bis(indole-3-ylmethyl)indole]. Grose and Bjeldanes (*20*) studied the effects of pH on the oligomerization of indole-3-carbinol in aqueous acid conditions corresponding to those encountered in gastric juice, and on the basis of HPLC analyses as well as NMR and MS experiments, they observed the formation of dimeric 2-substituted indole intermediates similar to those described by other authors (*17*). Moreover, depending upon the pH conditions, the formation of some cyclic or linear trimers substituted at position 2 or 3 was observed, and these products are thought to be responsible for the biological activity of the initial substances (*21*−*23*). In another work, while analyzing disposition and excretion of indole-3-carbinol in rats, Stresser (*18*) elucidated the structure of a novel indole-3-carbinol metabolite in liver extract. This dimeric compound [1-(3-hydroxymethyl)indolyl-3-indolylmethane] has been identified by means of ESI-MS and ^{1}H NMR and differs from 3,3′-diindolylmethane by the methylene attachment point.

Although it is well established in the literature that position 3 of indoles is the preferred site for an attack by electrophilic agents (*24*), substitutions have also been reported at position 1 (*18*) and position 2 of a 3-substituted indole (*17*). In the latter case, it has been described as the result of a direct attack (*17*) or as an indirect process involving a primary attack at position 3, yielding an indolenine intermediate, followed by a rearrangement (sigmatropic shift) leading to the 2,3-disubstituted indole (*20*, *25*, *26*).

Concerning the formation of **2**, one can thus postulate that in a first step, the thermal breakdown of glucobrassicin should yield indole-3-carbinol, which can easily lose water to give 3-methyleneindolenine. This latter species should subsequently attack an intact molecule of glucobrassicin to give 2-(3′-indolylmethyl)glucobrassicin as the final product. Taking into account steric factors on position 3 of the glucobrassicin indole nucleus, a reaction scheme involving a direct attack at position 2 (*17*) seems more likely than a mechanism involving a primary attack at position 3 as proposed by Grose and Bjeldanes (*20*).

To our knowledge, with respect to the thermal treatment of indole glucosinolates, this is the first observation of a degradation product in which the thioglucose moiety of the intact glucosinolate remains unchanged. The glucosinolate chemical structure has been described by various authors (*3*, *8*−*12*, *27*, *28*) as sensitive toward chemical (pH), enzymatic (myrosinase), and thermal degradation. In a previous study (*13*), we reported that **1** was stable with regard to chemical degradation and that **2** was the only thermal breakdown product observed after a 1 h thermal treatment of **1** at 100 °C; this was in contrast with the observation of several authors reporting formation of indole-3-carbinol and indole-3-acetonitrile as the result of the thermal treatment of raw vegetable materials containing glucobrassicin (*8*, *9*, *29*). The present work, conducted with ultrapure water at neutral pH and using a pure synthetic compound, suggests that **1** is not affected to a great extent by heat treatment as such and that, when raw materials are heated, the degradation of **1** may be attributed not only to thermal degradation but also to the contribution of several other factors such as enzymatic activities and/or chemical interactions. This suggests that whenever they form, these intermediate compounds (indole-3-carbinol and indole-3-acetonitrile) should immediately react with glucobrassicin to yield **2** in such a way that the *S*-(glucosyl)acetothiohydroxymate group at position 3 of the original glucobrassicin is not eliminated.

The occurrence of **2** after heat treatment of vegetables has never been mentioned. However, its formation during vegetable cooking cannot be totally excluded seeing that indole-3-carbinol is present together with unchanged **1**. The formation of **2** should then occur at the beginning of the cooking, that is, when myrosinase has degraded part of **1** into indole-3-carbinol according to previously established processes (*2*, *3*, *13*).

In conclusion, this work provides first evidence for the formation of a glucosinolate breakdown product in which the thioglucose moiety remains intact. If occurring during food consuming, the formation of compounds such as **2** may have an impact on the yield of chemoprotective indolic compounds because, on the one hand, **2** is no longer a substrate for myrosinase (*30*) and, on the other hand, its formation occurs to the detriment of other biologically active indolic compounds such as indole-3-carbinol.

ABBREVIATIONS USED

HPLC, high-performance liquid chromatography; LC-MS, liquid chromatography−mass spectrometry; ESI-MS, electrospray ionization mass spectrometry; CID, collision-induced dissociation; NMR, nuclear magnetic resonance.

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