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Ascorbigen as a precursor of 5,11-dihydroindolo[3,2-*b*]carbazole

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The formation of 5,11-dihydroindolo[3,2-*b*]carbazole, a polyaromatic hydrocarbon responsiveness-receptor agonist as well as an anti-initiator and promoter of carcinogenesis, from ascorbigen under conditions of chemical synthesis and during incubation with gastric juice at physiological conditions, is demonstrated. Ascorbigen, rather than indole-3-carbinol, is the most important indole derivative for the enzyme-inducing effects of dietary cruciferous vegetables. The concentration of ascorbigen in these vegetables is at least five times (in molar ratio) higher than that of indole-3-carbinol.

INTRODUCTION

A number of studies have demonstrated a decreased risk of cancer in subjects with a high consumption of cruciferous vegetables. Certain ingredients of a cruciferous diet modify the metabolism of carcinogens by induction of enzymes involved in xenobiotic metabolism (Wattenberg, 1971; McDanell *et al.*, 1989; Prochaska *et al.*, 1992). The active compounds in these vegetables were identified as transformation products of the alkaloid glucobrassicin. Enzymic breakdown of glucobrassicin (**1**) under the action of myrosinase affords skatylisothiocyanate (**2**). Attempts to isolate or trap **2** have proved to be unsuccessful (Hanley *et al.*, 1990). Compound **2** is supposed to be readily hydrolysed to indole-3-carbinol (**3**) (IC). IC (**3**) non-enzymatically interacts with the L-ascorbic acid to produce ascorbigen (**4**) (AG) (Scheme 1) (Gmelin & Virtanen, 1961; Kutacek *et al.*, 1969). The role of L-ascorbic acid in these processes is very important as the activity of myrosinase is enhanced at concentrations of L-ascorbic acid similar to those which exist in cruciferous vegetables (1·4–7·1 mM) (West *et al.*, 1977; Otte, 1991). IC (**3**) formed is then trapped by L-ascorbic acid.

The possibility of direct interaction of **2** or some other intermediate of **1** enzymatic transformation with L-ascorbate, with the release of SCN^-, remains to be elucidated.

When glucobrassicin is transformed by myrosinase in vitro in the absence of L-ascorbic acid IC (**3**) is the main product. This led to the incorrect conclusion that IC is the major indole-derived product in cruciferous vegetables. This was a result of analyses of cruciferous extracts using unsuitable solvents (CH_2Cl_2, acetonitrile), in which ascorbigen is poorly soluble (Bradfield & Bjeldanes, 1987; Prochaska *et al.*, 1992).

Separations using an HPLC method have demonstrated that IC is absent in cruciferous extracts or that its concentration is at least five times (in molar ratio) lower than that of AG (**4**) (McDanell *et al.*, 1987; Aleksandrova *et al.*, 1992). Unfortunately these data have been neglected by most investigators.

In a series of publications IC was considered to be the most potent inductor of enzymes involved in carcinogen metabolism among all products of glucobrassicin transformation. As already shown (McDanell *et al.*, 1988), in many cases the effects of IC were observed at doses which were considerably higher than those obtainable from brassica-containing diets (Loub *et al.*, 1975; Bailey *et al.*, 1987; Michnovicz & Bradlow, 1990; Morse *et al.*, 1990). In these experiments biological activities of IC and AG as well as of some other indole derivatives were compared at equal or at similar concentrations while in reality AG concentrations are higher than concentrations of any other indole-derived compounds in cabbages.

Thus the problem of search for the compound(s) responsible for anticarcinogenic properties of cruciferous diets was diverted to the inadequate problem of study of chemical and biochemical properties of IC.

IC is unstable in the acidic environment of the stomach and yields a plethora of products among which di(indol-3-yl)methane (**5**), the cyclotrimer (5,6,11,12,17,18-hexahydrocyclonona-(1,2-*b*:4,5-*b*':7,8-*b*"]tri-indole) (**6**),

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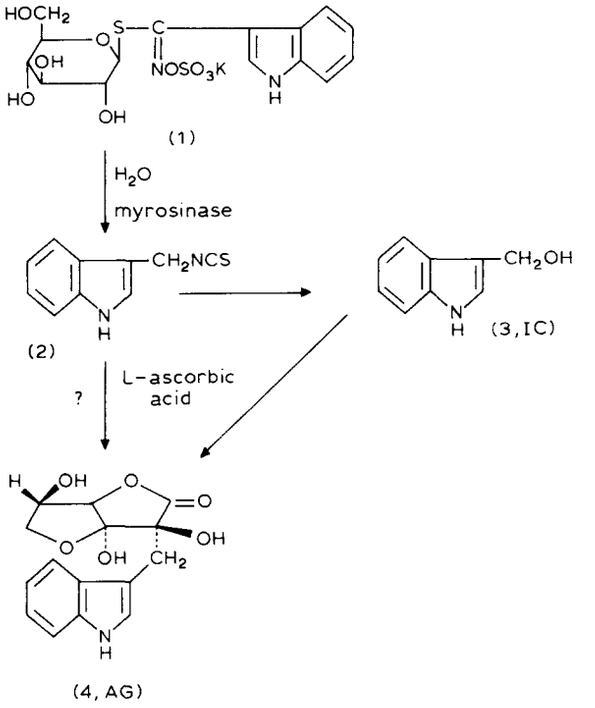


Scheme 1. Enzymic breakdown of glucobrassicin: (**1**) glucobrassicin; (**2**) skatylisothiocyanate; (**3**) indole-3-carbinol; (**4**, AG) ascorbigen.

2,3-bisskatylindole (**7**) and 5,11-dihydroindolo[3,2-*b*]carbazole (**8**) (ICZ) are the most important (De Kruif *et al.*, 1991; Grose & Bjeldanes, 1992). ICZ appears to be responsible for the enzyme-inducing effects of IC (Scheme 2). Its binding affinity to aromatic hydrocarbon responsiveness-receptor is only a factor of $3{\cdot}7 \times 10^{-2}$ lower than that of the highly toxic contaminant and cancer promoter 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (Gillner *et al.*, 1985; Bjeldanes *et al.*, 1991).

AG also gives rise to a number of products in acidic environments. Recently we have shown that it releases L-ascorbic acid; the (indol-3-yl)methyl (skatyl) moiety is trapped by a secondary molecule of ascorbigen to give 2'-skatylascorbigen (ascorbigen 'dimer') (**9**) and some other ascorbigen-derived products, including ascorbigen 'trimer' (**10**) (Scheme 3) (Korolev *et al.*, 1991; Preobrazhenskaya *et al.*, 1991). By the interaction of IC and L-ascorbic acid at pH 4–5, additionally to synthetic ascorbigen, identical to natural product **4**, small amounts of so-called Ascorbigen B were isolated, which was supposed to be the 2-epimer of **4** (Kiss & Neukom, 1966). We have demonstrated that Ascorbigen B is a mixture of compounds, where natural ascorbigen 'dimer' (**9**) was the major component, and **4** and its 'trimer' (**10**) were the minor components (Korolev *et al.*, 1991; Aleksandrova *et al.*, 1992).

A goal of the present work was to determine if ICZ can be formed from ascorbigen under acidic conditions (those under chemical synthesis as well as those under physiological conditions). The possibility of 5,11-dimethylinidolo[3,2-*b*] (**11**) formation from 1'-methylascorbigen (**12**) and from the 1'-methylascorbigen 'dimer' (**13**) was similarly studied (Scheme 3).

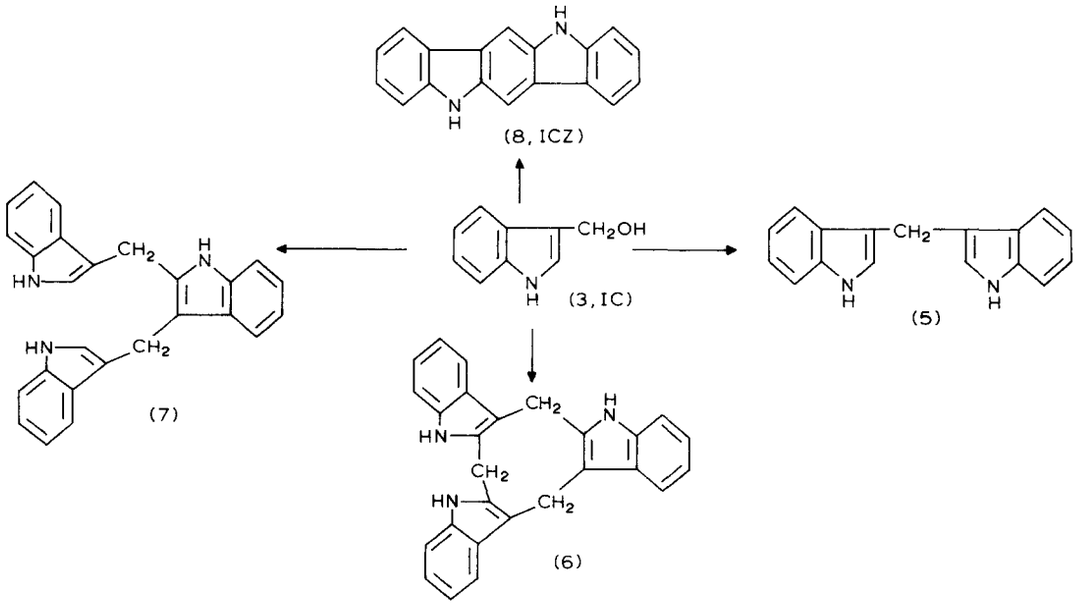
MATERIALS AND METHODS

Chemicals

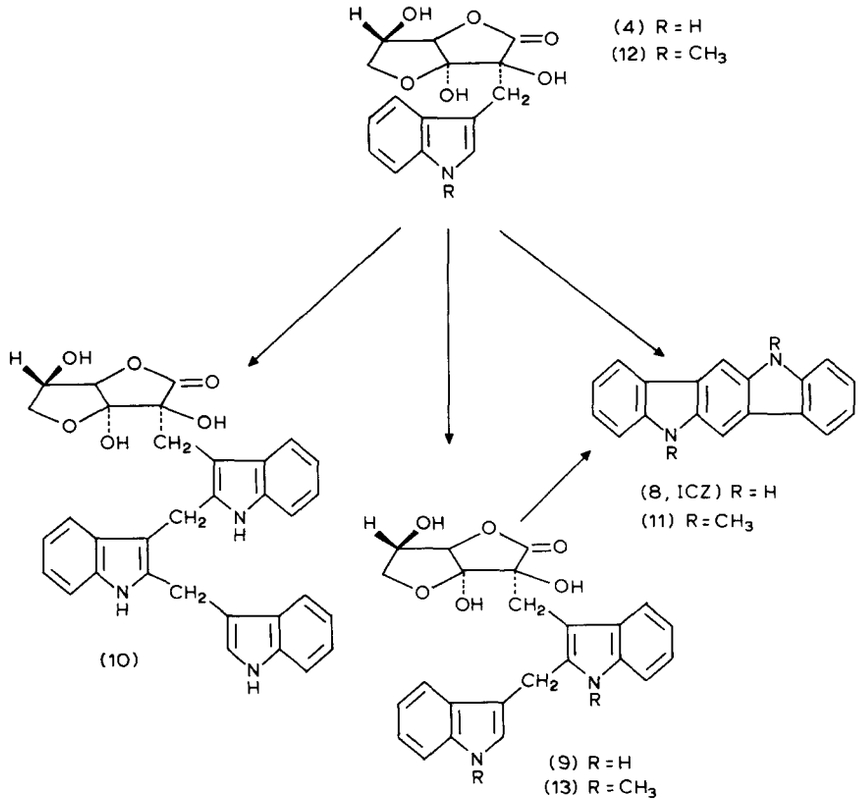
Silica gel 60 HF_{254} was used for TLC. HPLC grade acetonitrile and ammonium acetate were purchased from Merck (Darmstadt, Germany). Deionized water was obtained by the use of the Millipore Q system.

Instruments

HPLC analyses were performed on a Spectra-Physics Model 8000 HPLC (Spectra-Physics instruments, USA) fitted with a Zorbax 4·6 mm × 250 mm ODS column



Scheme 2. Transformations of indole-3-carbinol (**3**, IC) in acidic media: (**5**) di(indol-3-yl)methane; (**6**) cyclotrimer; (**7**) 2,3-bisskatylindole; (**8**, ICZ) 5,11-dihydroindolo-[3,2-*b*]carbazole.



Scheme 3. Transformations of ascorbigen (**4**, AG) or 1'-methylascorbigen (**12**) in acidic media: (**8**, ICZ) 5,11-dihydroindolo[3,2-*b*]-carbazole; (**9**) ascorbigen 'dimer'; (**10**) ascorbigen 'trimer'; (**11**) 5,11-dimethylindolo[3,2-*b*]carbazole; (**12**) 1'-methylascorbigen; (**13**) 1'-methylascorbigen 'dimer'.

(particle size 5 μm, Zorbax, DuPont, UK). Compounds were eluted in a reversed-phase gradient system with a percentage of acetonitrile in 0·1 M ammonium acetate buffer (pH 5·7) increasing from 40 to 100% over a period of 30 min. The flow rate was 1 ml/min, oven temperature 35°C. Compounds were detected by their absorption at 280 nm (SP8490 UV detector). HPLC profiles were obtained by injection of 20 μl at 0·136 AUFS.

^{1}H-NMR spectra were recorded on a Varian VXR-400 spectrometer with DMSO-d_6 as internal standard (δ 2·49 ppm).

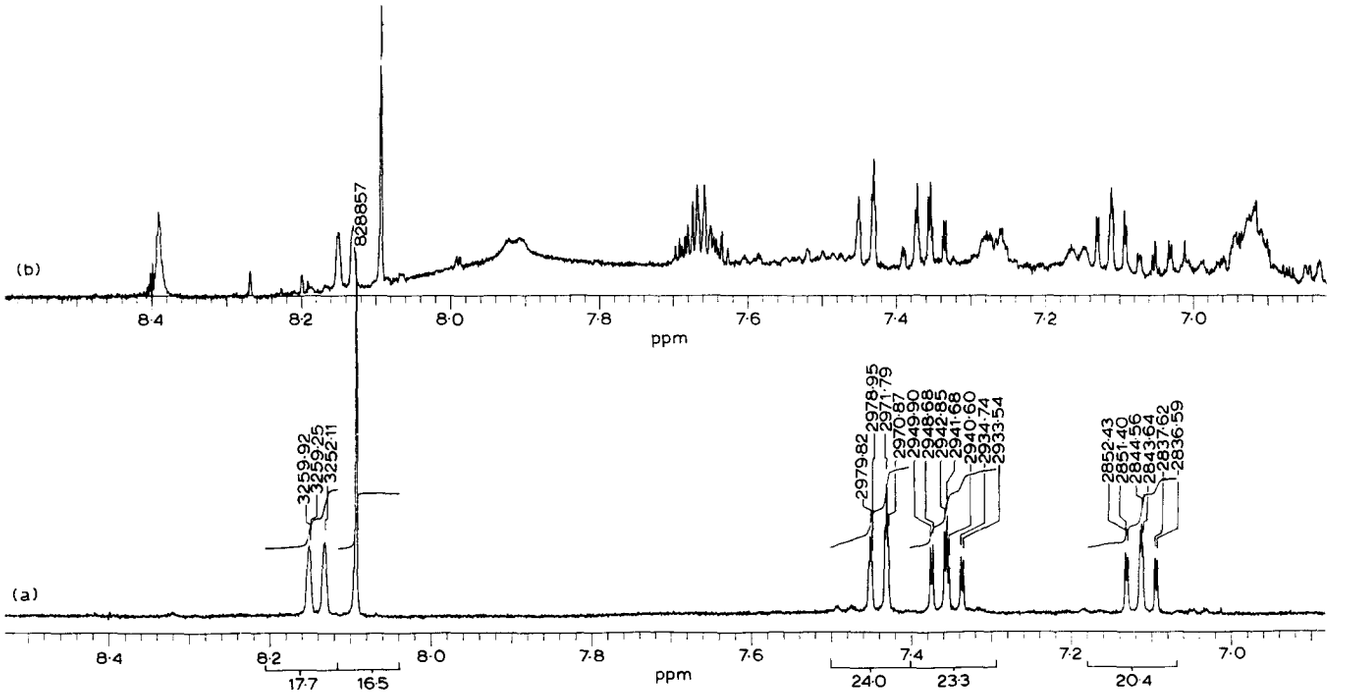


Fig. 1. (a) ^{1}H-NMR spectrum of authentic 5,11-dihydro-indolo[3,2-*b*]carbazole. (b) ^{1}H-NMR spectrum of the compounds isolated after chemical transformation of ascorbigen in MeOH–1 N HCl mixture.

Synthesis of indolocarbazoles from ascorbigens

5,11-Dihydroindolo[3,2-b]carbazole (8)
Ascorbigen (**4**) (1·0 g) was dissolved in 60 ml of MeOH–1 N HCl mixture (1 : 1) and the stirred solution was heated at 50°C for 5 h. The reaction mixture was extracted with ether, dried over Na_2SO_4 and evaporated *in vacuo*. The residue was dissolved in methanol and purified by TLC on silica gel using toluene as eluent. The compounds with R_f 0·65 were collected, eluted with methanol to give a mixture of compounds (0·0083 g), the main component being 5,11-dihydroindolo[3,2-*b*]-carbazole (< 1%), which was demonstrated by ^{1}H-NMR (see Fig. 1) and HPLC methods (HPLC R_t 20·1 min) as well as by comparison with an authentic sample).

^{1}H-NMR [in DMSO-d_6, δ ppm (J Hz)]: 8·14 (2H, d, J 8·0, $H_{1,7}$); 8·09 (2H, s, $H_{6,12}$); 7·44 (2H, d, J 8·2, $H_{4,10}$); 7·36 (2H, ddd, J 8·2, J 8·1, J 1·1, $H_{3,9}$); 7·11 (2H, ddd, J 7·9, J 7·9, J 1·0, $H_{2,8}$).

5,11-Dimethylindolo[3,2-b]carbazole (11)
Method A. 1'-Methylascorbigen (**12**) (0·5 g) was dissolved in 40 ml of MeOH–H_2O (1 : 1) acidified with HCl to pH 1·0 and heated at 50°C for 5 h. The red precipitate formed was collected and dissolved in ethyl acetate. The filtrate was extracted with ethyl acetate; combined extracts were washed with water, dried over $NaSO_4$ and evaporated *in vacuo*. The residue was dissolved in $CHCl_3$ and purified by TLC on silica gel in toluene. The substance with R_f 0·89 (0·0066 g, 1·5%) was eluted by methanol. ^{1}H-NMR, IR and UV spectra showed that the compound obtained was identical with an authentic sample of 5,11-dimethylindolo[3,2-*b*]carbazole (HPLC R_t 27·0 min).

^{1}H-NMR [in DMSO-d_6, δ ppm (J Hz)]: 8·31 (2H, s, $H_{6,12}$); 8·26 (2H, d, J 8·0, $H_{1,7}$); 7·56 (2H, d, J 8·2, $H_{4,10}$); 7·46 (2H, ddd, J 8·2, J 8·2, J 1·2, $H_{3,9}$); 7·19 (2H, ddd, J 8·0, J 8·0, J 1·0, $H_{2,8}$); 3·96 (6H, s, $Me_{5,11}$).

Method B. 1'-Methylascorbigen dimer (**13**) (0·5 g) produced 0·0085 g (2·8%) of **11** by the method described for **12**.

Incubation of ascorbigen or indole-3-carbinol with gastric juice

Ascorbigen (305 mg, 1 mM) or indole-3-carbinol (146 mg, 1 mM) was dissolved in 45 ml of gastric juice (pH 0) and incubated at 36–37°C for 5 h. The solution was extracted with ether (25 ml × 2): ether was evaporated and dried *in vacuo*. The residues were dissolved in mobile phase (0·1 mg/ml in both cases), then they were filtered and analyzed by HPLC.

RESULTS AND DISCUSSION

Initially we studied the possibility of ICZ formation under conditions of chemical synthesis. Earlier it had been demonstrated that the formation of condensation products from indole-3-carbinol under acidic conditions is facilitated by high methanol concentrations in the reaction medium (Amat-Guerri *et al.*, 1984). The presence of methanol was also crucial for production of ICZ from AG in chemical conditions. As a sample for comparison we used ICZ obtained by the method described (Bergman, 1970). Interaction of AG with acids results in a very complex mixture. ICZ could not be isolated in pure form: however, appropriate fractions isolated by TLC contained ICZ as a main component as demonstrated by ^{1}H-NMR (see Fig. 1). HPLC analysis also indicated ICZ.

Similarly, incubation of 1'-methylascorbigen (**12**) in an acidic water–methanol mixture produced 5,11-dimethylindolo[3,2-*b*]-carbazole (**11**) in 1·5% yield. An authentic sample of **11** was synthesized as described by Hünig and Steinmetzer (1976). The 1'-methylascorbigen 'dimer' (**13**) similarly gave **11** in 2·8% yield, indicating that dimers **9** or **13** might be intermediate in the transformations of ascorbigen derivatives into the indolocarbazoles **8** (ICZ) or **11**.

Experimental observations were obtained supporting the idea that ascorbigen is converted to ICZ under the low-pH conditions in the stomach. Extracts of gastric juice incubated with IC or AG were investigated by HPLC using the gradient solvent programme. Identification of components was based on retention times which remained constant during the course of the studies described here. Figure 2 demonstrates HPLC of extracts of gastric juice incubated with AG (a) or IC (b) in comparison with a blank sample (c). AG, as well as IC, both gave rise to very complex mixtures containing ICZ. Chromatographic analysis showed that spiking of the reaction mixtures with authentic ICZ appropriately increased the areas of the presented ICZ peaks, and no additional peaks were produced (data not shown). The concentration of ICZ in gastric juice after incubation with AG was ~20 times higher than after incubation with IC. Comparison of chromatograms of mixtures obtained from IC (Fig. 2(b)) and from AG (Fig. 2(a)) demonstrates that transformation of AG in acidic media produces more polar compounds, supposedly containing an L-ascorbic acid moiety. TLC demonstrates that this mixture contains ascorbigen 'dimer' (**9**) and ascorbigen 'trimer' (**10**) (data not shown). It suggests that the mixture obtained after incubation of IC in gastric juice contained components similar to those which were identified in the mixture obtained after incubation of IC in acids (Grose & Bjeldanes, 1992).

As it is already known that IC generates ICZ in vivo in intestine of rats, our data allow us to conclude that AG generates ICZ in the stomach. Hence AG is a main source of anti-initiator and a promoter of carcinogenesis by ICZ in cruciferous vegetables.

Anticarcinogenic properties of AG may depend not only on induction of enzymes involved in the metabolism of carcinogens. The *N*-methylated analogue of AG—1'-methylascorbigen (**12**)—is a highly active immunomodulator which enhances resistance of animals to bacterial and viral infections (Efimov, 1989; Malkova *et al.*, 1991). Some other 2-C-substituted L-ascorbic acid derivatives with structural features in common

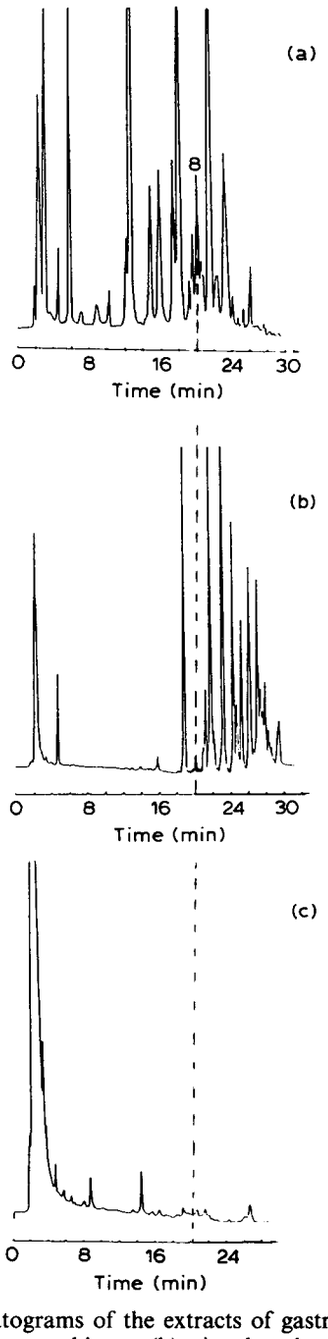


Fig. 2. Chromatograms of the extracts of gastric juice: (a)—incubated with ascorbigen; (b)—incubated with indole-3-carbinol; (c)—blank sample.

with ascorbigen have also been identified as immuno-modulators (Veltri *et al.*, 1986). The immuno-modulatory activity of natural ascorbigen remains to be investigated. It may be an additional factor of importance for the anticarcinogenic properties of a cruciferous diet.

This study may show new approaches to the understanding of the anticarcinogenic properties of a cruciferous diet on a molecular level.

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