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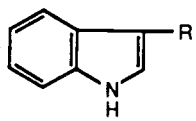
# INDOLES IN EDIBLE MEMBERS OF THE CRUCIFERAE

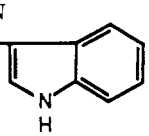
MONROE E. WALL,* HAROLD TAYLOR, PREMILA PERERA, and MANSUKH C. WANI*

*Research Triangle Institute, Research Triangle Park, North Carolina 27709*

ABSTRACT.—Antimutagenic fractions from collards yielded indole-3-carboxaldehyde [**4**] and traces of indole-3-acetonitrile [**2**]. The compounds had no antimutagenic activity. An analytical procedure for various indoles in plants was developed based on reversed-phase hplc. The indoles studied included the 3-carbinol **1**, the acetonitrile **2**, the carboxaldehyde **4**, the 3-carboxylic acid **5**, and the 3-acetic acid **6**. Many Cruciferae and non-Cruciferae were analyzed. The latter did not contain measurable quantities of these compounds. In the case of the Cruciferae—with the exception of collards, which consistently indicated the presence of the aldehyde **4**—the major indole found was the nitrile **2**. Although a particularly careful search for the carbinol **1** was conducted, only trace levels were noted. A review of the literature indicates that the content and occurrence of this indole in plants have been heavily overestimated. Because of the low levels found in the Cruciferae, our studies indicate that the role of the compound as a dietary factor may be questionable.

Wattenberg (1) has shown that various edible members of the Cruciferae when fed to rats as 20% of the diet produced significant enhancement of aryl hydrocarbon hydroxylase (AHH) activity. Subsequently, Wattenberg *et al.* (2) found several indoles, including indole-3-carbinol [**1**], indole-3-acetonitrile [**2**], 3,3′-diindolylmethane [**3**], and indole-3-carboxaldehyde [**4**], in such cruciferous vegetables as Brussels sprouts, cabbage, and cauliflower. Various biological activities have been attributed to these indoles. The carbinol **1** has been found to be an inhibitor of carcinogen-induced neoplasms, whereas the nitrile **2** was less effective. The carbinol **1** has also been found to be a general microsomal inducer (4–7) and to protect against covalent binding of benzo[a]pyrene metabolites to mouse liver DNA and protein (8). Recently it has been found that the carbinol **1** is inactive as an antimutagen (9) and was not only ineffective as an inhibitor of the induction of ornithine decarboxylase (ODC) activity in mouse epidermis by 12-*O*-tetradecanolylphorbol-13-acetate (TPA) but actually acted as a promoter (9). The various biological activities of the carbinol **1** and of the nitrile **2** are such that the level of these compounds in edible vegetables both in Cruciferae and non-Cruciferae would be of interest.



**1** R=$CH_2OH$
**2** R=$CH_2CN$
**3** R= 

**4** R=CHO
**5** R=COOH
**6** R=$CH_2COOH$

Recently, utilizing the procedure of Birt *et al.* (9), we made a study of inhibition of the mutagenic activity of 2-aminoanthracene (2AA) by a number of extracts of edible members of the Cruciferae family. Collards showed particular activity in the assay. In

the course of the chromatographic purification of collard extracts we isolated several indoles and developed an hplc analysis for a number of indoles that was applied to a large number of cruciferous and noncruciferous vegetables. Indole-3-carbinol could not be positively identified in any vegetable studied, and at the most only trace concentrations were noted. No indoles were found in noncruciferous plants.

This report presents our experimental findings. It suggests that regardless of the interesting physiological properties of pure **1**, the total absence or at best trace levels of **1** in cruciferous plants make the role of this indole questionable in the inhibition of induced carcinogenesis observed after various cultivars of *Brassica oleracea* L. were administered to rats as a large percentage of the total diet (1,2).

## EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Melting points were determined on a Kofler hot-stage microscope. Ir spectra (KBr disc) were recorded with a Perkin-Elmer 267 spectrophotometer. Uv spectra were measured in MeOH using a Varian 2290 spectrophotometer. $^1$H-nmr spectra were obtained with a Bruker WM 250 MHz spectrometer, using TMS as an internal standard in $CDCl_3/CD_3OD$ solution. $^{13}$C-nmr spectra were recorded with a JEOL 100 instrument. Mass spectra were obtained with an Associated Electrical Industries MS-902 instrument.

Tlc analyses were carried out on glass plates precoated with Si gel 60 F254 (E. Merck) (0.5-mm thickness for preparative tlc, 0.25-mm for analytical tlc). Solvent systems used for tlc analysis were (A) 5% MeOH in $CH_2Cl_2$, (B) 10% MeOH in $CH_2Cl_2$, and (C) 100% $CH_2Cl_2$. Hplc analysis was carried out with a Waters model 6000A solvent delivery system provided with a model U-6k Waters injector and Kratos model SF 770 spectroflow monitor (measuring wavelength 240 nm). The hplc separation was performed on a Zorbax ODS C-18 column (4.6 mm × 250 mm) with the solvent 65% $KH_2PO_4$ buffer (pH = 6.4) in MeOH (hplc grade B&J) at a flow rate of 1 ml/min. Chromatograms were recorded with a Omni Scribe Houston instrument recorder with a chart speed of 0.5 cm/min. Reference calibration mixtures were prepared fresh every day, and reference calibration curves were obtained several times each day when analyzing unknown samples. The indole content of each plant extract could then be calculated from the ratio of the area of the known indole to the area of the unknown found at the appropriate retention time.

PLANT MATERIAL.—The various cruciferous vegetables that were studied are shown in Table 1. Collards (*B. oleracea*) were collected from a local North Carolina farm and also purchased in local markets. All others came from local markets. The noncruciferous vegetables analyzed were celery, spinach, carrot leaves and roots, tomato, lettuce, okra, eggplant, and endive.

EXTRACTION OF COLLARDS.—Collard leaves (40 kg) were extracted with 95% EtOH in three batches. In each batch a total of 13.3 kg of collards as macerated in a Waring blender and allowed to soak in EtOH overnight. The EtOH solution was drained, and fresh EtOH was added to the plant material and allowed to soak overnight. The combined EtOH extracts (ca. 32 liters) were concentrated in vacuo. The concentrated EtOH extracts were partitioned between $CHCl_3$ and $H_2O$, and the combined $CHCl_3$ phases were

TABLE 1. Major and Minor Constituents of Some Cruciferous Vegetables.

| Name of Plant | Major (mg/100 g fresh wt) | Minor (<0.1 mg/100 g fresh wt) | Trace or Absent |
|---|---|---|---|
| Collards | **4** (0.5–2.7), **2** (trace–1.1) | **5** | **1**[a], **6**[a] |
| Broccoli | **4** (0.2), **2** (0.2) | **5** | **1**[a], **6** |
| Brussels sprouts | **2** (0.9) | **4**, **5** | **1**[a], **6** |
| Cauliflower | **2** (1.2) | **4**, **5** | **1**[a], **6** |
| Turnip top | **2** (2.3) | **4** | **1**, **6** |
| Turnip root | — | **4** | **1**, **6** |
| Kale | **2** (3.0) | **4**, **5** | **1**[a], **6** |
| Chinese cabbage | **2** (1.8) | **4**, **5** | **1**, **6** |
| Cabbage | **2** (0.6) | **4** | **1**, **6** |
| Mustard greens | **2** (0.3) | — | — |

[a]Trace.

evaporated under reduced pressure. The remaining extract was vacuum-dried (wt ca. 50 g). Vacuum-dried $CHCl_3$ extract was dissolved in equal volumes of petroleum ether (bp 38.7°–56.5°) and MeOH-$H_2O$ (9:1) and partitioned between petroleum ether and MeOH/$H_2O$. Both the petroleum ether phase and the MeOH/$H_2O$ phase were evaporated to dryness under reduced pressure. The MeOH/$H_2O$ phase was the only fraction that proved active in the antimutagen assay. The total weight of this fraction from 40 kg of collard tops was ca. 43 g.

ISOLATION OF INDOLE-3-CARBOXALDEHYDE [**4**].—Flash chromatography grade Si gel (100 g, Baker) was packed into a column in $CHCl_3$-MeOH (98:2). The column was washed with the same solvent. The crude MeOH/$H_2O$ fraction (10 g) from collards was dissolved in the same solvent mixture and applied to the column. The elution of the column was started with the same solvent system using $N_2$ pressure. The solvent was changed stepwise to $CHCl_3$-MeOH (97:3 and 95:5). Tlc was performed on collected fractions. Combinations were made of like fractions according to tlc patterns. The $CHCl_3$-MeOH (98:2) fractions from two such chromatographies were combined (wt 2.1 g) and chromatographed on Florisil (200 g, 100–200 mesh) packed in $CH_2Cl_2$. The elution started with $CH_2Cl_2$ and 15-ml fractions were collected. The separation was monitored by tlc using solvent system B. MeOH was added to the eluent stepwise, increasing to 20% MeOH in the final eluent. Fractions with the same tlc profiles were pooled and the solvent evaporated to dryness in vacuo.

Fractions 8–50 obtained with 4%–8% MeOH in $CH_2Cl_2$ contained crystalline material. These fractions were combined, and solvent was evaporated. The residue was dissolved in a minimal amount of MeOH. After allowing the solution to stand overnight, crystals were removed by filtration and recrystallized twice from MeOH (10 mg). Hrms of this compound showed $[M]^+$ to be *m/z* 145.0527 (calcd for $C_9H_7NO$, 145.0528). It was identified as indole-3-carboxaldehyde by comparison of mp, tlc, $^1$H-nmr, $^{13}$C-nmr, and mass spectra with those of a synthetic reference compound.

ISOLATION OF INDOLE-3-ACETONITRILE [**2**].—A less polar fraction was obtained from the crude MeOH/$H_2O$ collard extract described above by elution with $CH_2Cl_2$-MeOH (99:1). This product was further purified by means of preparative tlc in solvent system C. This yielded a pure compound in trace amounts. Hrms of this compound showed $[M]^+$ to be 156.0685 (calcd for $C_{10}H_8N_2$, 156.0687). Some other fragments were observed at *m/z* 155 and 130. On co-tlc with an authentic sample of indole-3-acetonitrile the unknown compound showed identical $R_f$ values and color reaction with Ehrlich spray reagent. Identity was further confirmed by hplc analysis which gave the same retention time for isolated **2** as found for an authentic sample.

EXTRACTION OF VEGETABLES FOR HPLC ANALYSIS.—Plant material (200 g) was ground twice in a Waring blender with 750 ml of cold EtOH-$H_2O$ (50:50). The combined extracts were filtered and concentrated under reduced pressure until all the EtOH was removed. The remaining aqueous phase was then acidified to pH ca. 3 with HCl. The acidified aqueous phase was then extracted twice with 500-ml portions of $CH_2Cl_2$; the $CH_2Cl_2$ phases were combined and concentrated to 100 ml. A portion of the $CH_2Cl_2$ phase (10 ml) was dried under $N_2$. The dried sample was dissolved in 1.0 ml of MeOH, and the solution was passed through a Waters Sep-pak C-18 cartridge. The Sep-pak column was then washed with 1.0 ml of MeOH. The combined MeOH eluates were dried under $N_2$ and the completely dried sample dissolved in 0.5 ml of MeOH. A sample (2.0 μl) of this solution was injected on the hplc column. Concentrations of indole-3-carboxaldehyde [**4**] and indole-3-acetonitrile [**2**] in each extract were calculated from the area of the hplc peak of the unknown compared to a standard calibration curve prepared daily from a mixture of the corresponding pure indoles (Figure 1). Indole-3-carboxaldehyde and indole-3-acetonitrile were determined quantitatively. Minor or trace constituents such as indole-3-carboxylic acid [**5**] or indole-3-carbinol [**1**] were given only qualitative analysis.

COOKING.—A 200-g batch of vegetable was heated with $H_2O$ (200 ml) in a pressure cooker for 3 min, and a similar batch was heated for 30 min. $H_2O$ was drained, and an equal amount of EtOH was added. Total volume was adjusted to 750 ml with EtOH-$H_2O$ (50:50), and the cooked plant was ground in a Waring blender. Plant material was ground twice with 750 ml of EtOH-$H_2O$ (50:50). The extract was then filtered and concentrated until all the EtOH was removed. The aqueous residue was then treated the same way as uncooked plant material and an aliquot analyzed by hplc.

## RESULTS AND DISCUSSION

Several 3-substituted indoles were isolated from chromatographic fractions obtained from collard leaves (*B. oleracea* var. *ocephala*). Although the crude fractions were antimutagenic to the 2AA (9), the isolated indoles were inactive in this assay. Crystalline compounds isolated were indole-3-carboxaldehyde [**4**] and indole-3-acetonitrile [**2**]. Trace amounts of indole-3-carboxylic acid [**5**] were identified by tlc retention time

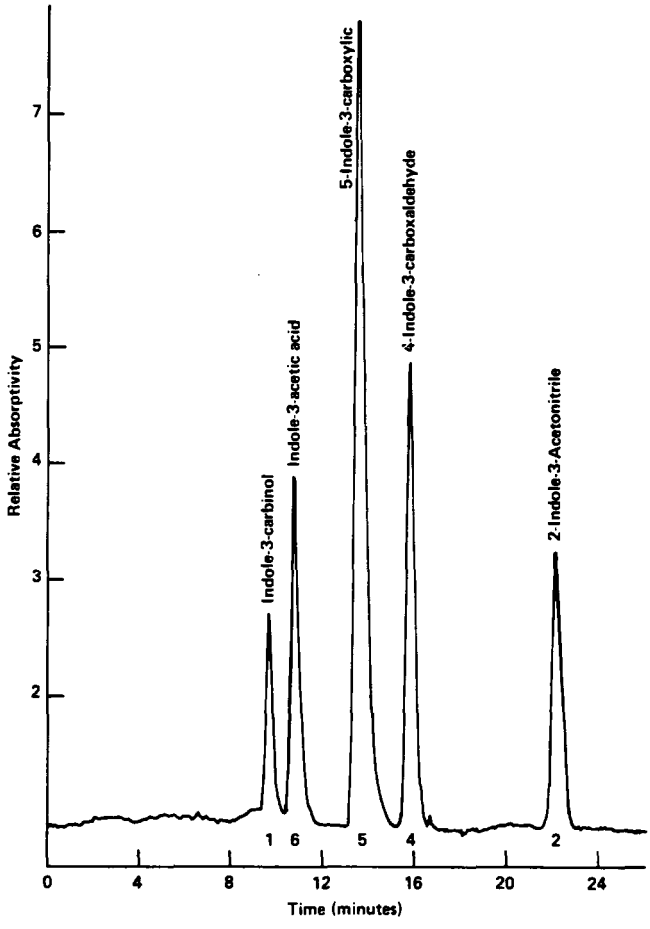



FIGURE 1. Hplc separation of 3-indole reference compounds **1**, **2**, **4**–**6**.

with known reference compounds. Indole-3-carbinol [**1**] was not found. The reversed-phase hplc procedure gave excellent separation of the indoles **1**, **2**, **4**, **5**, and **6**, both as mixtures of pure reference compounds (Figure 1) and in various plant extracts (Figures 2–4).

Recovery of the various indoles when added to plant extracts that do not contain indoles varied from 80% to 90%. Little or no interference from other plant constituents was noted for the nitrile, aldehyde, or carboxylic acid, which had the longest retention times. In most cases interference in the region of the 3-carbinol was minimal, but at times small impurities were noted just prior to the retention time of the 3-carbinol at the 240-nm wavelength selected. The absorptivity at 240 nm for the aldehyde and acids was about double that observed for the carbinol and nitrile. The use of the $KH_2PO_4$ buffer was required in order to obtain good peak shape.

The results of the analyses of cruciferous vegetables are shown in Table 1. Many noncruciferous plants were analyzed but did not contain indoles. As shown in Table 1, the 3-acetonitrile **2** was by far the most common and the major indole in most of the Cruciferae studied. Kale, turnip greens, and Chinese cabbage had the highest content, 2–3 mg/100 g fresh wt. With the exception of collard leaves, much lower concentrations of the aldehyde were noted. In the case of collards, samples freshly picked at a North Carolina farm contained almost 3 mg of the aldehyde **4** per 100 g of the sample

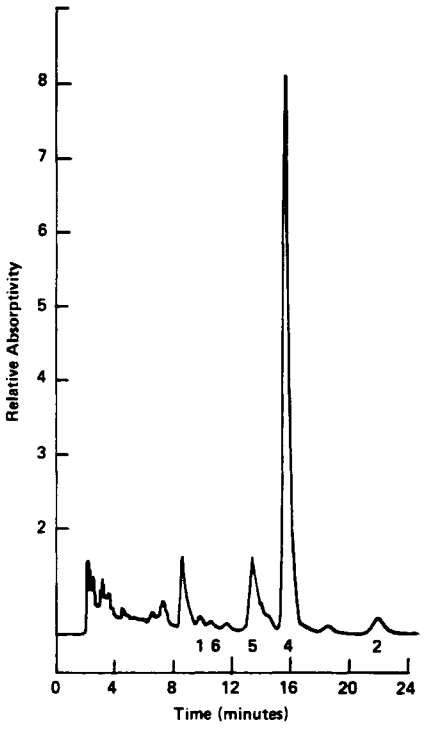



FIGURE 2. Hplc analysis of collards. Numbers correspond to reference compounds and are located at retention times noted for reference mixture obtained immediately following analysis of unknown.

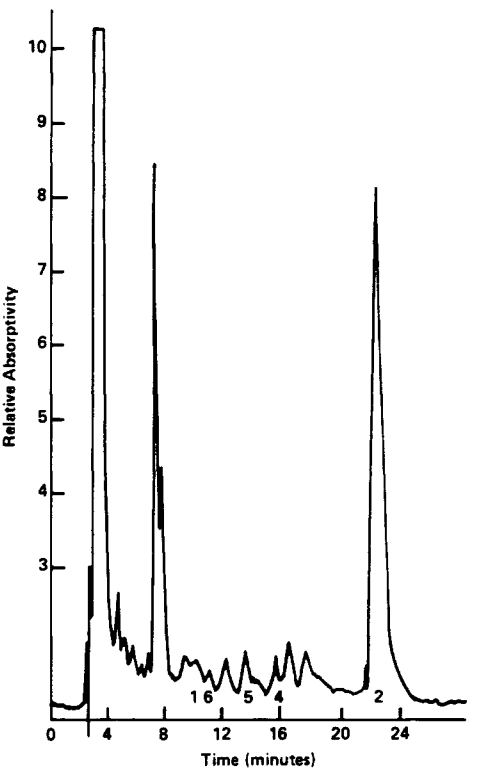



FIGURE 3. Hplc analysis of brussels sprouts. Numbers correspond to reference compounds and are located at retention times noted for reference mixture.

and only a small quantity of the nitrile **2**. However, in other instances collards purchased in markets contained major quantities of nitrile and lower concentrations of aldehyde. In no instance could the 3-carbinol be observed except as a somewhat questionable trace that, in our system, would be <0.01 mg/100 g.

According to Virtanen (12), the precursor of indoles found in *Brassica* is a sulfur-containing conjugate called glucobrassicin that enzymatically cleaves to the nitrile **2** under acidic conditions. The carbinol **1** is formed by another mechanism and, in any case, was not observed under our conditions. The origin of the aldehyde is less clear, but it could arise from an enzymatic oxidation (2,11).

In a few cases, collards and kale, the plant material after initial analysis was cooked in a pressure cooker for 3 min and 30 min. The results are shown in Table 2. The results obtained from two different collard and kale collections show in general an increase after 3 min, particularly of the nitrile **2**, which in one case increased greatly after 30 min heating. Virtanen states that boiling will release $SCN^-$ ion from glucobrassicin. It is conceivable that such a process is occurring during cooking. However, this complex issue was beyond the scope of our investigation. The significant point is that a major increment, particularly in content of **2**, occurs after cooking. Cooking had no effect on **1**, which was present only at trace levels.

The literature regarding the level of indole-3-carbinol in plants is confusing. We believe in some cases gross errors or inexactitudes exist. Classic studies by E.R.H. Jones and co-workers resulted in isolation and characterization from cabbage of the nitrile **2**,

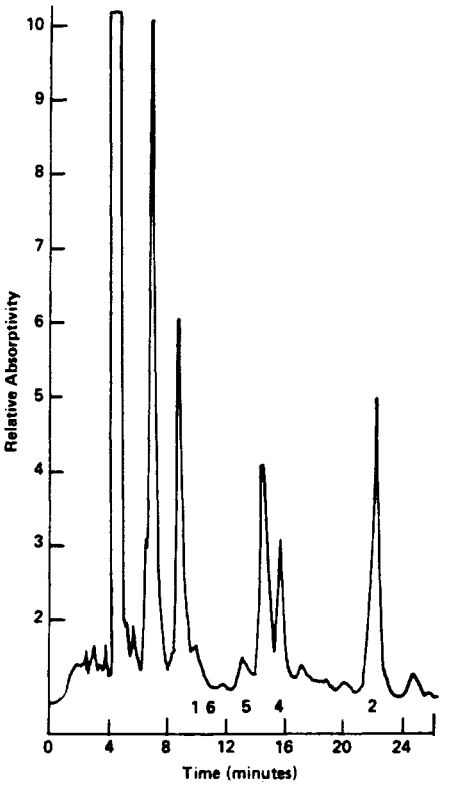



FIGURE 4. Hplc analysis of turnip tops. Numbers correspond to reference compounds and are located at retention times noted for reference mixture.

the aldehyde **4**, and the 3-carboxylic acid **5** by impeccable, classical procedures (10,11). The nitrile is a major product of the enzymatic decomposition of indolylmethylglucosinolate (12); the aldehyde may occur as a product of the enzymatic α oxidation of indole-3-acetic acid (11). The carbinol **1** was not found by Jones and co-workers (10,11). Wattenberg *et al.* (2) isolated from Brussels sprouts **2** and **4** as crystalline, well-characterized compounds in the ratio of 5:1, respectively. The carbinol **1** was not isolated, but its presence was indicated by tlc techniques. Thus, it can be assumed that **1** was present only in trace amounts in the Brussels sprouts, the nitrile being the major indole. Our hplc analyses of Brussels sprouts confirm this, the ratio of **2** to **4** being about 15:1 (Figure 3). The carbinol **1**, if indeed present, was noted in only trace amounts.

TABLE 2. Effect of Pressure Cooking on Indole Content[a] of Collards and Kale.

| Plant | Uncooked | | Cooked 3 min | | Cooked 30 min | |
|---|---|---|---|---|---|---|
| | **4** | **2** | **4** | **2** | **4** | **2** |
| Collards . . . . . | 0.5 | 1.0 | 0.9 | 3.7 | 0.2 | 3.0 |
| Collards . . . . . | 0.4 | 0.4 | 0.6 | 2.3 | 1.1 | 1.1 |
| Kale . . . . . | — | 2.6 | — | 2.6 | — | 0.7 |
| Kale . . . . . | — | 1.2 | — | 2.9 | — | 7.7 |

[a]In mg/100 g fresh wt.

Because the carbinol is much more active than the other indoles, as an AHH inducer or in the inhibition of induced carcinogenesis, it has been the subject of many studies (2–8). The fact that it is probably a trace constituent was not emphasized or possibly was overlooked by Wattenberg and co-workers (2,3,13), although this is clearly evident by inspection of their experimental data that state that the nitrile **2** and the aldehyde **4** were isolated, yields determined, and structures obtained by standard procedures, whereas the only evidence for the presence of the carbinol **1** was a comparison of the tlc characteristics of **1** with a reference compound (2). Other workers have incorrectly stated that the carbinol either was a major indole in cruciferous plants (5,6), or was present in many cruciferous plants (8), or was isolated from Brussels sprouts (2,7).

The trace presence or non-existence of the indole-3-carbinol in cruciferous plants makes it questionable that it can be the effective plant constituent in the reported inhibition of chemical carcinogenesis (1,2,13).

Moreover, recent studies at our laboratory[1] show that after oral administration of the aldehyde, nitrile, and carbinol to rats, all are converted to the 3-carboxylic acid almost immediately after oral administration. Thus, after 15 min and at all times thereafter, no aldehyde and only the 3-carboxylic acid could be found in rat plasma. After 30 min and at all times thereafter, separate studies with the carbinol, nitrile, and aldehyde showed that no starting compound and only the 3-carboxylic acid or its conjugate were found in urine. Our analytical and metabolic studies indicate that the role of the indole-3-carbinol may have been overestimated. Because of its trace occurrence in the Cruciferae and because of its rapid metabolic conversion to the corresponding 3-carboxylic acid, we believe that the role of this indole may be questionable in the inhibition of induced carcinogenesis when cruciferous plants are ingested by animals. Similar statements are probably correct in regard to the AHH activity. An interesting but unresolved question is whether the effects noted when the pure indole-3-carbinol [**1**] is administered to animals are due to a metabolic product.

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