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Antimutagenic Constituents of *Casimiroa edulis* with Potential Cancer Chemopreventive Activity

Aiko Ito,[†,‡] Lisa A. Shamon,[†,‡,§] Boyang Yu,[†] Eugenia Mata-Greenwood,[†] Sang Kook Lee,[†] Richard B. van Breemen,[†] Rajendra G. Mehta,[∥] Norman R. Farnsworth,[†] Harry H. S. Fong,[†] John M. Pezzuto,[†,∥] and A. Douglas Kinghorn*,[†]

Program for Collaborative Research in the Pharmaceutical Sciences and Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, and Department of Surgical Oncology, College of Medicine, University of Illinois at Chicago, Chicago, Illinois 60612

An ethyl acetate extract derived from the seeds of the medicinal and food plant *Casimiroa edulis* inhibited mutagenicity induced by 7,12-dimethylbenz[*a*]anthracene (DMBA) with *Salmonella typhimurium* strain TM677. It also showed complete inhibition of DMBA-induced preneoplastic lesions with an in vitro mouse mammary gland organ culture system at a concentration of 10 μg/mL. Bioassay-guided phytochemical investigation of this extract using antimutagenicity as a monitor led to the isolation of four furocoumarins, constituted by the known compounds phellopterin (**1**) and isopimpinellin (**2**) and the novel compounds (*R*,*S*)-5-methoxy-8-[(6,7-dihydroxy-3,7-dimethyl-2-octenyl)oxy]psoralen (**3**) and (*R*,*S*)-8-[(6,7-dihydroxy-3,7-dimethyl-2-octenyl)oxy]psoralen (**4**). Four known alkaloids, casimiroin (**5**), 4-methoxy-1-methyl-2(1*H*)-quinolinone (**6**), 5-hydroxy-1-methyl-2-phenyl-4-quinolone (**7**), and γ-fagarine (**8**), and two known flavonoids, zapotin (**9**) and 5,6,2′-trimethoxyflavone (**10**), were also isolated. Of these isolates, compounds **3** and **5** showed the most potent antimutagenic effects in the forward mutagen assay utilizing *S. typhimurium* strain TM677, whereas casimiroin (**5**) and 5,6,2′-trimethoxyflavone (**10**) significantly inhibited the formation of DMBA-induced preneoplastic lesions in mouse mammary gland organ culture.

Keywords: *Casimiroa edulis; Rutaceae; furocoumarins; (R,S)-5-methoxy-8-[(6,7-dihydroxy-3,7-dimethyl-2-octenyl)oxy]psoralen; (R,S)-8-[(6,7-dihydroxy-3,7-dimethyl-2-octenyl)oxy]psoralen; alkaloids; flavonoids; antimutagens; mouse mammary organ culture assay; cancer chemoprevention*

INTRODUCTION

Casimiroa edulis Llave et Lex. (Rutaceae) is a tree that is widely distributed throughout Mexico and Central America. The edible fruits, commonly known as "zapote blanco", have a pleasant flavor and are consumed as a tropical dessert (Kincl et al., 1956). The Pharmacopeia of Mexico once recognized both the fruit and seed of *C. edulis*, with the former used as an anthelmintic and the latter as a vulnerary (Power and Callan, 1911). In previous work, the seeds of *C. edulis* have afforded alkaloids (Power and Callan, 1911; Aebi, 1956; Djerassi et al., 1956, 1958; Kincl et al., 1956; Meisels and Sondheimer, 1957; Sondheimer and Meisels, 1958; Randolph and Friedrich, 1958; Mechoulam et al., 1961; Raman et al., 1962; Toube et al., 1967; Romero et al., 1983; Rizvi et al., 1985), coumarins (Dreyer, 1968; Enríquez et al., 1984), flavonoids (Kincl et al., 1956; Dreyer and Bertelli, 1967; Garratt et al., 1967; Dreyer, 1968; Romero et al., 1983), and limonoids (Sondheimer et al., 1959; Dreyer, 1968; Murphy et al., 1968). From the bark of *C. edulis*, alkaloids and flavonoids were also reported as constituents (Iriarte et al., 1956; Sondheimer and Meisels, 1960). Imidazole alkaloid derivatives have been isolated from the leaves of *C. edulis* (Romero et al., 1983). There have been a number of synthetic studies published on the various constituents of *C. edulis* (Meisels and Sondheimer, 1957; Weinstein and Hylton, 1964; Dreyer and Bertelli, 1967; Panzica and Townsend, 1973).

In early pharmacological work, the seeds of *C. edulis* were shown to be devoid of hypnotic effects when an alcoholic extract was administered to dogs (Power and Callan, 1911). There have been a number of reports on the hypotensive, hypnotic, and sedative activities of the seeds of *C. edulis*. Subsequent in vivo studies have shown that an aqueous extract of the seeds of *C. edulis* can lower blood pressure in a variety of animal species, and this effect was associated with the presence of imidazole alkaloid constituents that stimulate histamine receptors (Magos and Vidrio, 1991; Vidrio and Magos, 1991; Magos et al., 1995). The aqueous extract of the leaves has shown anticonvulsant and sedative activity (Navarro et al., 1995). Furoquinoline alkaloids, such as γ-fagarine, a known *C. edulis* constituent from the bark (Iriarte et al., 1956), have been examined and were shown to be responsible for various physiological, pharmacological, and biological effects. They have been found to possess antiarrhythmic, antidiuretic, spasmolytic, and vasoconstrictive activities (Petit-Paly et al., 1982) as well as sedative and hypothermic effects (Achenbach, 1977).

* Author to whom correspondence should be addressed [telephone (312) 996-0914; fax (312) 996-7107; e-mail kinghorn@uic.edu].

[†] College of Pharmacy.

[‡] These authors contributed equally to the work.

[§] Present address: Geraldine Brush Cancer Research Institute, California Pacific Medical Center, 2330 Clay St., San Francisco, CA 94115.

[∥] College of Medicine.

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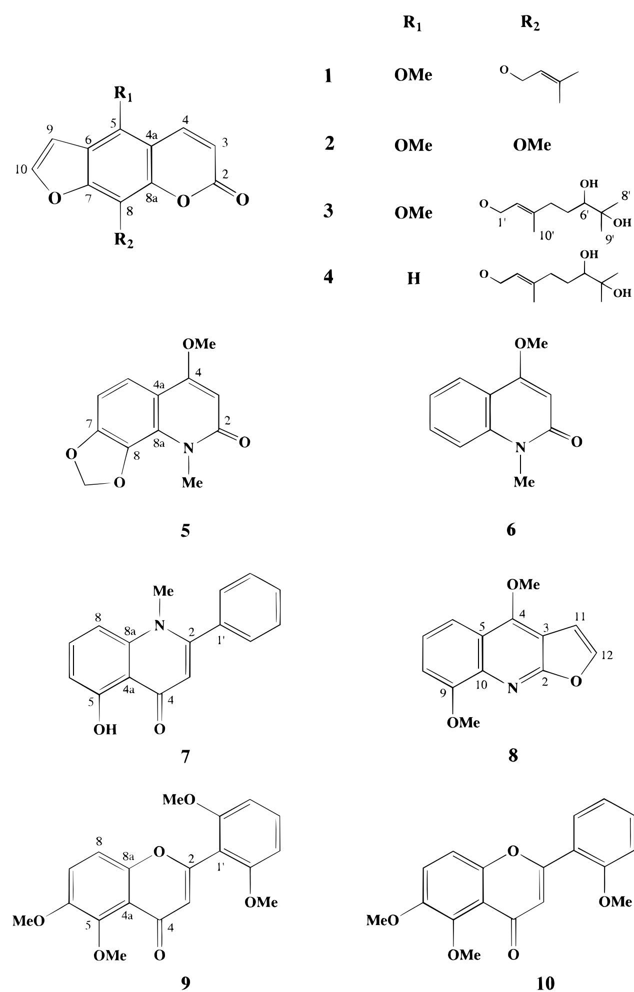
Cancer chemoprevention generally refers to the prevention, delay, or reversal of carcinogenesis in humans by the ingestion of dietary or pharmaceutical agents (Sporn et al., 1976; Pezzuto, 1995). Certain constituents of culinary herbs, fruits, spices, and vegetables have been shown to prevent the development of cancer in animal models (Huang et al., 1994; Ho et al., 1994), and clinical intervention trials are underway with selected plant constituents (Kelloff et al., 1994). As a part of our current work on cancer chemoprevention, a battery of mechanism-based in vitro assays is employed to detect potential cancer chemopreventive agents (Pezzuto, 1995). In the present study, an ethyl acetate extract of the seeds of *C. edulis* was prepared and found to inhibit 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced mutation in *Salmonella typhimurium* strain TM677. Inhibition of mutagenesis is a potentially effective mechanism for the prevention/suppression of human cancers resulting from DNA damage caused by exposure to environmental mutagens and carcinogens (Shamon et al., 1994). Bioassay-guided phytochemical investigation using antimutagenicity as a monitor led to the isolation of four furocoumarins, namely, phellopterin (**1**), isopimpinellin (**2**), and the novel compounds **3** and **4**, along with four alkaloids, casimiroin (**5**), 4-methoxy-1-methyl-2(1*H*)-quinolinone (**6**), 5-hydroxy-1-methyl-2-phenyl-4-quinolone (**7**), and γ-fagarine (**8**),



and two flavonoids, zapotin (**9**) and 5,6,2′-trimethoxyflavone (**10**).

To further evaluate the activity of these compounds as potential chemopreventive agents, they were tested in a mouse mammary organ culture assay (Mehta et al., 1991; Pezzuto, 1995). In addition, their activities were tested in an ethoxyresorufin *O*-deethylase (EROD) assay using rat liver microsomes (Ahn et al., 1996), an ornithine decarboxylase (ODC) induction assay mediated by the tumor promoter 12-*O*-tetradecanoylphorbol 13-acetate (TPA) in murine epidermal 308 cells (Lichti and Gottesman, 1982), and a cellular differentiation assay with HL-60 human leukemia cells (Suh et al., 1995). This paper deals with the isolation, structural identification, and biological evaluation of these compounds.

MATERIALS AND METHODS

^{1}H NMR, ^{13}C NMR, and ^{1}H−^{1}H COSY (including APT) spectra were measured on a Varian XL-300 instrument operating at 300 and 75.6 MHz, respectively. Compounds were analyzed in $CDCl_3$, with tetramethylsilane (TMS) as internal standard. A General Electric Omega 500 NMR spectrometer, operating at 499.9 MHz, was used to perform HMQC and HMBC experiments. ^{13}C NMR multiplicity was determined using APT and DEPT experiments. The DEPT experiment was conducted on a Nicolet NMC-360 instrument, operating at 90.8 MHz for ^{13}C. High-resolution electron impact mass spectra (EIMS) were obtained using a Finnigan MAT (Bremen, Germany) MAT-90 mass spectrometer. Electrospray mass spectra (ESMS) were acquired using a Micromass (Manchester, U.K.) Quattro II triple-quadrupole mass spectrometer. For ESMS, samples were dissolved in 50% aqueous methanol containing either 1% ammonia or acetic acid to facilitate the formation of negative or positive ions, respectively. Each sample was infused at 10 μL/min, and mass spectra were acquired at a scan speed of 2 s over the mass range m/z 50−800. IR spectra were taken on a Nicolet MX-1 FT-IR spectrophotometer. UV spectra were measured on a Beckman DU-7 spectrometer. Melting points were determined using a Fisher-Johns melting point apparatus and are uncorrected. Optical rotations were obtained on a Perkin-Elmer model 241 polarimeter.

Thin-layer chromatographic (TLC) analysis was performed on Kieselgel 60 F_{254} (Merck, Darmstadt, Germany) plates (silica gel, 0.25 mm layer thickness), with compounds visualized by spraying with 10% (v/v) H_2SO_4 followed by charring at 110 °C for 10 min. Silica gel (Merck 60A, 70−230 mesh ASTM) and Sorbisil C_{18} reversed-phase silica gel (Phase Separation, Ltd., Deeside, Clywd, U.K.) were used for column chromatography. Preparative TLC was performed on Kieselgel 60 F_{254} (Merck) plates (silica gel, 0.50 mm layer thickness). HPLC separations were performed on a Beckman model 100A system equipped with a Shimadzu SPD 6AV UV detector and employing a YMC-Pack ODS-AQ (250 × 4.6 mm) column (YMC, Wilmington, NC). All solvents used for chromatographic separations were purchased from Fisher Scientific (Fair Lawn, NJ) and distilled before use.

Plant Material. The seeds of *C. edulis* were obtained from Eli Lilly Co., Indianapolis, IN, through the courtesy of the late Dr. Gordon H. Svoboda. A voucher specimen is deposited at the University of Illinois Pharmacognosy Field Station, Lisle, IL.

Antimutagenicity Assay. The procedure used to detect forward mutation to 8-azaguanine resistance in *S. typhimurium* strain TM677 was performed as previously described (Grüter et al., 1990; Shamon et al., 1994). TM677 was derived from Ames *S. typhimurium* strain TA1535, which lacks the uvrA DNA repair mechanism. In addition, the strain carries the "R-factor" plasmid pKM101, which enhances an error-prone DNA repair system and thereby increases the mutation rate (Skopek et al., 1978; Maron and Ames, 1983). For analysis, a frozen aliquot of TM677 was quickly thawed and added to minimal essential medium (50-fold dilution). The culture was incubated for 1 h in a 37 °C shaking water bath

to yield a viable cell concentration of $\sim 1 \times 10^7$ bacteria/mL. Incubation mixtures were then prepared, consisting of 0.77 mL of bacteria in minimal essential medium, 0.1 mL of S9 liver homogenate derived from Aroclor 1254-pretreated rats, and 0.11 mL of a NADPH-generating system, distributed into 15 mL conical centrifuge tubes. To monitor antimutagenic activity, test substances (40 μg/mL final concentration added in 10 μL of DMSO) were added to duplicate incubation mixtures concurrently with DMBA (80 μM final concentration added in 10 μL of DMSO). Following incubation in a rotating dry-air incubator (2 h, 37 °C), the reaction mixture was quenched by the addition of 4 mL of phosphate-buffered saline (PBS). The bacteria were recovered by centrifugation, resuspended in 5 mL of PBS, diluted, and plated (in triplicate) on minimal agar in the presence or in the absence of 8-azaguanine. The plates were then incubated (48 h, 37 °C) and scored with an automatic colony counter (Imaging Products International, Chantilly, VA). The results were expressed as a mutant fraction, that is, the average number of colonies capable of growing in the presence of 8-azaguanine divided by the average number of colonies capable of growing in the absence of 8-azaguanine, after correcting for dilution factors. After the spontaneous (DMSO control) mutant fraction was subtracted from each treatment group, the percent inhibition was calculated relative to controls treated with only DMBA. Test compounds were initially screened at a fixed concentration of 40 μg/mL. Those demonstrating inhibitory activity $\geq$50% were subjected to dose−response studies, as described above. Utilizing three to five test concentrations (depending on availability), in addition to the solvent control value (no inhibition), semilog dose−response curves were constructed, and IC_{50} values were calculated by linear regression analysis. The values used for the calculation of mean mutant fractions agreed within 5%.

Mouse Mammary Organ Culture Assay. The inhibition of lesion formation in mouse mammary organ culture was performed as previously described (Mehta et al., 1991). Briefly, BALB/c female mice (4 weeks old, Charles River, Wilmington, MA) were pretreated for 9 days with 1 μg of estradiol and 1 mg of progesterone. On the 10th day, the mice were sacrificed and the second pair of thoracic mammary glands were dissected on silk and transferred to 60 mm culture dishes containing 5 mL of Waymouth's 752/1 MB medium supplemented with 100 units/mL streptomycin, 100 units/mL penicillin, and 35 μg/mL glutamine. The glands were incubated for 10 days (37 °C, 95% O_2 + 5% CO_2) in the presence of growth-promoting hormones (5 μg of insulin, 5 μg of prolactin, 1 μg of aldosterone, and 1 μg of hydrocortisone per milliliter of medium). Glands were exposed to 2 μg/mL DMBA between 72 and 96 h. After the exposure, glands were rinsed and transferred to new dishes with fresh medium. The fully differentiated glands were then permitted to regress by withdrawing all hormones, except insulin, for 14 additional days. To evaluate the inhibition of lesion formation, potential chemopreventive agents were present in the medium during days 1−10 of culture.

EROD Assay. The incubation mixture, containing microsomal protein (200 μg) derived from the liver of Aroclor 1254-induced rats (Maron and Ames, 1983), with 10 μM 7-ethoxyresorufin (20 μL of a 1 mM solution in DMSO), 10 μL of DMSO or test sample, and 0.1 M phosphate buffer (pH 7.6), was equilibrated for 1 min at 37 °C. The reaction was initiated by the addition of 250 μM NADPH (10 μL of a 50 mM solution) (final volume = 2 mL). After a 5 min incubation period, the reaction was terminated by the addition of methanol (1 mL), and the fluorescence of resorufin was quantified (560 nm excitation and 586 nm emission wavelengths) using a Perkin-Elmer fluorescence spectrophotometer (Burke et al., 1975, 1985; Guo et al., 1992; Ahn et al., 1996). A standard calibration was made by determining the fluorescence of authentic resorufin. For the determination of IC_{50} values, three to five concentrations of test samples (depending on availability) were evaluated in triplicate, and percent inhibition was calculated relative to DMSO-treated control groups. Mean inhibitory values were used for the construction of semilog dose−response curves, and IC_{50} values were calculated by linear regression analysis.

HL-60 Cell Differentiation Assay. Induction of cellular differentiation using HL-60 cells was assessed as previously described (Suh et al., 1995). HL-60 (human promyelocytic leukemia) cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 units of penicillin/mL, and 100 μg of streptomycin/mL. Incubations were performed at 37 °C in a humidified atmosphere of 5% CO_2 in air. For testing, cells in the log phase of growth were diluted to 1.2×10^5 cells/mL and preincubated for 18 h (2×10^5 cells/mL). Samples dissolved in DMSO were prepared in 24-well plates, and HL-60 cells were then added, with the final DMSO concentration maintained at 0.1% v/v. After 4 days of incubation, the cells were analyzed to determine the percentage exhibiting functional and enzymatic markers of differentiated cells, as judged by three assays, which are described in the following paragraphs.

(a) Nitroblue Tetrazolium (NBT) Reduction. This assay was used to evaluate the ability of sample-treated HL-60 cells to produce superoxide when challenged with 12-*O*-tetradecanoylphorbol 13-acetate (TPA). A 1:1 (v/v) mixture of a cell suspension (10^6 cells) and freshly prepared TPA/NBT solution (phosphate-buffered saline solution containing 2 mg/mL NBT and 1 μg/mL TPA) was incubated for 1 h at 37 °C. Cells were then smeared on glass slides and counterstained with 0.3% (w/v) safranin O in methanol. Positive cells are able to reduce NBT, yielding intracellular black-blue formazan deposits, which can be determined by microscopic examination of a minimum of 200 cells. The results were expressed as a percentage of positive over total cells. NBT-reducing ability was detected in both granulocytic and monocytic lineages.

(b) Nonspecific/Specific Acid Esterase Activity. Assays for α-naphthyl acetate esterase (nonspecific acid esterase, NSE) and naphthyl AS-D chloroacetate esterase (specific acid esterase, SE) were performed using cytochemical kits from Sigma Chemical Co. (91-A and 91-C). Differentiated cells were assessed by microscopic examination of a minimum of 200 cells (in duplicate) for each experiment. NSE is detected primarily in monocytes, macrophages, and histocytes and is virtually absent in granulocytes. Monocytes should show black granulation. Lymphocytes may occasionally exhibit enzyme activity. SE is usually considered specific for cells of granulocytic lineage. Sites of activity showed bright red granulation, with little or no activity in monocytes and lymphocytes.

(c) Inhibition of [^{3}H]Thymidine Incorporation. The level of HL-60 cell proliferation can be measured by determining [^{3}H]thymidine incorporation into DNA. Sample-treated cells (100 μL) were removed and placed into 96-well plates, and 0.5 μCi/mL [^{3}H]thymidine (65 Ci/mmol) was added. After 16 h of further incubation, cells were harvested and counted using a Microbeta counter. The percentage of [^{3}H]thymidine incorporation per 10^6 cells was calculated by dividing the sample counts per minute (cpm) by the DMSO-treated control cpm.

Ornithine Decarboxylase (ODC) Assay. ODC activity was assayed by measuring the release of [^{14}C]CO_2 from L-[1-^{14}C]ornithine as described previously (Lichti and Gottesman, 1982; Gerhäuser et al., 1995). Mouse epidermal cells 308 (5×10^4/2 mL) were plated in 24-well plates. After 18 h (cell density of 2×10^5 cells/mL), cells were treated with 100 ng/mL TPA in 2 μL of 100% DMSO and test agents at various concentrations for 6 h. Cells were then washed with PBS and frozen at −80 °C until assayed for ODC activity. Cells were lysed by two cycles of freezing and thawing, and the plates were placed on ice. A substrate and cofactor mixture [200 μL containing 2 μL of L-[1-^{14}C]ornithine (200 nCi, 56 mCi/mmol, 100 μCi/mL, from Moravek), 50 μL of sodium phosphate buffer (0.2 M in phosphate, pH 7.2), 16 μL of ethylenediaminetetraacetic acid (EDTA; 12.5 mM adjusted to pH 8.0 with NaOH), 10 μL of dithiothreitol (DTT; 50 mM in water), 4 μL of pyridoxal phosphate (PLP; 5 mM in 10 mM NaOH), and 118 μL of cold L-ornithine (78 μg/mL, final 365 μM)] was added to each well. The plates were then briefly agitated on a rotary shaker and covered with 3.5 in. × 5.25 in. sheets of Parafilm

in which holes had been punched, such that the holes were centered over each well. The Parafilm was firmly pressed against the rims of the wells, and 0.5 in. diameter paper disks were placed over each hole and moistened with 30 μL of 1 M NaOH. The dishes were returned to the shaking water bath for another 1 h to allow absorption of $[^{14}C]CO_2$ by NaOH. The disks were then transferred to scintillation vials, dried, and counted. Protein content of each well was determined according to the procedure of Lowry et al. (1951) using bovine serum albumin as a standard. Specific activity was expressed as nanomoles of product per hour per milligram of protein.

Extraction and Isolation Procedure. The seeds of *C. edulis* (735 g) were extracted with MeOH (1.4 L × 2) at room temperature, and the solution was evaporated in vacuo. The dried MeOH extract was resuspended in 10% MeOH in H_2O and partitioned with petroleum ether. The aqueous MeOH layer was dried, redissolved in H_2O, and partitioned with EtOAc. In the antimutagenicity assay with *S. typhimurium* strain TM677, the EtOAc extract produced 78.6% inibition of DMBA-induced mutations at a concentration of 40 μg/mL. The extract, at 10 μg/mL, also showed 100% inhibition of DMBA-induced preneoplastic lesions with the mouse mammary organ culture assay system. The EtOAc extract was subjected to silica gel column chromatography and eluted with increasing concentrations of MeOH in $CHCl_3$ to give six fractions. Fractions 1 and 2 were active in the antimutagenicity assay. Additional chromatographic separation of bioactive fraction 1 (82.4% inhibition of DMBA-induced mutation at 40 μg/mL) over silica gel with increasing concentrations of EtOAc in petroleum ether yielded five subfractions (fractions 1A–1E). Further chromatography of subfraction 1A over silica gel with 0–20% EtOAc/petroleum ether yielded two pure furocoumarins (**1** and **2**; 15 and 20 mg, respectively) after purification by preparative TLC (20% EtOAc/petroleum ether as solvent). Alkaloids **5** (10 mg) and **6** (8 mg) were obtained from subfraction 1D using silica gel column chromatography (50% EtOAc/petroleum ether) and reversed-phase low-pressure liquid chromatography over C_{18} silica gel (70% MeOH in H_2O as solvent). Subfraction 1E was subjected to silica gel column chromatography, eluted with 60% EtOAc/petroleum ether, to afford another two alkaloids, **7** (15 mg) and **8** (4 mg). Flavonoids **9** (27 mg) and **10** (15 mg) were obtained from subfraction 1C using silica gel column chromatography by elution with 25% EtOAc/petroleum ether. From active fraction 2 (68.2% inhibition at 40 mg/mL), when eluted with increasing concentrations of EtOAc in hexane, the novel compounds **3** (9 mg) and **4** (3 mg) were obtained using silica gel column chromatography. These compounds were finally purified by HPLC with a YMC-Pack ODS-AQ column (250 × 4.6 mm) using MeOH/H_2O (3:7) at a flow rate of 1 mL/min.

*Phellopterin (**1**):* prisms ($CHCl_3$/MeOH); mp 99–100 °C; UV, IR, ^1H NMR, and EIMS consistent with literature values (Dreyer, 1968); ^{13}C NMR ($CDCl_3$, 75.6 Hz) δ 160.6 (s, C-2), 150.8 (s, C-7), 145.1 (d, C-10), 144.4 (s, C-5), 144.3 (s, C-8a), 139.7 (s, C-3′), 139.5 (d, C-4), 126.8 (s, C-8), 119.8 (d, C-2′), 114.5 (s, C-6), 112.7 (d, C-3), 107.5 (s, C-4a), 105.1 (d, C-9), 70.4 (t, C-1′), 25.8 (q, C-4′), 18.1 (q, C-5′).

*Isopimpinellin (**2**):* yellow needles ($CHCl_3$/MeOH); mp 147–148 °C; UV, IR, ^1H NMR, and ^{13}C NMR data consistent with literature values (Kincl et al., 1956; Elgamal et al., 1979).

*(R,S)-5-Methoxy-8-[(6,7-dihydroxy-3,7-dimethyl-2-octenyl)-oxy]psoralen (**3**):* needles ($CHCl_3$/ MeOH); mp 76–78 °C; $[\alpha]^{20}_{\lambda}$ 0° (*c* 2.3, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ϵ) 238 (3.65), 250 (3.61), 267 (3.71), 312 (3.56) nm; IR (dry film) ν_{max} 3445, 2963, 2854, 1717, 1588, 1479, 1371, 1149 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 8.13 (1H, d, J = 9.8 Hz, H-4), 7.63 (1H, d, J = 2.3 Hz, H-10), 7.00 (1H, d, J = 2.3 Hz, H-9), 6.28 (1H, d, J = 9.8 Hz, H-3), 5.66 (1H, br dd, J = 7.0, 7.0 Hz, H-2′), 4.88 (2H, m, H-1′), 4.18 (3H, s, OMe-5), 3.24 (1H, br d, J = 10.4 Hz, H-6′), 2.26 (1H, m, H-4′), 2.12 (1H, m, H-4′), 1.68 (3H, s, H-10′), 1.55 (1H, m, H-5′), 1.38 (1H, m, H-5′), 1.17 (3H, s, H-8′), 1.13 (3H, s, H-9′); ^{13}C NMR ($CDCl_3$, 75.6 Hz) δ 160.7 (s, C-2), 150.9 (s, C-7), 145.1 (d, C-10), 144.5 (s, C-5), 144.4 (s, C-8a), 142.6 (s, C-3′), 139.5 (d, C-4), 126.7 (s, C-8), 120.2 (d, C-2′), 114.5 (s, C-6), 112.7 (d, C-3), 107.5 (s, C-4a), 105.1 (d, C-9), 77.6 (d, C-6′), 73.0 (s, C-7′), 70.3 (t, C-1′), 60.7 (q, OMe-5′), 36.4 (t, C-4′), 29.2 (t, C-5′), 26.4 (q, C-8′), 23.0 (q, C-9′), 16.3 (q, C-10′); EIMS m/z M^+ missing, 232 (100), 217 (42), 189 (9), 161 (5); negative-ion ESMS m/z 401.10; positive-ion ESMS m/z 403.15.

*(R,S)-8-[(6,7-Dihydroxy-3,7-dimethyl-2-octenyl)oxy]psoralen (**4**):* needles ($CHCl_3$/MeOH); mp 92–94 °C; $[\alpha]^{20}_{\lambda}$ 0° (*c* 1.8, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ϵ) 238 (3.61), 250 (3.58), 267 (3.68), 312 (3.53) nm; IR (dry film) ν_{max} 3445, 2963, 2920, 2854, 1717, 1588, 1402, 1149, 1088 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.78 (1H, d, J = 9.6 Hz, H-4), 7.70 (1H, d, J = 2.2 Hz, H-10), 7.38 (1H, s, H-5), 6.82 (1H, d, J = 2.2 Hz, H-9), 6.37 (1H, d, J = 9.6 Hz, H-3), 5.67 (1H, br dd, J = 7.0, 7.0 Hz, H-2′), 5.03 (1H, dd, J = 7.0, 2.4 Hz, H-1′), 3.27 (1H, br d, J = 10.5 Hz, H-6′), 2.27 (1H, m, H-4′), 2.13 (1H, m, H-4′), 1.71 (3H, s, H-10′), 1.55 (1H, m, H-5′), 1.42 (1H, m, H-5′), 1.17 (3H, s, H-8′), 1.13 (3H, s, H-9′); ^{13}C NMR ($CDCl_3$, 75.6 Hz) δ 160.7 (s, C-2), 148.7 (s, C-7), 146.7 (s, C-10), 144.5 (d, C-4), 143.9 (s, C-8a), 142.8 (s, C-3′), 131.6 (s, C-8), 125.9 (d, C-6), 120.1 (d, C-2′), 116.5 (s, C-4a), 114.7 (d, C-3), 113.4 (s, C-5), 106.7 (d, C-9), 77.7 (d, C-6′), 73.0 (s, C-7′), 70.1 (t, C-1′), 36.4 (t, C-4′), 29.2 (t, C-5′), 26.4 (q, C-8′), 23.0 (q, C-9′), 16.5 (q, C-10′); EIMS m/z M^+ missing, 232 (100), 217 (41), 189 (8), 161 (5); negative-ion ESMS m/z 371.14; positive-ion ESMS m/z 373.13.

*Casimiroin (**5**):* needles ($CHCl_3$/MeOH); mp 202–203 °C; exhibited spectral (UV, IR, EIMS) data comparable to published values (Kincl et al., 1956; Toube et al., 1967); ^1H NMR ($CDCl_3$, 300 MHz) δ 7.53 (1H, d, J = 8.6 Hz, H-5), 6.78 (1H, d, J = 8.6 Hz, H-6), 6.04 (2H, s, H-9), 5.89 (1H, s, H-3), 3.91 (3H, s, OMe), 3.84 (3H, s, N-Me); ^{13}C NMR ($CDCl_3$, 75.6 Hz) δ 164.1 (s, C-2), 162.7 (s, C-4), 149.9 (s, C-7), 133.5 (s, C-8), 126.5 (s, C-8a), 118.0 (d, C-5), 113.0 (s, C-4a), 104.3 (d, C-6), 101.0 (d, C-9), 94.6 (d, C-3), 55.8 (q, OMe), 29.1 (q, NMe).

*4-Methoxy-1-methyl-2(1H)-quinolinone (**6**):* needles ($CHCl_3$/MeOH); mp 99–100 °C; exhibited spectral (UV, IR, ^1H NMR, EIMS) data comparable to published values (Nayar et al., 1971); ^{13}C NMR ($CDCl_3$, 75.6 Hz) δ 163.9 (s, C-2), 162.7 (s, C-4), 139.7 (s, C-8a), 131.2 (d, C-7), 123.4 (d, C-6), 121.7 (d, C-5), 116.5 (s, C-4a), 114.1 (d, C-8), 96.5 (d, C-3), 55.8 (q, OMe), 29.1 (q, NMe).

*5-Hydroxy-1-methyl-2-phenyl-4-quinolone (**7**):* needles ($CHCl_3$/MeOH); mp 174–175 °C; exhibited spectral (UV, IR, ^1H NMR, EIMS) data comparable to published values (Hart et al., 1968); ^{13}C NMR ($CDCl_3$, 75.6 Hz) δ 181.7 (s, C-4), 162.8 (d, C-5), 155.5 (s, C-2), 142.7 (s, C-8a), 135.3 (s, C-1′), 134.3 (d, C-7), 129.9 (d, C-4′), 128.9 (d, C-2′,6′), 128.4 (d, C-3′,5′), 113.9 (s, C-4a), 110.9 (d, C-6), 109.8 (d, C-8), 104.6 (d, C-3), 37.9 (q, NMe).

*γ-Fagarine (**8**):* yellow prisms ($CHCl_3$/MeOH); mp 142–143 °C; exhibited spectral (UV, IR, ^1H NMR, EIMS) data comparable to published values (Iriarte et al., 1956; Narasimhan and Mali, 1974); ^{13}C NMR ($CDCl_3$, 75.6 Hz) δ 163.2 (s, C-2), 156.8 (s, C-4), 154.5 (s, C-9), 143.9 (d, C-12), 137.4 (s, C-10), 123.4 (d, C-7), 119.6 (s, C-3), 114.0 (d, C-6), 107.6 (d, C-8), 104.5 (d, C-11), 103.8 (s, C-5), 59.0 (q, OMe-4), 55.9 (q, OMe-9).

*Zapotin (**9**):* prisms ($CHCl_3$/MeOH); mp 147–148 °C; exhibited spectral (UV, IR, ^1H NMR, EIMS) data comparable to published values (Kincl et al., 1956; Dreyer and Bertelli, 1967; Garratt et al., 1967); ^{13}C NMR ($CDCl_3$, 75.6 Hz) δ 178.2 (s, C-4), 158.8 (s, C-2), 158.5 (s, C-2′, 6′), 152.6 (s, C-5), 149.6 (s, C-4a), 147.8 (s, C-6), 132.0 (d, C-4′), 119.4 (s, C-8a), 118.8 (d, C-7), 115.2 (d, C-3), 113.8 (d, C-8), 111.4 (s, C-1′), 103.9 (d, C-3′, C-5′), 61.8 (q, OMe-5), 52.7 (q, OMe-6), 56.0 (q, OMe-2′, OMe-6′).

*5,6,2′-Trimethoxyflavone (**10**):* prisms ($CHCl_3$/MeOH); mp 125–126 °C; exhibited spectral (UV, IR, ^1H NMR, ^{13}C NMR, EIMS) data comparable to published values (Dreyer and Bertelli, 1967; Wollenweber et al., 1990).

RESULTS AND DISCUSSION

Through an antimutagenicity-guided bioassay procedure conducted with the ethyl acetate-soluble fraction of a methanol extract of the seeds of *C. edulis*, involving silica gel column chromatography and HPLC, 10 compounds were isolated. These substances comprised two

novel furocoumarins (**3** and **4**) along with two known furocoumarins [phellopterin (**1**) and isopimpinellin (**2**)], four alkaloids [casimiroin (**5**), 4-methoxy-1-methyl-2(1*H*)-quinolinone (**6**), 5-hydroxy-1-methyl-2-phenyl-4-quinolinone (**7**), and γ-fagarine (**8**)], and two flavonoids [zapotin (**9**) and 5,6,2′-trimethoxyflavone (**10**)].

Compounds **1**−**4** displayed similar UV spectra (λ_{max} 238, 250, 267, 312 nm), typical of compounds having a furocoumarin skeleton (Dreyer, 1968; Enríquez et al., 1984). This was supported by the ^{13}C NMR data of **1**−**4**, in addition to their ^{1}H NMR spectra, which showed characteristic resonances for two lactone protons (H-3 and H-4) and two furan protons (H-9 and H-10). The EIMS of compounds **1** and **2** did not exhibit the expected molecular ion (*m*/*z* 300 and 402, respectively), but in both cases a major ion at *m*/*z* 232 was observed along with three ions at *m*/*z* 217, 189, and 161, consistent with a 5,8-disubstituted psoralen-type furocoumarin skeleton (Saiki et al., 1974). Further structural features were determined by ^{1}H−^{1}H COSY, ^{13}C−^{1}H COSY, HMQC, and HMBC spectra. Therefore, **1** and **2** were identified as 5-methoxy-8-[(3-methyl-2-butenyl)oxy]psolaren (phellopterin) and 5,8-dimethoxypsolaren (isopimpinellin), respectively, by comparison of their physical and spectroscopic data with literature values (Dreyer, 1968; Elgamal et al., 1979). The ^{13}C NMR data for phellopterin (**1**) are reported for the first time.

The NMR spectra of compound **3** were very similar to those of **1** and **2**, except for the side chain attached at the C-8 position. The ^{13}C NMR spectrum revealed that the 10-carbon side chain contained a double bond, three methyl groups, three methylenes, and two methine carbons. In the ^{1}H NMR spectrum of **3**, a signal at δ 4.88 was assigned to the methylene protons at C-1′. The vinyl proton at C-2′ appeared as a broad doublet of doublets at δ 5.66. The signal at δ 1.68 was assigned to a vinyl methyl group at C-3′, and that at δ 3.24 was assigned to the proton attached to C-6′. The C-4′ and C-5′ methylene group signals appeared at δ 2.26 and δ 2.12, 1.55, and 1.38, respectively, and two terminal methyl groups appeared as singlets at δ 1.17 and 1.13. The structure of the side chain at C-8 was confirmed by HMBC NMR spectral observations, which showed cross-peaks between H-1′ and C-8 and C-2′, between H-2′ and C-4′ and C-10′, between H-6′ and C-5′, C-8′, and C-9′, between CH_3-8′ and C-6′, between CH_3-9′, and C-6′, and between CH_3-10′ and C-2′. Although EIMS (Saiki et al., 1974), CIMS, and FABMS did not afford of the molecular ion of **3**, the negative- and positive-ion ESMS analysis of **3** showed molecular ion peaks at *m*/*z* 401.10 and 403.15, respectively. Owing to a lack of any optical rotation ($[\alpha]_\lambda = 0°$), **3** was assigned as a racemic substance. The structure of **3** was characterized therefore as (*R,S*)-5-methoxy-8-[(6,7-dihydroxy-3,7-dimethyl-2-octenyl)oxy]psoralen.

The ^{1}H and ^{13}C NMR spectral data of compound **4** indicated the lack of any methoxyl group. The ^{13}C NMR spectrum of **4**, when compared with that of **3**, showed a significant upfield shift for the C-5 signal of 31.1 ppm (δ 113.4), accompanied by a downfield shift of the C-4a, C-6, and C-8 signals of 9.0, 11.4, and 4.9 ppm, respectively. In the ^{1}H NMR spectrum, a signal at δ 7.38 (1H, s) was assigned to the aromatic proton at C-5. Upfield shifts for the H-4 and H-9 signals of 0.35 and 0.18 ppm, respectively, were observed in the ^{1}H NMR spectrum of **4**, when compared with that of **3**. The negative- and positive-ion ESMS analyses of **4** showed molecular ion peaks at *m*/*z* 371.14 and 373.13, respectively. Like **3**, compound **4** was found to be optically inactive ($[\alpha]_\lambda = 0°$). Therefore, the structure of **4** was characterized as (*R,S*)-8-[(6,7-dihydroxy-3,7-dimethyl-2-octenyl)oxy]psoralen. The structure proposed for **4** was fully supported by its HMBC NMR spectrum.

Table 1. Evaluation of the Inhibitory Potential of Compounds 1−10

compd	*S. typhimurium*[a]	MMOC[b]	EROD[c]
1	7.9	20	1.8
2	10.6	0	1.5
3	1.9	25	>50
4	23.7	57	>50
5	3.6	80	>50
6	37.1	34	>50
7	10.5	36	16.3
8	nd[d]	nd	nd
9	>40	50	nd
10	9.7	80	<10[e]

[a] Inhibition of DMBA-induced mutagenesis with *S. typhimurium* strain TM677. Results are expressed as IC_{50} values (μg/mL). [b] Inhibition of DMBA-induced preneoplastic lesions with MMOC. Compounds were tested at a concentration of 10 μg/mL, and results are expressed as percent inhibition. On the basis of historical controls, inhibition of >60% is considered significant. [c] Inhibition of EROD activity determined with microsomes from the liver of Aroclor 1254-pretreated rats. Results are expressed as IC_{50} values (μg/mL). [d] nd, not determined. [e] Due to limited test material, EROD was tested at a fixed concentration of 10 μg/mL. At this test concentration, 87% inhibition was observed.

In a previous paper, 5-methoxy-8-geranylpsoralen was reported from *C. edulis* (Enríquez et al., 1984). Accordingly, it is possible that the side chains of compounds **3** and **4** are biosynthesized from geraniol as a biogenetic precursor via 6′,7′-epoxygeraniol. As a result of this process, racemization at the C-6′ position might occur.

Compounds **5**−**8** were positive for Dragendorff's reagent and identified as the known alkaloids 4-methoxy-1-methyl-7,8-methylenedioxy-2(1*H*)-quinolinone (casimiroin), 4-methoxy-1-methyl-2(1*H*)-quinolinone, 5-hydroxy-1-methyl-2-phenyl-4-quinolone, and 4,9-dimethoxyfuro[2,3-*b*]quinoline (γ-fagarine), respectively, by comparison of their physical and spectroscopic data with literature values (Iriarte et al., 1956; Kincl et al., 1956; Toube et al., 1967; Hart et al., 1968; Nayar et al., 1971; Narasimhan and Mali, 1974). ^{13}C NMR data are reported for these compounds in the present study.

The UV and NMR data of compounds **9** and **10** suggested a fully aromatic flavone ring system that was substituted by several methoxyl groups in each case. Compounds **9** and **10** were identified as 5,6,2′,6′-tetramethoxyflavone (zapotin) and 5,6,2′-trimethoxyflavone, respectively, by comparison of their physical and spectroscopic data with literature values (Kincl et al., 1956; Dreyer and Bertelli, 1967; Garratt et al., 1967; Wollenweber et al., 1990). The ^{13}C NMR data of **9** have not been reported before.

As indicated by the current paper (Table 1), and as previously demonstrated by Wall and co-workers (Wall, 1992; Wall et al., 1988), coumarins can serve as antimutagens, and 8-*O*-substitution enhances inhibitory potential. Antimutagens can be classified as desmutagens, those that interact with mutagens outside of the cell and deactivate them through chemical or enzymatic reactions (De Flora and Ramel, 1988; Kada et al., 1981), or as bioantimutagens, which can act on cells after mutagen-induced DNA damage has occurred (Kada et al., 1986), by interfering with cellular processes involved in the "fixation" of mutation, such as

error-prove DNA repair (Kuroda and Inoue, 1988) or replication. Other antimutagens can react with the mutagen or modulate (inhibit or enhance) metabolism of the mutagen (De Flora and Ramel, 1988). Because *S. typhimurium* strain TM677 lacks the uvrA DNA repair mechanism and contains pKM101, it may be speculated that the coumarins investigated in this paper function as desmutagens. However, because the test organism was treated simultaneously with DMBA and potential antimutagens, additional studies with various temporal sequences of exposure are required to unequivocally classify the mode of action [cf. De Flora et al. (1992)].

The present studies were conducted with liver preparations derived from Aroclor 1254-pretreated rats. Aroclor 1254 induces a broad spectrum of cytochrome P-450 (CYP) isozymes, including the CYP2 family, which appears to be involved in the metabolism of DMBA (O'Dowd et al., 1988). Furocoumarins **1**−**4**, each bearing substituents at the 8-position, inhibited DMBA-induced mutation with *S. typhimurium* strain TM677 (Table 1). Antimutagenic potential with *S. typhimurium* has previously been demonstrated with compound **2** (Schimmer et al., 1991), and both **1** and **2** were shown to inhibit the metabolic activity of CYP (Shin and Woo, 1990; Cai et al., 1993). This may relate to the mechanism of the antimutagenic response, as both **1** and **2** were also found to inhibit EROD activity. However, alternate mechanisms may also apply, because **3**, the most active antimutagen isolated during the course of this study, was not active as an inhibitor of EROD activity. Furthermore, because compound **4**, a weaker antimutagen, was inactive as an inhibitor of EROD activity, the nature of the R_2-substituent seems to have a strong bearing on this latter activity.

These data are of interest from a drug metabolism point of view. As shown previously, bergamottin, which contains a 5-geranyl substitution, is a potent inhibitor of EROD activity, which suggests inhibition of the CYP1A family. On the other hand, 6′,7′-dihydroxybergamottin was recently discovered in grapefruit juice as an inhibitor of CYP3A (Edwards et al., 1996), a human CYP family involved in the metabolism of drugs such as cyclosporin (Ducharme et al., 1995) and the carcinogen aflatoxin B_1 (Shimada and Guengerich, 1989). Preliminary studies indicate that **4** may also inhibit cyclosporin metabolism in rats (S. M. Chung, personal communication). Thus, the 6′,7′-dihydroxy moiety may confer specificity for cytochromes P-450 other than the CYP1A family, and further studies in this area are warranted.

However, isolates **1**−**4** do not appear very promising as cancer chemopreventive agents. As summarized in Table 1, these isolates did not mediate impressive inhibitory activity in the mouse mammary organ culture (MMOC) system, wherein a good correlation has been established between agents capable of inhibiting preneoplastic lesions and preventing mammary carcinogenesis in full-term animal studies (Mehta et al., 1991). Furthermore, it is known that many furocoumarins are phototoxic. Whereas this property is useful in a therapeutic setting, as in psoriasis photochemotherapy, in which psoralens are administered to patients followed by exposure to UVA light at the site of the disease (Melski et al., 1977), it is not desirable for long-term administration to a healthy population. However, **1** is not a human phototoxin (Kavli et al., 1983), and no specific data are available for the other three compounds. Nonetheless, lack of significant response in the MMOC system does not support the development of these isolates as chemopreventive agents.

Three alkaloids with varying antimutagenic activities, **5**, **6**, and **7**, were also isolated during the course of this study, with **5** being the most active (Table 1). A fourth alkaloid, γ-fagarine (**8**), was also isolated, but this substance is a known mutagen. For example, treatment of human lymphocytes with **8** induced sister-chromatid exchange (Schimmer and Leimeister, 1988). Furoquinoline alkaloids act as promutagens in *S. typhimurium* (Mizuta and Kanamori, 1985; Kanamori et al., 1986; Paulini et al., 1987), primarily by the frameshift mechanism (Hafele and Schimmer, 1988; Paulini et al., 1989). Because our studies with *S. typhimurium* TM677 confirmed the mutagenic potential of **8**, no further characterization of biological activity was performed.

Of the alkaloids isolated from *C. edulis* and tested, in addition to functioning as the most potent antimutagen, **5** significantly inhibited DMBA-induced lesions with the MMOC system. Compound **6** was ∼10-fold less active as an antimutagen and weakly active in the MMOC system. Neither compound inhibited EROD activity. However, compound **5** was further investigated and found to be ineffective in the process of cell differentiation as judged by treatment of HL-60 cells (ED_{50} > 4 μg/mL) and unable to inhibit the induction of ODC by treatment of mouse 308 cells with TPA (ED_{50} > 4 μg/mL). Therefore, the mode of action in the MMOC system remains unknown. Alkaloid **7**, although active as an antimutagen and an inhibitor of EROD activity, was not active in the process of inhibiting DMBA-induced preneoplastic lesions.

Last, two structurally related flavonoids were isolated, one of which (**10**) was an active antimutagen bearing promising cancer chemopreventive activity as judged by the MMOC system (Table 1). Under a limited set of test conditions, inhibition of EROD activity was observed. Moreover, **10** induced cell differentiation with cultured HL-60 cells (ED_{50} = 1.4 μg/mL) and inhibited TPA-induced ODC activity with mouse 308 cells (ED_{50} = 0.3 μg/mL). On the basis of this activity profile, further studies are warranted to more fully characterize the chemopreventive potential of this agent.

The cancer chemopreventive ability of *C. edulis* and its constituents must be confirmed by in vivo carcinogenesis studies. In terms of dietary material, the resulting response will obviously depend on the overall profile of activity mediated by the sum total of the constituents. It is difficult to speculate on the nature of such a response, but it is clear that certain constituents (especially **5** and **10**) show promise as chemopreventive agents. Future studies will be directed toward further characterization of the biologic potential of these materials.

ABBREVIATIONS USED

CYP, cytochrome P-450; DMBA, 7,12-dimethylbenz[*a*]anthracene; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EIMS, electron impact mass spectrum; EROD, ethoxyresorufin *O*-deethylase; ESMS, electrospray mass spectrum; HL-60, human promyelocytic leukemia; MMOC, mouse mammary organ culture; NADP, β-nicotinamide adenine dinucleotide phosphate; NADPH, β-nicotinamide adenine dinucleotide phosphate, reduced form; NBT,

nitroblue tetrazolium; NSE, nonspecific acid esterase; ODC, ornithine decarboxylase; PBS, phosphate-buffered saline; PLP, pyridoxal phosphate; SE, specific acid esterase; TMS, tetramethylsilane; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

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