Myristicin: A Potential Cancer Chemopreventive Agent from Parsley Leaf Oil

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Parsley (*Petroselinum sativum* Hoffm.) is used extensively as a culinary herb for garnishing and seasoning. Bioassay-directed fractionation of parsley leaf oil has resulted in the isolation of an active compound named myristicin. Myristicin and other fractions were tested for their ability to induce increased activity of the detoxifying enzyme system glutathione *S*-transferase in several mouse target tissues. Myristicin showed high activity as a GST inducer in the liver and small intestinal mucosa. To establish preliminary structure–activity relationship, the olefinic bond of myristicin was reduced by catalytic hydrogenation to yield dihydromyristicin. The latter compound retained high activity in the liver and small intestinal mucosa. Thus, the isolated double bond is not required for the enzyme-inducing activity. Since the ability to induce an increase in the detoxifying enzyme activity by anticarcinogenic natural products has been found to correlate with their activity in the inhibition of tumorigenesis, myristicin may be considered a potential chemopreventive agent.

The isolation of naturally occurring secondary metabolites as inhibitors of carcinogenesis from edible plants offers a high potential for obtaining chemopreventive agents that can be useful in reducing the incidence of cancer in humans (Loub et al., 1975; Pantuck et al., 1976). Essential oils are a particularly interesting group of natural products. They occur widely in food consumed by humans (Kesterson et al., 1971; Salzer, 1977; Shaw, 1979). As part of our continuing program to discover novel chemopreventive agents from natural and synthetic sources (Lam and Zheng, 1991a,b; Zheng et al., 1991a–d), a large number of essential oils were subjected to investigation. In the preliminary screening, parsley leaf oil exhibited high biological activity that warrants further investigation (Lam and Zheng, 1991a).

Parsley (*Petroselinum sativum* Hoffm.) is a small biennial flowering plant (2–3 ft high) bearing greatly divided pinnately compound leaves. Parsley is extensively employed as a culinary herb for garnishing and seasoning. All parts of the plant contain a volatile or essential oil that is responsible for the pronounced odor and flavor of parsley. Commercially there exist two types of parsley oil, which are steam-distilled from the seeds and the fresh leaves, respectively. Typical yield for the leaf oil is about 500 g of oil/ton of the plant material (Shaath et al., 1986). Parsley leaf oil possesses a superior taste and aroma of the plant and is used primarily in flavoring meat, sauces, canned food, seasonings, etc., and to a lesser extent in perfumery (Shaath et al., 1986).

A short-term enzyme assay for the screening of potential inhibitors of chemical carcinogenesis has been employed successfully in the bioassay-directed isolation of active natural products in our laboratories (Lam and Hasegawa, 1989b; Lam and Zheng, 1991b). This rapid screening assay is based on the determination of the induction of the detoxifying enzyme system glutathione *S*-transferase (GST) (Habig et al., 1974; Jakoby and Habig, 1980). GST is a phase II enzyme that detoxifies xenobiotics (Chasseaud, 1979). GST catalyzes the reaction of glutathione (GSH) with electrophiles including activated carcinogens to form less toxic conjugates which are readily eliminated by excretion. An enhancement of GST activity suggests an increase in the ability to detoxify carcinogens. Thus, any compounds that induce an increase in the activity of this detoxifying enzyme system may be potential inhibitors of chemically induced tumorigenesis. The correlation of the induction of increased GST activity with the inhibition of carcinogenesis has been well documented. A number of known inhibitors that elicit GST enzyme activity have been found to inhibit chemically induced tumorigenesis in laboratory animals (Lam and Hasegawa, 1989a,b; Lam and Zheng, 1990b; Sparnins and Wattenberg, 1981; Wattenberg and Lam, 1984).

Preliminary screening studies carried out in our research laboratories indicated the presence of highly active inducers of detoxifying enzymes in parsley leaf oil (Lam and Zheng, 1991a). The GST enzyme activity in the mouse liver and small intestinal mucosa induced by the oil at a 30-mg dose was 2.29 and 3.75 times higher than that of the control, respectively. If such high elevation of GST activity is confirmed to correlate with the inhibition of tumorigenesis, the active compounds present in the oil can be considered potential chemopreventive agents. Recently, using GST bioassay as a guide, we have isolated a highly active compound named myristicin from parsley leaf oil. Myristicin appears to be a major chemical constituent of the oil and is responsible for the high GST-inducing activity observed in the preliminary screening. In this paper, we report the isolation, identification, and biological evaluation of myristicin.

MATERIALS AND METHODS

General Procedure. Low- and high-resolution electron impact mass spectra (EI-MS) were obtained on a Kratos MS-25 and an AEI MS-30 mass spectrometer, respectively. Nuclear magnetic resonance (NMR) spectra (δ, J in hertz) were recorded on a Nicolet NT-300 NMR spectrometer. Tetramethylsilane (TMS) was used as the internal reference (δ 0.00) for ^{1}H NMR spectra measured in $CDCl_3$. The solvent $CDCl_3$ was also used as the internal reference (δ 77.00) for ^{13}C NMR spectra. The signal multiplicities were described using the abbreviations s for singlet, d for doublet, t for triplet, q for quartet, and m for multiplet. Preparative liquid chromatography was performed on a Waters PrepLC/system 500A instrument equipped with two connected PrepPak-500/silica cartridges purchased from Millipore Waters Associates (Milford, MA).

Chemicals. Parsley leaf oil was obtained from Berjé Chemical Co. (Bloomfield, NJ). Glutathione was purchased from Sigma Chemical Co. (St. Louis, MO) with approximately 98% purity.

0021-8561/92/1440-0107$03.00/0

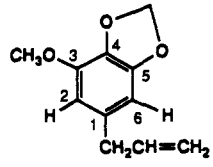
Table I. Effects of Parsley Oil Fractions on the Activity of Glutathione S-Transferase in Target Tissues of A/J Mice

		liver		small intestinal mucosa		forestomach		lung		large intestinal mucosa	
fraction	dosage,[a] mg	GST sp act.[b]	ratio[c]	GST sp act.[b]	ratio[c]	GST sp act.[b]	ratio[c]	GST sp act.[b]	ratio[c]	GST sp act.[b]	ratio[c]
control		1.41 ± 0.16		0.74 ± 0.14		1.00 ± 0.12		0.34 ± 0.13		0.64 ± 0.37	
A	20	3.14 ± 0.32	2.23[d]	2.06 ± 0.45	2.78[d]	0.85 ± 0.25	0.85	0.38 ± 0.12	1.12	0.37 ± 0.08	0.58
B	20	2.52 ± 0.23	1.79[d]	1.96 ± 0.20	2.65[d]	1.32 ± 0.84	1.32	0.48 ± 0.07	1.41	0.41 ± 0.05	0.64
C	10	3.61 ± 0.76	2.56[d]	1.92 ± 0.53	2.59[d]	0.95 ± 0.24	0.95	0.35 ± 0.05	1.03	0.37 ± 0.03	0.58
	5	3.14 ± 0.45	2.23[d]	1.17 ± 0.24	1.58	1.17 ± 0.22	1.17	0.44 ± 0.14	1.29	0.54 ± 0.10	0.84
	2.5	2.28 ± 0.21	1.62[d]	0.97 ± 0.03	1.31	0.95 ± 0.10	0.95	0.36 ± 0.04	1.06	0.39 ± 0.06	0.61
D	20	4.21 ± 0.38	2.99[d]	2.42 ± 0.56	3.27[d]	1.02 ± 0.44	1.02	0.39 ± 0.06	1.15	0.44 ± 0.14	0.69

[a] Dissolved in 0.3 mL of cottonseed oil and given once every 2 days for a total of three doses. [b] μmol min^{-1} (mg of protein)$^{-1}$. [c] Test/control. [d] All P values were obtained by Student's t-test (n = 4), $P < 0.005$.

1-Chloro-2,4-dinitrobenzene (CDNB) was obtained from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were of reagent grade or purer and purchased from Aldrich unless noted otherwise.

Myristicin (1). A 9.35-g portion of the parsley leaf oil was subjected to liquid chromatographic separation on two connected silica gel cartridges using hexane–ethyl acetate (99:1, 10 L) as the solvent system. Fractions were collected and analyzed by silica



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gel TLC (hexane–AcOEt 98:2). Fractions with the same R_f values were combined to yield four major fractions (A–D). Fraction C showed high GST-inducing activity (Table I) and afforded an oily compound upon removal of the solvent. The crude compound was purified by distillation in vacuo (10 mmHg) at 96–98 °C to afford compound 1 as a colorless oil (1.32 g): high-resolution EI-MS, calcd for $C_{11}H_{12}O_3$ (M^+) 192.0785, obsd 192.0779; ^{1}H NMR ($CDCl_3$) δ 3.30 (d, 2 H, J = 6.7 Hz, $CH_2CH{=}CH_2$), 3.88 (s, 3 H, OCH_3), 5.06 (d, 1 H, J = 9.6 Hz, cis-coupled CH=CH_2), 5.08 (d, 1 H, J = 18.9 Hz, trans-coupled CH=CH_2), 5.92 (br m, 1 H, CH=CH_2), 5.92 (s, 2 H, OCH_2O), 6.35 (d, 1 H, J = 1.5 Hz, H-2), 6.38 (d, 1 H, J = 1.5 Hz, H-6); ^{13}C NMR ($CDCl_3$) δ 40.18 (t, $CH_2CH{=}CH_2$), 56.44 (q, OCH_3), 101.16 (t, OCH_2O), 102.59 (d, C-6), 107.61 (d, C-2), 115.76 (t, CH=CH_2), 133.41 (s, C-4), 134.52 (s, C-1), 137.31 (d, CH=CH_2), 143.43 (s, C-5), 148.78 (s, C-3); EI-MS m/z (relative intensity) 192 (M^+, 100), 191 (M^+ – H, 14), 165 (M^+ – CH=CH_2, 21), 161 (M^+ – CH_3O, 10), 151 (M^+ – CH_2-CH=CH_2, 24), 133 (m/z 165 – CH_3OH, 13), 113 (21), 101 (11), 95 (11), 91 (8), 83 (11), 77 (5), 70 (19), 69 (23), 51 (65).

Dihydromyristicin (2). Myristicin (1, 2.2 g, 11.5 mmol) dissolved in methanol (5 mL) was hydrogenated in the presence of the catalyst palladium on activated carbon at room temperature for 2 h. The catalyst was removed by filtration through

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a Celite pad and washed with methanol. The combined filtrate and washings were evaporated under reduced pressure. The crude product was then loaded on a silica gel pipet column and eluted with 1% ethyl acetate in hexane to afford compound 2 as a colorless oil (2.2 g, 99% yield): TLC analysis, single spot (R_f 0.17) on a 10% $AgNO_3$–silica gel plate developed with 2% ethyl acetate in hexane; high-resolution EI-MS, calcd for $C_{11}H_{14}O_3$ (M^+) 194.0943, obsd 194.0934; ^{1}H NMR ($CDCl_3$) δ 0.96 (t, 3 H, J = 7.5 Hz, $CH_2CH_2CH_3$), 1.61 (6 lines, 2 H, J = 7.5 Hz, $CH_2CH_2CH_3$), 2.52 (t, 2 H, J = 7.5 Hz, $CH_2CH_2CH_3$), 3.92 (s, 3 H, OCH_3), 5.95 (s, 2 H, OCH_2O), 6.36 (d, 1 H, J = 1.5 Hz, H-2), 6.40 (d, 1 H, J = 1.5 Hz, H-6); EI-MS: m/z (relative intensity) 194 (M^+, 27), 165 (M^+ – C_2H_5, 100), 152 (M^+ – C_3H_6, 2), 120 (4), 92 (5), 77 (14), 69 (13), 53 (15), 51 (17).

Animals. Female A/J mice, 7 weeks of age, were obtained from the NIH. Animals were housed in temperature-controlled animal quarters with a 12/12 h light/dark cycle. Water was given ad libitum. They were acclimated for 1 week after arrival before they were fed semipurified diet (ICN Nutritional Biochemicals, Cleveland, OH) until the end of the experiment. One week after the start of the semipurified diet they were divided into control and experimental groups with four mice per group. The experimental groups were given by gavage 20 mg per dose of the test compounds, suspended in 0.3 mL of cottonseed oil, once every 2 days for a total of three doses. The control group was given cottonseed oil alone. Twenty-four hours after the last administration, the mice were killed by cervical dislocation. The liver, forestomach, lung, bladder, and mucosa of the proximal one-third of the small intestine and the large intestine including the cecum were removed for enzyme preparation. The tissues were homogenized in cold 1.15% KCl solution (pH 7.4) by means of a Brinkman homogenizer. The homogenate was centrifuged at 9000g for 20 min, and the supernatant was centrifuged at 100000g for 1 h. The cytosolic fractions were kept frozen at –80 °C until use. Each sample represents one tissue from each individual animal.

Glutathione S-Transferase Assay. The activity of cytosolic GST was determined according to the method of Habig et al. (1974) using 1-chloro-2,4-dinitrobenzene as the substrate. Assays were performed at 30 °C in 0.1 M phosphate buffer (pH 6.5), in the presence of 5 mM GSH, 1 mM CDNB, and 20 μL of the cytosol. The reaction was monitored at 340 nm on a Beckman DU-65 spectrophotometer equipped with a temperature-controlled cell compartment. Complete assay mixture without the cytosolic enzyme was used as the control. Data were analyzed according to Student's t-test, and P values were obtained in comparison to the control. The protein concentration of these samples was determined according to the method of Lowry and co-workers (Lowry et al., 1951).

RESULTS AND DISCUSSION

Parsley leaf oil was fractionated to search for the active constituents using GST assay as a guide. Four fractions were collected from silica gel column chromatography and were tested for their ability to elicit GST activity (Table I). Fraction C, which contained mainly one compound, showed high enzyme-inducing activity in the liver (T/C 3.54) and small intestinal mucosa (T/C 3.75) at the 20-mg dose in a previous study. To obtain dose–response data, fraction C was evaluated at the dose levels of 10, 5, and 2.5 mg, respectively, while other fractions were tested at the 20-mg level. Of the five tissues examined, fraction C exhibited higher activity in a dose-dependent manner in the liver and small intestinal mucosa (Table I).

The active fraction C afforded compound 1 upon distillation in vacuo. Compound 1 gave a molecular ion as a base peak in the high-resolution EI mass spectrum at m/z 192.0779, which corresponded to the formula

Table II. Effects of Myristicin Compounds on the Activity of Glutathione S-Transferase in Target Tissues of A/J Mice

chemical	dosage,[a] mg	liver		small intestinal mucosa		forestomach		lung		large intestinal mucosa		bladder	
		GST sp act.[b]	ratio[c]	GST sp act.[b]	ratio[c]	GST sp act.[b]	ratio[c]	GST sp act.[b]	ratio[c]	GST sp act.[b]	ratio[c]	GST sp act.[b]	ratio[c]
control		0.92 ± 0.17		0.50 ± 0.15		0.46 ± 0.05		0.37 ± 0.09		0.21 ± 0.08		0.98 ± 0.65	
myristicin	20	3.74 ± 0.80	4.07[d]	1.40 ± 0.47	2.80	0.52 ± 0.14	1.13	0.34 ± 0.06	0.92	0.29 ± 0.28	1.38	1.78 ± 0.60	1.82
dihydro-myristicin	20	4.28 ± 0.31	4.65[d]	1.44 ± 0.40	2.88[d]	0.47 ± 0.05	1.02	0.36 ± 0.05	0.97	0.27 ± 0.03	1.29	0.93 ± 0.05	0.95
control		0.78 ± 0.07		0.28 ± 0.09		0.73 ± 0.08		0.30 ± 0.02		0.31 ± 0.04		2.51 ± 0.43	
myristicin	10	3.33 ± 0.52	4.27[d]	0.91 ± 0.07	3.25[d]	0.87 ± 0.22	1.19	0.34 ± 0.06	1.13	0.42 ± 0.05	1.35	3.53 ± 1.62	1.41
dihydro-myristicin	10	2.65 ± 0.25	3.40[d]	0.62 ± 0.09	2.21[d]	1.11 ± 0.43	1.52	0.52 ± 0.04	1.73[d]	0.40 ± 0.03	1.29	2.47 ± 0.31	0.98

[a] Dissolved in 0.3 mL of cottonseed oil and given every 2 days for a total of three doses. [b] μmol min^{-1} (mg of protein)$^{-1}$. [c] Test/control. [d] All P values were obtained by Student's t-test ($n = 4$), $P < 0.005$.

$C_{11}H_{12}O_3$ (calcd 192.0785). The high degree of unsaturation and intense molecular ion peak suggested that 1 could be a stable aromatic compound. The mass spectrum also showed significant ions at m/z 165 and 151 resulting from the loss of vinyl and allyl groups, respectively, from the three-carbon side chain. Other important ions in the spectrum generated by the loss of a methoxyl group suggested the presence of this oxygenated substituent. The ^{1}H NMR spectrum of 1 confirmed the presence of a methoxyl group at δ 3.88 and a methylenedioxyl group at δ 5.92. The proton spectrum also showed the presence of a pair of meta-coupled aromatic protons. The characteristic allyl proton signals consisting of an A_2MXY system appeared at δ 3.30 (A_2), 5.92 (M), 5.04 (X), and 5.09 (Y). The nuclear Overhauser effect (NOE) experiments led to the establishment of the substitution on the benzene ring. Irradiation of the methoxyl group at δ 3.88 enhanced only an aromatic signal at δ 6.35, suggesting the location of the methoxyl between an aromatic proton (H-2) and a substituent (OCH_2O). Irradiation of the methylene at δ 3.36 (allyl group) enhanced H-2 and H-6, indicating the presence of the allyl substituent between the two aromatic protons. This led to the conclusion that the methylenedioxyl group must be located at the 4- and 5-positions. The ^{13}C NMR spectrum of 1 confirmed the presence of a highly substituted phenol ether derivative of allylbenzene. The aromatic ring carbon signals were assigned by comparison with model compounds and by application of substituent effect rules. The carbon spectrum also showed typical signals for methoxyl and methylenedioxyl groups at δ 56.44 (q, OCH_3) and 101.16 (t, OCH_2O). The signal at δ 40.18 (t), 115.76 (t), and 137.31 (d) could be assigned to the allyl group. Thus, compound 1 was identified as myristicin or 3-methoxy-4,5-methylenedioxy-1-allylbenzene. Myristicin was previously found in parsley leaf oil (Shaath et al., 1986). The qualitative and quantitative analysis by GC/MS indicated that this compound constituted 6.32% of the leaf oil. Myristicin is also present in different contents in other edible plant oils, such as dill herb oil (7.63%) (Huopalahti and Linko, 1983) and celery seed oil (0.18%) (Formacek and Kubcezka, 1982).

To determine the significance of the isolated olefinic bond toward the enzyme-inducing activity, dihydromyristicin (2) was prepared by catalytic hydrogenation of 1. This compound has not been found in parsley. Compound 2 showed a molecular ion at m/z 194.0934, corresponding to the elemental composition $C_{11}H_{14}O_3$ (calcd 194.0943) in the high-resolution EI mass spectrum. The EI mass spectrum showed a base peak at m/z 165 formed by loss of an ethyl group from the side alkyl chain, which is a characteristic cleavage for the propylbenzene structure. The ^{1}H and ^{13}C NMR spectra of 2 indicated the disappearance of the olefinic double bond on the side chain, which further confirmed the structure of 2 as dihydromyristicin.

The GST-inducing activity of compounds 1 and 2 was determined in the liver, lung, forestomach, bladder, colon, and small bowel mucosa of A/J mice (Table II). Two dose levels of the compounds were used. At the higher dose (20 mg), both compounds appeared to increase GST activity at least 4 times greater than the control in the liver and about 2.5 times over the control in the small intestinal mucosa. In the colon the GST enzyme activity induced by the two compounds was about 30% higher than that of the control level. No increase of GST activity in either the lung or the forestomach was observed with the two compounds. In the bladder myristicin showed significant GST-inducing activity, while dihydromyristicin was inactive. No significant difference in activity between these two compounds was observed at the 20-mg dose in all of the tissues except the bladder. Dihydromyristicin showed slightly higher activity than myristicin in the liver and small bowel, while the reverse was observed in the forestomach and colon. This suggests that the existence of the isolated olefinic double bond in the allyl side chain is not critical for the activity. At the lower dose (10 mg), myristicin and its dihydro compound still showed higher activity in the liver and small bowel. Furthermore, myristicin appeared to be more active than dihydromyristicin in the two tissues.

Parsley or parsley leaf oil is frequently and widely used in human food. On the basis of bioassay-directed isolation, myristicin appears to be responsible for the high GST-inducing activity observed with the oil in the preliminary screening. The present results indicate that compounds 1 and 2 are capable of inducing increased activity of the detoxifying enzyme GST when given orally. To establish the correlation between GST-inducing activity and tumor inhibition by the two compounds, the determination of their ability to inhibit BP-induced tumorigenesis in mice is underway. Since an increase of GST activity is associated with anticarcinogenic potential, myristicin and its derivatives show promise as useful chemopreventive agents.

ACKNOWLEDGMENT

We thank Thomas P. Krick and Nan Wang, College of Biological Sciences, University of Minnesota, for their assistance in obtaining spectra, Keith Berg and Frank Yue for their technical assistance, and Aimee Larson for the preparation of the manuscript. This research was supported by a grant from the National Cancer Institute (USPHS CA 47720).

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Received for review July 29, 1991. Accepted October 8, 1991.

Registry No. 1, 607-91-0; 2, 52811-28-6; glutathione *S*-transferase, 50812-37-8.