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Received for review November 28, 1988. Accepted April 25, 1989. Published with the approval of the Oregon State University Experiment Station as Technical Paper No. 8708. This study was supported in part by Public Health Service Research Grant DK 38306 from the National Institute of Diabetes and Digestive and Kidney Diseases.

Isolation of Compounds with Antimutagenic Activity from Savoy Chieftain Cabbage

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Dried leaves of Savoy Chieftain cabbage (*Brassica oleracea*) were extracted with methanol, methylene chloride, or petroleum ether. Soluble extracts were fractionated by flash chromatography on silica gel columns. Nonacosane, 15-nonacosanone, pheophytin *a*, and β-sitosterol were isolated and examined for their ability to inhibit the mutagenicity of *N*-methyl-*N*-nitrosourea (MNU) and 2-aminoanthracene (2-AA) in the Ames bacterial and V79 cell mammalian mutagenicity assays. In the Ames assay, nonacosane and pheophytin were without inhibitory action against either 2-AA- or MNU-induced mutagenesis. 15-Nonacosanone was more effective than β-sitosterol. In the V79 assay, all four compounds were active against the mutagenicity of 2-AA but only nonacosane, 15-nonacosanone, and β-sitosterol were active against that of MNU. Other crude extracts were identified with antimutagenic activity, but their compositions were not determined.

Epidemiological data have shown an inverse relationship between vegetable consumption and the relative risk of developing colon and stomach cancers (Graham et al., 1972; Modan et al., 1975). A lower incidence of stomach cancer occurred in nonsmokers who consistently ate vegetables than in those who did not (Hirayama, 1977). An inverse relationship between the consumption of cruciferous vegetables (e.g., cabbage) and the incidence of colon (Graham et al., 1978), stomach (Haenszel et al., 1976), and breast and prostatic (Phillips, 1975) cancers has been shown.

Laboratory studies have shown that vegetables or their extracts can inhibit carcinogenesis and interfere with the metabolism and mutagenicity of carcinogens (Birt and Bresnick, 1988). Addition of naturally occurring indoles to the diet reduced the incidences of 7,12-dimethylbenz[*a*]anthracene (DMBA) induced mammary and 3,4-benzo[*a*]pyrene (BP) induced gastric tumors by at least 55% (Wattenberg and Loub, 1978). Cinnamic acid derivatives (Wattenberg, 1983) and flavonoid-like compounds (Sparnins et al., 1982) reduced the incidence of chemically induced tumors. Many naturally occurring compounds act as antioxidants (Sparnins et al., 1982) or stimulate glutathione (GSH) synthesis or the levels of those compounds involved in maintaining GSH levels. A positive effect on these processes would facilitate the formation of GSH derivatives of the electrophiles of chemical carcinogens, representing a major detoxification pathway. Coffee bean extracts induced GSH *S*-transferase activity in vivo (Lam et al., 1982). A diet supplemented with broccoli stimulated GSH *S*-transferase and arylhydrocarbon hydroxylase (AHH) activities in rat liver and significantly altered the profile of BP metabolites (Aspry and Bjeldanes, 1983). Diets containing cabbage, Brussels sprouts, or alfalfa similarly affected BP metabolism in mouse liver (Hendrich and Bjeldanes, 1983). AHH activity was stimulated by plant phenols, leading to changes in BP metabolism by epidermal microsomal preparations (Das et al., 1987).

The mutagenicities of BP and 3-methylcholanthrene (3-MC) (Lai et al., 1980), DMBA, MNU, BP, and 4-nitroquinoline oxide (Kimm et al., 1982), and BP and a cigarette smoke condensate (Terwel et al., 1985) were reduced by vegetable extracts. The reduction correlated with the chlorophyll content of the extracts. An antimutagenic component of these extracts was identified as chlorophyllin (Lai et al., 1980; Kimm et al., 1982; Ong et al., 1986).

In the present study, dried leaves of Savoy Chieftain cabbage were extracted with organic solvents. Each extract was tested for antimutagenic activity in a mammalian mutagenicity (Jenssen, 1984) system using 2-aminoanthracene (2-AA) (indirect acting) and MNU (direct acting) as mutagens.

MATERIALS AND METHODS

Animals. Eight-week-old male Syrian golden hamsters [Unei(SYR)] from the Eppley Institute colony were used as a source of hepatocytes in the V79 assay.

Isolation of Extracts. Savoy Chieftain cabbage (*Brassica oleracea*), grown by the Department of Horticulture, University of Nebraska, was dried by either (i) air-drying individual leaves at 4 °C, (ii) forced air-drying of individual leaves at 40 °C, or (iii) freeze-drying shredded cabbage. The type of drying did not affect the nature of the extracts. The harvested material was about 7% of the wet weight. Dried cabbage was stored at −20 °C until extracted. Of the dried cabbage 30–50 g was exhaustively extracted with petroleum ether, methylene chloride, or methanol in a Soxhlet, yielding 1.6%, 1.0%, and 7.6% of the starting material, respectively. These extracts were subjected to further organic extractions, typical examples of which are described below.

(*a*) *Petroleum Ether Extracts.* The dried petroleum ether extract was dissolved in warm acetone and the resultant mixture cooled and filtered to remove some of the lipids. Acetone was

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0021-8561/89/1437-1363$01.50/0

removed by evaporation, yielding a dark green oil that was flash chromatographed on a silica column, eluted with hexane, hexane–ethyl acetate (9:1, 4:1, and 7:3, v/v) and ethyl acetate. Fractions of similar composition (monitored by thin-layer chromatography (TLC)) were combined and tested for antimutagenic activity. Three groups of combined fractions were active, and their compositions were determined. The first group was eluted with hexane and purified by recrystallization from hexane to yield nonacosane: mp 63.5–65.5 °C (lit. mp 62.7–62.8 °C; Channon and Chibnall, 1929); ^{1}H NMR ($CDCl_3$) δ 1.2 (t, 54 H), 0.82 (t, 6 H); mass spectrum, m/e (relative intensity) 408.4695 (0.23), 337.3821 (0.30), 309.3515 (0.42), 225.2576 (0.78), 71.0864 (62.44), 57.0722 (100.00); m/e calculated for $C_{29}H_{60}$, 408.4695. The second group was eluted with hexane–ethyl acetate (4:1, v/v) and yielded 15-nonacosanone. On recrystallization from hexane–ethyl acetate, it yielded the following data: mp 80–81 °C (lit. mp 80.5–81.0 °C; Channon and Chibnall, 1929); ^{1}H NMR ($CDCl_3$) δ 2.39 (t, 4 H), 1.57 (m, 4 H), 1.29 (s, 44 H), 0.89 (t, 6 H); ^{13}C NMR ($CDCl_3$) 211.52, 42.87, 31.96, 29.70, 29.67, 29.53, 29.47, 29.40, 29.36, 23.99, 22.72, 19.32, 14.11 ppm; mass spectrum, m/e (relative intensity) 422.4505 (2.7), 253.2603 (14.8), 241.2520 (55.0), 240.2461 (28.5), 225.2214 (100); m/e calculated for $C_{29}H_{58}O$, 422.4522. The third group was eluted with hexane–ethyl acetate (4:1, v/v). The green color was removed with acetone. The acetone-insoluble residue yielded β-sitosterol (23,25-dihydrostigmasterol): mp 139–142 °C (lit. mp 137–139 °C; Georg, 1954); IR (CH_2Cl_2) ν_{max} 3610, 1610 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 5.31 (d, 1 H), 3.48 (m, 1 H), 2.24 (m, 2 H), 2.1–0.5 (m, 46 H), with s at 0.96, 0.89, 0.86, 0.80, 0.78, 0.77, 0.75, 0.63; ^{13}C NMR ($CDCl_3$) 11.8, 12.0, 18.8, 19.0, 19.4, 19.9, 21.1, 23.0, 24.3, 26.1, 28.2, 29.1, 31.6, 31.9, 33.9, 36.1, 36.5, 37.2, 39.8, 42.2, 42.3, 45.8, 50.1, 56.7, 71.8, 121.7, 140.7 ppm; mass spectrum, m/e (relative intensity) 414.3868 (31), 400.3684 (18), 399.3625 (16), 936.3574 (20), 385.3468 (7), 381.3517 (16), 367.3362 (8), 329.3203 (19), 315.3046 (9), 303.3053 (29), 273.2220 (17), 255.2111 (27), 231.1745 (21), 213.1645 (41); m/e calculated for $C_{29}H_{50}O$, 414.3890. Pheophytin a (3,7,11,15-tetramethyl-1,2-hexadecenyl ester of 3-phorbinepropanoic acid) was isolated from these extracts but was more readily isolated from the initial methylene chloride extract.

(*b*) *Methylene Chloride Extracts.* The methylene chloride extract was flash chromatographed on silica gel, eluted with hexane–ethyl acetate (7:3, 5:3, and 1:1, v/v), ethyl acetate, ethyl acetate–methanol (9:1, 4:1, and 2:3, v/v), and methanol. Fractions of similar composition, as determined by TLC, were combined and tested for antimutagenic activity. Four active fractions, all eluted with hexane–ethyl acetate (7:3, v/v), comprised pheophytin a or a mixture of predominantly pheophytin a and pheophytin a'. A fifth active fraction was not identified. Pheophytin a, purified by preparative TLC, contained a small trace of pheophytin a' and was characterized as follows: ^{1}H NMR (DTHF) δ 9.28 (s), 9.07 (s), 8.67 (s), 3.52 (s), 3.27 (s), 2.92 (s), 2.9–0.8 (m); ^{13}C NMR (DTHF) 182.17, 172.88, 172.79, 169.99, 162.32, 155.77, 151.64, 150.08, 145.50, 142.40, 142.26, 138.73, 136.78, 136.58, 136.44, 132.25, 130.33, 130.03, 129.07, 122.29, 119.67, 107.02, 104.51, 97.79, 93.88, 65.51, 61.48, 52.44, 52.37, 50.87, 40.52, 38.27, 38.22, 38.15, 37.50, 33.67, 33.54, 31.76, 30.58, 28.81, 26.06, 25.96, 23.30, 22.99, 20.05, 20.00, 19.71, 17.47, 16.24, 11.95, 11.67, 11.94 ppm; mass spectrum, m/e (FAB) 871 (35), 691 (10), 593 (20), 533 (12), 397 (100); IR, ν_{max} (α) 406 (29), 468 (6.4), 504 (12.2), 532 (10.4), 560 (4.8), 610 (8.5).

Thin-Layer Chromatography. Thin-layer chromatography of isolated fractions was performed on silica plates (Altech, HLF) eluted with ethyl acetate–hexane. The properties of the eluates was consistent with the eluting solution used to obtain the particular fractions from the flash chromatography columns. Plates were stained with aniline (3 g) in ethanol (100 mL) and concentrated sulfuric acid (3 mL), blotted, and heated at 150 °C for 1 min to visualize the spots.

Mutagenicity Assays. *Bacterial Assay.* The modification (Yahagi et al., 1977) of the procedure of Marron and Ames (1983) was used with strains TA98 and TA100 of *Salmonella typhimurium*. A hepatic S9 fraction from Arochlor 1254 treated male Sprague–Dawley rats was used as the activating system with 2-AA as the indirect-acting mutagen. Rats were given one injection of Arochlor 1254 (500 mg/kg, i.p.) in corn oil 5 days before isolation of the liver S9 fraction. Test compounds were added according to the procedure of Birt et al. (1986). The mutagens used were MNU and 2-AA, 99.2 and 2.5 μg/plate, respectively. The extracts were added at 150, 300, and 600 μg/plate before the mutagens.

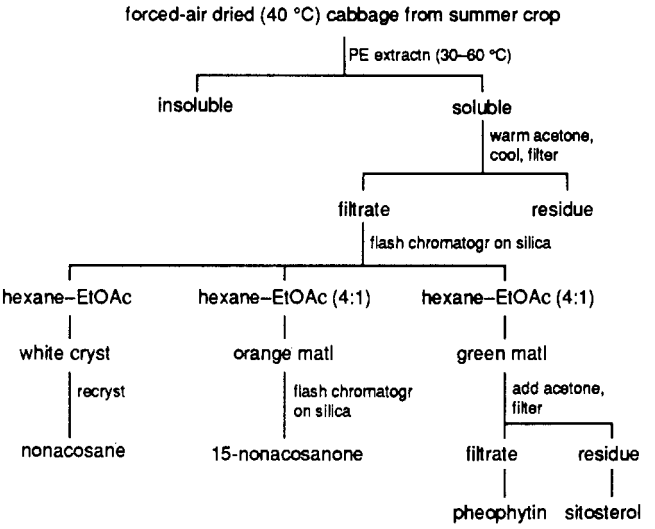

Figure 1. Flow diagram of representative extraction of force-dried cabbage leading to the extraction of nonacosane, 15-nonacosanone, pheophytin, and β-sitosterol.

Mammalian Assay. The mammalian (V79) mutagenicity procedure of Langenbach et al. (1978) was used. Hamster hepatocytes (about 10^7), isolated by the method of Laishes and Williams (1976), were added in 4 mL of Williams medium E (WE) to 25-cm^3 T-flasks that had been seeded with V79 cells (4×10^5) 24 h earlier. The hepatocytes were allowed 4 h to attach at which time the medium was replaced and the extract and 2-AA were added. Approximately 2×10^6 hepatocytes attached. Hepatocytes were not used when MNU was the mutagen. Twenty-four hours later the V79 cells were plated out into two groups in 100-mm Petri dishes in 4 mL of WE. One group (five dishes), which was used for the measurement of absolute survival plating efficiency, was harvested 7 days later when the number of colonies was counted. The other group (15 dishes) was used for the measurement of mutational frequency and was harvested 21 days after ouabain treatment (2 mM). Ouabain was added 2 days after plating the V79 cells. The development of resistance to ouabain was used as the measure of mutagenicity (Arlett et al., 1975) induced by MNU and 2-AA. The extracts were added before the mutagens at 0.05 and 0.25 mg/plate. Incubations were performed at 37 °C at 80% relative humidity in 95% air–5% CO_2. Mutagenicity is expressed as mutants/10^6 survivors.

RESULTS

A representative of dried cabbage extracted with petroleum ether is shown in Figure 1. It produced an insoluble and a soluble fraction. The former was solubilized and flash chromatographed on a silica column, which was eluted with hexane–ethyl acetate (4:1, v/v). Nonacosane was isolated from the hexane–ethyl acetate eluate and 15-nonacosanone, pheophytin, and β-sitosterol were isolated from the hexane–ethyl acetate (4:1, v/v) eluates. The first fraction, eluted in hexane, yielded nonacosane. It was identified by its melting point (Channon and Chibnall, 1929), NMR (^{13}C and ^{1}H), and MS data. MS established that the molecular ion was $C_{29}H_{60}$. The fragmentation pattern showed an initial loss of 15 amu, typical of an alkane. The proton spectrum showed two peaks in the ratio 9:1. The ^{13}C spectrum showed seven peaks in the alkane region. The material from the second group of active fractions was eluted with hexane–ethyl acetate (4:1, v/v) and yielded 15-nonacosanone. MS confirmed the formula $C_{29}H_{58}O$ and showed peaks corresponding to α, β, and γ cleavage at C_{15} (Laseter et al., 1968; Notting and Macey, 1971). The strong peak at 241 is consistent with $\beta + 1$ cleavage. The third group comprised later fractions

Table I. Inhibition of the Mutagenicity of 2-AA (25 mg/mL) in the Heptatocyte V79 Assay by Compounds Isolated from Savoy Chieftain Cabbage[a]

modifier	dose, μg/mL	mutants/10^6 survivors[b]	% inhibn of mutagenicity
none		18 (15)	0
nonacosane	250	0 (16)	100
	50	3 (20)	84
nonacosanone	250	2 (30)	89
	50	18 (30)	0
sitosterol	250	0 (57)	100
	50	0 (71)	100
chlorophyll[c]	250	4 (31)	78
	50	6 (67)	67

[a] 2-AA was activated by hamster hepatocytes (about 2×10^6/flask). [b] Absolute survival plating efficiencies in percent are shown in parentheses. [c] Chlorophyll was an authentic standard and was *not* isolated from the cabbage. It is included for comparison.

Table II. Inhibition of the Mutagenicity of MNU (50 μg/mL) in the V79 Assay by Compounds Isolated from Savoy Chieftain Cabbage

modifier	dose, μg/mL	mutants/10^6 survivors[a]	% inhibn of mutagenicity
none		11 (59)	
nonacosane	50	6 (90)	45
nonacosanone	50	6 (75)	45
sitosterol	50	11 (63)	0
pheophytin *a*	250	6 (34)	45
	50	9 (39)	18
chlorophyll[b]	250	0 (27)	100
	50	5 (36)	55

[a] Absolute survival plating efficiencies, in percent, are shown in parentheses. [b] Chlorophyll was an authentic standard and was *not* isolated from cabbage. It is included for comparison.

eluted with hexane–ethyl acetate (4:1, v/v) and contained β-sitosterol. This was identified by its melting point and MS and NMR data (Morimoto et al., 1967; Wright et al., 1978; Ricca et al., 1978; Koizumi et al., 1979). These fractions also contained pheophytin *a*.

The major product isolated from the methylene chloride extracts was pheophytin *a*. It was identified by its visible spectrum (Smith, 1975) and ^1H and ^{13}C spectra (Close et al., 1963; Smith et al., 1984; Lotjonnen and Hynninen, 1983). It had a molecular ion at 871 corresponding to $C_{55}H_{74}N_4O_5$ and was slightly contaminated with pheophytin *a'*.

The percent inhibition of the mutagenicity of 2-AA and MNU by the four compounds in the V79-hepatocyte assay is shown in Tables I and II, respectively. Nonacosane, 15-nonacosanone, and β-sitosterol were comparably effective against 2-AA mutagenicity at 250 μg/mL (Table I). Pheophytin *a* was without effect against 2-AA. Nonacosane, 15-nonacosanone, and β-sitosterol were too toxic in combination with MNU at 250 μg/mL (Table II). At 50 μg/mL, nonacosane and 15-nonacosanone were comparably efficient, producing a 45% inhibition of MNU mutagenicity; β-sitosterol was without effect. Pheophytin *a* was as effective as the alkanes but at a higher dose (250 μg/mL). An authentic sample of chlorophyll (from Dr. D. Wheeler) was used for comparison. It was less effective than nonacosane, 15-nonacosanone, and β-sitosterol against the mutagenicity of 2-AA but was more effective than any of the four compounds against that of MNU.

The mutagenicities (revertants/plate) of 2-AA (in TA98) and MNU (in TA100) in the presence of increasing concentrations (150, 300, 600 μg/mL) of the four isolated compounds are shown in Tables III and V, respectively. The toxicity of these compounds in TA98 and TA100 in the absence of the mutagens is shown in Tables IV and VI, respectively. 15-Nonacosanone and β-sitosterol reduced the mutagenicity of 2-AA by about 25 and 30%, respectively, at the highest dose (600 μg/mL). The isolated compounds were not toxic in TA98 (Table IV). 15-Nonacosanone was not available for testing in the toxicity assay. All four compounds inhibited the mutagenicity of MNU in TA100 (Table V) although that produced by nonacosane and pheophytin was slight, probably no more than 5%, allowing for the spread in mutation frequencies. 15-Nonacosanone was the most effective inhibitor. These compounds were not toxic to TA100 in the absence of MNU (Table VI). 15-Nonacosanone was not available for testing in the toxicity assay. An authentic sample of chlorophyll was without effect against either 2-AA or MNU in our Ames assay.

Table III. Inhibition of the Mutagenicity of 2-AA (2.5 μg/Plate) in *S. typhimurium* TA98 in the Ames Assay by Compounds Isolated from Savoy Chieftain Cabbage[a]

dose, μg/mL	revertants/plate[b,c]			
	nonacosane	nonacosanone	pheophytin *a*	sitosterol
0	1389 ± 28	1389 ± 28	2472 ± 159	1389 ± 28
150	1583 ± 175	1270 ± 96	2743 ± 159	1248 ± 94
300	1508 ± 414	1004 ± 92	2526 ± 100	1064 ± 113
600	1268 ± 74	978 ± 17	2388 ± 70	789 ± 82

[a] 2-AA was activated with rat liver S9. [b] Mean ± standard error of three measurements. [c] Background count = 29 ± 2 revertants/plate.

Table IV. Toxicity of Compounds Isolated from Savoy Chieftain Cabbage in *S. typhimurium* TA98[a]

extract	revertants/plate,[b] mg extract/plate		
	0.15	0.3	0.6
nonacosane	22 ± 2	24 ± 1	26 ± 3
pheophytin *a*	32 ± 3	31 ± 2	25 ± 5
β-sitosterol	28 ± 1	28 ± 2	25 ± 3

[a] Hepatic S9 was added. Mean ± standard error of three measurements. 29 ± 2 revertants/plate were found in plates treated with S9 alone.

Table V. Inhibition of the Mutagenicity of MNU (99.2 μg/Plate) in *S. typhimurium* TA100 in the Ames Assay by Compounds Isolated from Savoy Chieftain Cabbage

dose, μg/mL	revertants/plate[a,b]			
	nonacosane	nonacosanone	pheophytin *a*	sitosterol
0	2672 ± 33	2981 ± 113	3061 ± 131	2672 ± 33
150	2389 ± 59	1185 ± 166	3005 ± 118	2647 ± 438
300	1936 ± 150	823 ± 23	2810 ± 95	1959 ± 81
600	2101 ± 175	737 ± 24	2680 ± 85	1226 ± 96

[a] Mean ± standard error of three measurements per dose. [b] Background count = 147 ± 9 revertants/plate.

Table VI. Toxicity of Compounds Isolated from Savoy Chieftain Cabbage in *S. typhimurium* TA100[a]

extract	revertants/plate,[b] mg extract/plate		
	0.15	0.3	0.6
nonacosane	131 ± 3	145 ± 7	127 ± 2
pheophytin *a*	126 ± 1	172 ± 2	149 ± 3
β-sitosterol	146 ± 3	130 ± 10	133 ± 6

[a] Mean ± standard error of three measurements. [b] Background count = 147 ± 9 revertants/plate.

DISCUSSION

The data presented here show that four compounds with antimutagenic activity can be isolated from cabbage. Other crude fractions had antimutagenic activity but did not contain the compounds discussed here and were not characterized. Two of the isolated compounds or types of compounds have been shown to have anticarcinogenic or antimutagenic activity in other studies. β-Sitosterol,

a plant steroid, inhibited MNU (Deschner et al., 1982) and azoxymethane (Nigro et al., 1982) carcinogenicity in the rat intestine. It reduced the number of crypt cells and mature crypt cells actively synthesizing DNA in the colon (Deschner et al., 1982). β-Sitosterol is poorly absorbed and would be expected to be active in the colon (Baxter, 1968). Vegetarian members of the Seventh Day Adventist church had significantly higher serum levels of β-sitosterol than nonvegetarians, which might explain their lower incidence of intestinal cancer (Nair et al., 1984). In our studies, β-sitosterol was active against the mutagenicity of 2-AA, which required activation, but was inactive against that of MNU, a direct-acting mutagen. Since β-sitosterol was given immediately before the mutagen, it is unlikely that it induce any detoxifying enzyme(s). Consequently, its mechanism of action must involve either the stimulation of the repair of mutagenic DNA adducts produced by 2-AA or the inhibition of the activation of 2-AA. 2-AA would be metabolized by a mechanism involving an aminium radical intermediate (Boyland, 1986). Steroids can be metabolized by radical-dependent reactions to inhibit the metabolism of other substrates that are similarly metabolized (Georgellis et al., 1987). β-Sitosterol competitively inhibits some cytochrome P-450 mediated reactions (Shefer et al., 1988), which could account for its inhibitory action against 2-AA mutagenicity. Alternatively, it could repair mutagenic lesions on the V79 DNA, although there is no available evidence for such a mechanism.

Pheophytin *a* belongs to a class of compounds that are related to chlorophyll and inhibit the mutagenicity of a variety of mutagens. If chlorophyll is placed in an acid environment, such as in the stomach, it loses the Mg^{2+} ion from the phorbine ring and forms pheophytin (Kimm et al., 1982). Chlorophylls are excreted as pheophytins (Lai et al., 1980). Chlorophylls *a* and *b* inhibited the mutagenicity of 3-MC (Kimm et al., 1982), BP and cigarette smoke condensate (Terwel et al., 1985), and extracts of dietary mixtures, e.g., of red wine, chewing tobacco, and fried pork (Ong et al., 1986). The antimutagenic activity correlated with the chlorophyll content of the extracts. It has been suggested that chlorophylls, which are antioxidants, are antimutagenic through radical scavenging (Brune and San Pietro, 1970). In our assay pheophytin *a* was without inhibitory activity against the mutagenicity of 2-AA (Table III) and MNU (Table IV) in the Ames assay and 2-AA in the V79 assay (Table I). It had some activity against MNU in the V79 assay (Table II). Overall, it was only poorly antimutagenic. Chlorophyll was significantly more active than pheophytin against the mutagenicity of 2-AA and MNU.

The alkanes nonacosane and 15-nonacosanone were the most effective against 2-AA and MNU in the V79 assay. Their effectiveness against MNU, which is direct-acting, indicates that they could be active without stimulating detoxifying enzymes. Their mode of action is not known, although they could inhibit the activation of 2-AA by noncompetitive inhibition of the activating enzyme(s) through hydrophobic bonding with the apoprotein moiety of the enzyme. Nonacosane was without inhibitory activity in the Ames assay while 15-nonacosanone was the most active of the four compounds. This is the reverse of the situation in the V79 assay and suggests that these compounds may act by more than one mechanism. The 15-keto group in 15-nonacosanone could enable this compound to be a reversible competitive inhibitor of cytochrome P-450 through reaction with the heme iron as well as noncompetitively inhibiting this action through hydrophobic bonding with the apoprotein.

We have shown that cabbage contains compounds with antimutagenic activity that can be isolated, enabling a study of their mode of action. It has been suggested that the antimutagenic activity is an antioxidant action. The limited ability of pheophytin *a* to inhibit indicates that other mechanisms are involved. Data not presented indicate that the compounds discussed do not represent the totality of antimutagenic substances in cabbage.

ABBREVIATIONS USED

2-AA, 2-aminoanthracene; HBSS, Hanks balanced salts solution; MNU, *N*-methyl-*N*-nitrosourea; TLC, thin-layer chromatography; WE, Williams medium E.

Registry No. Nonacosane, 630-03-5; 15-nonacosanone, 2764-73-0; β-sitosterol, 83-46-5; pheophytin *a*, 603-17-8.

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Received for review October 27, 1988. Revised manuscript received April 6, 1989. Accepted April 19, 1989. Supported by Grant CA 38150 from the National Institutes of Health, Laboratory Cancer Research Center Support Grant CA 36727 from the National Cancer Institute, and Institutional Grant SIG 16 from the American Cancer Society.

Aroma of Birch Syrup

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The composition of the aroma extract of Finnish birch syrup was studied by gas chromatography and gas chromatography–EI-mass spectrometry. A total of 70 volatile compounds were identified. The major compounds were 2,3-dihydro-3,5-dihydroxy-6-methylpyran-4*H*-one (11–45 mg/kg), acetic acid (4–7), 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (furaneol) (0.8–2.4), 2,6-dimethylpyrazine (0.8–2.3), 2-oxopropanol (0.7–2.0), 3-methyl-2(5*H*)-furanone (0.6–1.6), 2,3-butanediol (0.8–1.0), 4-aminophenol (0.4–0.9), and 2-hydroxy-3-methyl-2-cyclopenten-1-one (cyclotene) (0.2–0.5). When the syrup was heated, several furan and pyran derivatives together with cyclotene typically increased, whereas 2,6-dimethylpyrazine decreased. The total content of the identified aromatic compounds was low.

When carbohydrate-rich foodstuffs are heated, sugar-derived cyclic compounds are found in most of the products, regardless of the origin of raw materials or the unit operations of the processes.

2,3-Dihydro-3,5-dihydroxy-6-methylpyran-4*H*-one, 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (furaneol), and 2-hydroxy-3-methyl-2-cyclopenten-1-one (cyclotene) are all typical heat-accelerated reaction products of reducing sugars, or the mixture of reducing sugars and amino compounds in aqueous media (Mevissen and Baltes, 1983; Hayase and Kato, 1985; Ledl, 1984; Baltes and Mevissen, 1988). The presence of an amino acid does not greatly affect the formation rate of these compounds (Baltes and Mevissen, 1988). For the reaction rates the moisture–temperature conditions are of primary importance.

Furaneol is common in heated carbohydrate-rich foodstuffs such as roasted almonds (Takei and Yamanishi, 1974), soy sauce (Osaki et al., 1985), roasted coffee (Tressl et al., 1983), sukiyaki (Shibamoto et al., 1981), beef broth (Tonsbeek et al., 1968), green tea (Hara and Kubota, 1982), liquid smoke (Fiddler et al., 1970), maple syrup (Kallio, 1988), and tobacco volatiles (Matsukura et al., 1985). Because of its exceptionally low odor threshold value, 0.03 μg/L (Honkanen et al., 1980), furaneol seems to be one of the character impact compounds in many foodstuffs.

Besides its direct influence on the odor and aroma of food components, furaneol is a precursor of other volatiles. In a model experiment, when heating furaneol over various pH values at 160 °C, Shu et al. (1985, 1986) found several compounds being formed such as 3-hydroxy-2-butanone, 2,3-butanedione, 2,3-pentanedione, and 2,5-dimethyl-3-(2*H*)-furanone.

Both 2,3-butanedione and 2,3-pentanedione have been identified in heated foodstuffs: sweet potato (Tiu et al., 1985), textured soy protein (Ames and MacLeod, 1984), winged beans and soybeans (del Rosario et al., 1984), cooked meat (Shibamoto et al., 1981), molasses (Godshall et al., 1980), sour cream butter (Mick et al., 1982), and roasted coffee (Wang et al., 1983). The diones were also found in the aroma fraction of yeast extract (Ames and MacLeod, 1985) and Rooibos tea (Habu et al., 1985) when the Likens and Nickerson steam distillation and extraction method (boiling required) or headspace collection followed by thermal desorption of the volatiles from the Tenax trap was used.

Cyclotene is an aroma factor in many of the protein- and carbohydrate-based foodstuffs consumed daily: fried (Ho et al., 1983) and cooked bacon (Shu and Mokherjee, 1985), sukiyaki (Shibamoto et al., 1981), dried bonito (Yajima et al., 1983), soy sauce (Aishima, 1981), malt extract (Przybylski and Kaminski, 1983), "Wasanbon" sugar (Matsui and Kitaoka, 1981), bread crust (Folkes and Gramshaw, 1981), caramel color (Hardt and Baltes, 1987), roasted coffee (Tressl et al., 1983), and roasted tobacco flavor (Matsukura et al., 1985).

Although, 2,3-dihydro-3,5-dihydroxy-6-methylpyran-4*H*-one is odorless (Shaw et al., 1971), other compounds such as furaneol, may originate from the ring contraction of this pyranone (Mills, 1978), which indicates the importance of pyranone derivatives as aroma precursors of foodstuffs.

Birch syrup is a novel syrup innovation, the commercial production of which started in Finland in spring 1988. Its composition (Kallio and Ahtonen, 1987a,b), production

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0021-8561/89/1437-1367$01.50/0