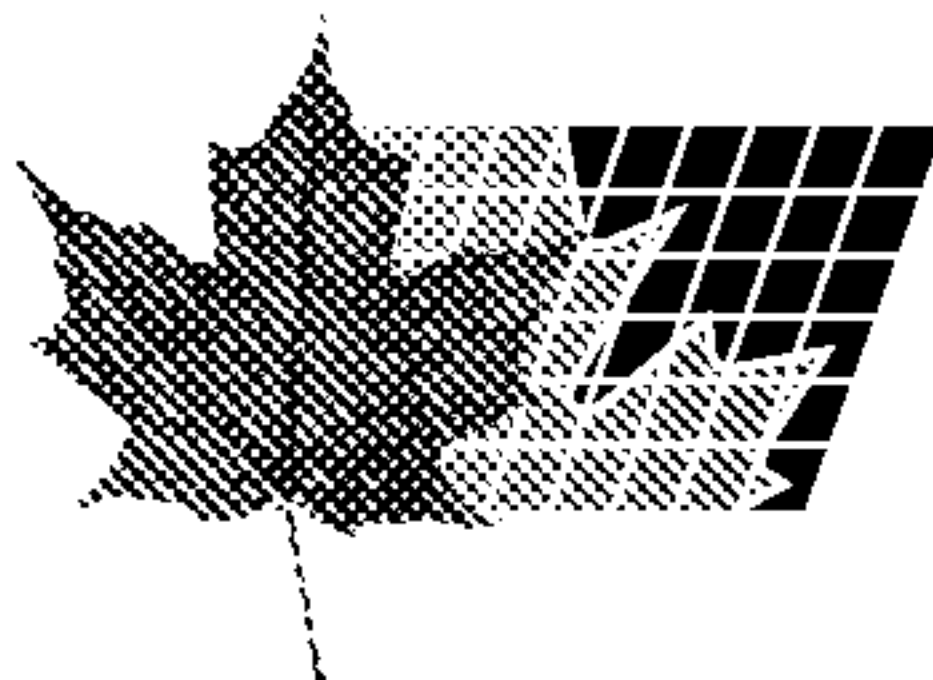




- (72) GUTHRIE, NAJLA, CA
(72) MANTHEY, JOHN A., US
(72) HOROWITZ, ROBERT M. (Deceased), (UNKNOWN)
(71) KGK SYNERGIZE INC., CA
(71) GUTHRIE, NAJLA, CA
(71) USDA-ARS-OTT, US
(51) Int.Cl.⁶ A61K 31/35
(30) 1998/10/06 (09/167,634) US
(54) **COMPOSITIONS ET PROCEDES POUVANT INHIBER DES
MALADIES NEOPLASTIQUES AU MOYEN DE COMPOSES
APPARENTES A LIMOCITRINE ET 5-DESMETHYL-
SINENSETINE**
(54) **COMPOSITIONS AND METHODS OF INHIBITING
NEOPLASTIC DISEASES WITH COMPOUNDS RELATED TO
LIMOCITRIN AND 5-DESMETHYL SINENSETIN**

(57) Compositions and methods for the prevention and treatment of neoplastic diseases and atherosclerosis are described. Individuals at a high risk of developing or having neoplasia or atherosclerosis undergoing conventional therapies may be treated with an effective dose of limocitrin compounds including, but not limited to, 3,5,7,4'-tetramethoxylimocitrin, limocitrin 3,5,7,4'-trimethyl ether 5-acetate, 3,5,7,4'-tetramethoxylimocitrin or 3,7,4'-trimethoxylimocitrin, and 5-desmethyl sinensetin.

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 31/35	A1	(11) International Publication Number: WO 00/19998 (43) International Publication Date: 13 April 2000 (13.04.00)
(21) International Application Number: PCT/US99/23238 (22) International Filing Date: 5 October 1999 (05.10.99) (30) Priority Data: 09/167,634 6 October 1998 (06.10.98) US (71) Applicants: KGK SYNERGIZE [CA/CA]; The University of Western Ontario, Suite 130, 100 Collip Circle, Research Park, London, Ontario N6G 4X8 (CA). USDA-ARS-OTT [US/US]; Building 005, BARC-W, Room 407, Beltsville, MD 20705 (US). (71)(72) Applicant and Inventor: GUTHRIE, Najla [CA/CA]; Suite 2401, 389 Dundas Street, London, Ontario N6B 3L5 (CA). (72) Inventors: MANTHEY, John, A.; 104 Gregory Circle, Auburndale, FL 33823 (US). HOROWITZ, Robert, M. (deceased). (74) Agent: KARMAI, Rashida, A.; Stroock & Stroock & Lavan LLP, 180 Maiden Lane, New York, NY 10038-4982 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: COMPOSITIONS AND METHODS OF INHIBITING NEOPLASTIC DISEASES WITH COMPOUNDS RELATED TO LIMOCITRIN AND 5-DESMETHYL SINENSETIN (57) Abstract Compositions and methods for the prevention and treatment of neoplastic diseases and atherosclerosis are described. Individuals at a high risk of developing or having neoplasia or atherosclerosis undergoing conventional therapies may be treated with an effective dose of limocitrin compounds including, but not limited to, 3,5,7,4'-tetramethoxylimocitrin, limocitrin 3,5,7,4'-trimethyl ether 5-acetate, 3,5,7,4'-tetramethoxylimocitrin or 3,7,4'-trimethoxylimocitrin, and 5-desmethyl sinensetin.		

WO 00/19998

PCT/US99/23238

COMPOSITIONS AND METHODS OF INHIBITING
NEOPLASTIC DISEASES WITH COMPOUNDS RELATED
TO LIMOCITRIN AND 5-DESMETHYL SINENSETIN

1. INTRODUCTION

The present invention relates to compositions and methods for the prevention and treatment of neoplastic cells and diseases, especially breast cancer, with compounds derived from limocitrin compounds. The present invention also relates to compositions and methods for the prevention and treatment of atherosclerosis, thrombosis, inflammatory diseases, allergies or viral diseases. These compounds are a group of naturally occurring and/or synthetic flavonoids including, but not limited to 5-desmethyl sinensetin, and the limocitrin-derived compounds, 3,5,7,4'-tetramethoxylimocitrin, 3,7,4'-trimethoxylimocitrin, 3,5,7,4'-tetraethoxylimocitrin or limocitrin-3,5,7,4'-trimethyl ether 5-acetate.

2. BACKGROUND

Limocitrin analogues are a group of citrus-derived flavonoids that are naturally occurring in the plant or are chemically synthesized. 5-desmethoxy sinensetin is chemically synthesized from sinensetin (Tatum, J.H. et al., 1972, Phytochemistry II: 2283-2288), although it has been reported to occur in trace quantities in mandarin leaves (Sugiyama, S. et al., 1993, chem. Pharm. Bull. 41:714-719. Flavonoids are polyphenolic compounds that occur ubiquitously in foods of plant origin. The major dietary sources of flavonoids are vegetables, fruits and beverages such as tea and red wine (Hertog, M.G.L. et al., 1993, J. Agric. Food Chem 41:1242-1246).

Plants have evolved flavonoids as protection against parasites, herbivores, pathogens and oxidative cell injury. In addition, flavonoids contribute to the color of fruits and vegetables. Cook, N.C. et al., 1996, J. Nutr. Biochem. 7:66-76. The average intake of dietary flavonoids in the United States has been estimated at 1g/day expressed as glycosides,

of which about 170 mg expressed as aglycones, consist of flavonols, flavonones and flavones (Kühnau, J., 1976, World Rev. Nutr. Diet 24:f117-191). Flavonol and flavone intake thus exceeds that of other dietary antioxidants such as beta-carotene (2-3mg/day) and vitamin E (7-10mg/day) and equals approximately one-third of vitamin C (70-100mg/day) (Nutrient intakes. Individuals in 48 states. Year 1977-1978. Report No. I-2. Consumer Nutrition Division, Human Nutrition Information Service. Hyattsville, M.D.: U.S.D.A. 1984). Flavonoids have been demonstrated to be the most potent dietary antioxidants and in light of the large dietary consumption, flavonoids make a major contribution to the antioxidant potential of the human diet. The main food sources of flavonols and flavones are black tea, onions, apples, herbs and spices, cloves or black pepper (Hertog, M.G.L., et al., 1992, J. Agric. Food Chem. 40:2379-2383).

2.1 Epidemiological Studies

Epidemiological studies consistently show an inverse association between the consumption of fruits and vegetables and cancer risk at various sites and a positive association with dietary factors such as fats and total calorie intake (Block, G., et al., 1992, Nutr. Cancer 17:1-29). With numbers of diet-related cancer deaths elevated and is still rising, use of nutraceuticals have become increasingly popular in North America. "Nutraceuticals" used herein are defined as any substance that may be considered a food or part of a food and provides medical or health benefits, including the prevention and treatment of disease. Until recently, natural medicine catered primarily to a limited population. Now, however, many health care organizations are giving new consideration to alternative therapies. A potential U.S. market for nutraceuticals has been estimated at two hundred fifty billion dollars, four times that for conventional medicine. Presently, the share of sales for nutraceuticals used as fortifiers accounts for nearly half of one percent of the estimated three hundred billion dollar

U.S. processed food and beverage industry, and that translates into a one and one half billion dollar market.

On the average, participants with the highest consumption of fruits and vegetables experience a fifty percent reduction in risk of cancers of the alimentary and respiratory tract compared to participants with the lowest intakes. Fruit and vegetable consumption could be a marker for other aspects of lifestyle which are responsible for the lower cancer rates, but it is possible that fruits and vegetables contain flavonoids such as quercetin that prevent cancer (Steinmetz, K.A., et al., 1991, Cancer Causes Control 2:325-357).

The association between quercetin and cardiovascular disease has been studied in prospective cohort studies and cross-cultural ecological studies. Flavonol and flavone intake was inversely associated with mortality from coronary heart disease and to a lesser extent with incidence of first myocardial infarction. These effects were independent of known risk factors for coronary heart disease such as serum cholesterol, body mass index, blood pressure, smoking and intake of antioxidant vitamins, alcohol, and fat. Flavonol and flavone intake (mainly quercetin) was also inversely associated with stroke risk (Hertog, M.G.L. et al., 1993, Lancet 324:1007-1011; Keli, S.O., et al., 1996, Arch. Inter. Med. 154:637-642). However, four thousand different types of flavonoids have been described and it is crucial that the active components be identified not only to make a positive impact on agriculture but also to more specifically use these nutraceuticals as anticancer agents and/or antithrombotic, anticoronary heart disease, antimyocardial infarction and/or antistroke agents.

2.2 Cancer Growth and Chemotherapy

Cancer is a disease of inappropriate tissue accumulation. Chemotherapeutic agents share one characteristic: they are usually more effective in killing or damaging

malignant cells than normal cells. However, the fact that they do harm normal cells indicates their potential toxicity. Animal tumor investigations and human clinical trials have shown that drug combinations produce higher rates of objective response and longer survival than single agents. Combination drug therapy is, therefore, the basis for most chemotherapy employed at present (DeVita, V.T. et al., 1995, Cancer 35:98).

Cancer treatment requires inhibition of a variety of factors including tumor cell proliferation, metastatic dissemination of cancer cells to other parts of the body, invasion, tumor-induced neovascularization, and enhancement of host immunological responses and cytotoxicity. Conventional cancer chemotherapeutic agents have often been selected on the basis of their cytotoxicity to tumor cells. However, some anticancer agents have adverse effects on the patient's immune system. Thus it would be greatly advantageous if a cancer therapy or treatment could be developed that would afford non-cytotoxic protection against factors that might lead to progression of tumors.

Because hormone therapy as well as chemotherapy is effective in controlling advanced breast cancer, it has been used as an adjuvant to mastectomy in primary breast cancer. Patients with estrogen receptor + (ER+) or estrogen receptor - (ER-) tumors benefit from adjuvant chemotherapy. However, tamoxifen used alone as an adjuvant to mastectomy for breast cancer shows benefit in extending disease-free and overall survival (Cummings, F.J. et al., 1985, Ann. Intern. Med. 103:324). While there are various methods for treating cancerous cells and diseases, there remains a need in the art for at least reducing replication of neoplastic cells, especially in disease states such as breast cancer. There also remains a need in the art for methods for reducing the incidences of cardiovascular diseases. The present invention is different from the related art and provides for methods and compositions for using limocitrin analogues and 5-desmethyl sinensetin in treating neoplastic cells, and reducing cardiovascular disease.

3. SUMMARY OF THE INVENTION

The present invention is directed to a method for the prevention and/or treatment of neoplastic diseases, which involves using a composition of limocitrin analogues and 5-desmethyl sinensetin to treat an individual at high risk for, or suffering from cancer.

The present invention is also directed to a method for the prevention and/or treatment of breast cancer, which involves using a composition of limocitrin analogues and 5-desmethyl sinensetin in individuals at high risk for breast cancer.

The present invention is also directed to a method for the prevention and/or treatment of atherosclerosis, myocardial infarction, stroke or thrombosis, which involves using a composition of limocitrin analogues and 5-desmethyl sinensetin in an individual in need thereof.

The present invention is further directed to a method for inducing anti-inflammatory, anti-allergic, anti-viral and/or estrogenic activity, which involves using a composition of limocitrin analogues and 5-desmethyl sinensetin in an individual in need thereof.

The present invention is directed to the use of limocitrin analogues and 5-desmethyl sinensetin and tamoxifen in an individual suffering from breast cancer.

The present invention is directed to a method for the prevention and/or treatment of neoplastic diseases, which involves using an effective dose of a combination of one or more limocitrin analogues and 5-desmethyl sinensetin, with or without conventional chemotherapy or hormonal and/or radiation therapy or surgery, in a patient suffering from cancer.

The present invention is also directed to a method for inhibiting the oxidation of low-density lipoproteins and platelet aggregation and adhesion, which involves using an effective dose of a composition of limocitrin compounds.

The present invention is also directed to a method of administering an effective amount of a limocitrin compound to cancerous cells to reduce proliferation of said cells in vitro and in vivo.

4. DETAILED DESCRIPTION OF THE INVENTION

The method of the invention involves administering an effective dose of a one or a combination of limocitrin analogues and 5-desmethyl sinensetin, tamoxifen or a chemotherapeutic agent, in an individual who is identified as being at enhanced risk for cancer and/or as having cancer, in order to prevent and/or treat cancer.

It may be that the ability of these compounds to inhibit tumor cell proliferation, to inhibit the metastatic spread of tumor cells or to prevent immuno-suppression induced by chemotherapeutic agents, contributes to their effectiveness in the prevention and treatment of neoplastic diseases. These possible mechanisms of action are in no way meant to limit the scope of the invention and are presented purely for explanatory and/or illustrative purposes.

The method of the invention also involves administering an effective dose of limocitrin analogues and 5-desmethyl sinensetin to an individual who is identified as being at enhanced risk for atherosclerosis or thrombosis and/or as having atherosclerosis, stroke, myocardial infarction or thrombosis, in order to prevent and treat coronary heart diseases.

It may be that the ability of the flavonoids to inhibit platelet aggregation and adhesion, contributes to their effectiveness in the prevention and treatment of atherosclerosis, stroke, myocardial infarction and thrombosis. These possible mechanisms of action are in no

way meant to limit the scope of the invention and are presented purely for explanatory and/or illustrative purposes.

Cancer

Cancer is the second leading cause of death in the United States, after heart disease (Boring, C.C. et al., 1993, CA Cancer J. Clin. 43:7), and develops in one in three Americans, and one of every four Americans dies of cancer. Cancer can be viewed as a breakdown in the communication between tumor cells and their environment, including their normal neighboring cells. Signals, both growth-stimulatory and growth-inhibitory, are routinely exchanged between cells within a tissue. Normally, cells do not divide in the absence of stimulatory signals, and likewise, will cease dividing in the presence of inhibitory signals. In a cancerous, or neoplastic state, a cell acquires the ability to "override" these signals and to proliferate under conditions in which normal cells would not grow.

In addition to unhindered cell proliferation, cells must acquire several traits for tumor growth to occur. For example, early on in tumor development, cells must evade the host immune system. Further, as tumor mass increases, the tumor must acquire vasculature to supply nourishment and remove metabolic waste. Additionally, cells must acquire an ability to invade adjacent tissue, and ultimately cells often acquire the capacity to metastasize to distant sites.

Breast cancer is a major health problem in most industrialized countries. It has been estimated that 184,300 new invasive cases of breast cancer occurred among women in the United States in 1996. In North American women, characteristics that are associated with a threefold to fourfold increase in risk for breast cancer include (1) first-degree female family members (mother and sisters) who had breast cancer, (2) prior breast cancer, (3) nulliparity, (4) age greater than 30 years at first pregnancy and (5) early menarche or late menopause (Sattin, R.W. et al., 1985, JAMA 253:1908). International studies have demonstrated a

positive correlation between per capita consumption of fat and alcohol and the incidence of breast cancer. (Carroll and Schatazkin A. et al., 1987, N. Engl. J. Med. 316:1169). Several studies have linked the consumption of fresh fruits and vegetables, and vitamin E with reduced risk of developing cancer, including breast cancer (Steinmetz, K.A. et al., 1991, Cancer Causes Control 2:427-442). Although this protective effect has been generally attributed to the antioxidant capacities of vitamin C and beta-carotene present in these foods, it may be related to other phytochemical constituents such as flavonoids. The use of analogues of limocitrins and of 5-desmethyl sinensetin alone or in combination with a cancer chemo-therapeutic agent has not been reported for the prevention and treatment of neoplastic diseases. During recent years, conventional therapy used in prevention and treatment of cancer has become increasingly supplemented with substances that are considered as nutraceuticals or considered as foods or parts of foods but which provide health benefits. As dietary components, the methoxylated flavones are anticipated to have very low cytotoxicity, and accordingly as cancer treating and/or preventing agents, the methoxylated and ethoxy flavones show very low toxicity compared with polyhydroxylated flavones. Thus, the methoxylated and ethoxylated limocitrin analogues and 5-desmethyl sinensetin of the present invention will also have this important advantage over other dietary anti-cancer flavonoids.

The present invention provides a number of different limocitrin compounds including but not limited to the two naturally occurring methoxylated limocitrin analogues (3,5,7,4'-tetramethoxylimocitrin and 3,7,4'-trimethoxylimocitrin) and two synthetic limocitrin analogues (limocitrin 3,7,4'-trimethyl ether 5 acetate and limocitrin 3,5,7,4'-tetraethyl ether). The present invention further provides the structurally-related compound, 5-desmethyl sinensetin.

Cancers that can be prevented and/or treated by the compositions and methods of the present invention include, but are not limited to, human sarcomas and carcinomas, e.g.

carcinomas, e.g., colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chondroma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma, retinoblastoma; leukemias, e.g., acute lymphocytic leukemia and acute myelocytic leukemia (myeloblastic, promyelocytic, myelomonocytic, monocytic and erythroleukemia); chronic leukemia (chronic myelocytic (granulocytic) leukemia and chronic lymphocytic leukemia); and polycythemia vera, lymphoma (Hodgkin's disease and non-Hodgkin's disease), multiple myeloma, Waldenstrom's macroglobulinemia, and heavy chain disease. Specific examples of such cancers are described in the sections below.

4.1 Atherosclerosis thrombosis and stroke

In the United States, the complications of atherosclerosis account for about one half of all deaths and for about one third of deaths in persons between 35 and 65 years of age. Atherosclerosis, or the development of atheromatous plaques in large and medium-sized arteries, is the most common form of arteriosclerosis. Many factors are associated with the acceleration of atherosclerosis, regardless of the underlying primary pathogenic change, for example, age, elevated plasma cholesterol level, high arterial blood pressure, cigarette

smoking, reduced high-density lipoprotein (HDL) cholesterol level, or family history of premature coronary artery disease.

The risk of death from coronary artery disease has a continuous and graded relation to total serum cholesterol levels greater than 180 mg/dl (Stamler, J. et al., 1986, JAMA 256 : 2823). Approximately one third of adults in the United States have levels that exceed 240 mg/dl and, therefore, have a risk of coronary artery disease that is twice that of people with cholesterol levels lower than 180mg/dl. Acceleration of atherosclerosis is principally correlated with elevation of LDL, or beta fraction, which is rich in cholesterol but poor in triglycerides. Elevation of HDL or alpha fraction, has a negative correlation with atherosclerosis (Castelli, W.P. et al., 1986, JAMA 256 : 2835). HDL exerts a protective effect and the ratio of total cholesterol to HDL cholesterol is a better predictor of coronary artery disease than the level of either alone. Total cholesterol levels are classified as being desirable (<200 mg/dl), borderline high (200-239 mg/dl), or high (>240 mg/dl) (Report of the National Education Program Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults, 1988, Arch. Intern. Med. 148 : 36).

Advances in the study of cholesterol metabolism and coronary disease have initiated an era of increased emphasis on preventive therapy. New guidelines for the detection and treatment of high blood cholesterol in adults recommend that patients with high cholesterol levels or with borderline-high levels and two or more additional risk factors should have a measurement of LDL. LDL cholesterol levels are then classified as borderline-high risk (130-159 mg/dl) or high risk (≥ 160 mg/dl). Dietary treatment is recommended for those patients with high-risk levels of LDL and for those with borderline-high risk levels who have two or more additional risk factors. Drug treatment is recommended for all patients with LDL levels greater than 189 mg/dl and for those patients with LDL cholesterol levels between 159 and 189 mg/dl who have two or more additional risk factors. Among the many

drugs that have been used to reduce serum cholesterol levels are cholestyramine, colestipol, clofibrate, gemfibrozil and lovastatin. The use of limocitrins or 5-desmethyl sinensetin alone or in combination with a cholesterol-lowering drug has not been reported for the treatment of hypercholesterolemia.

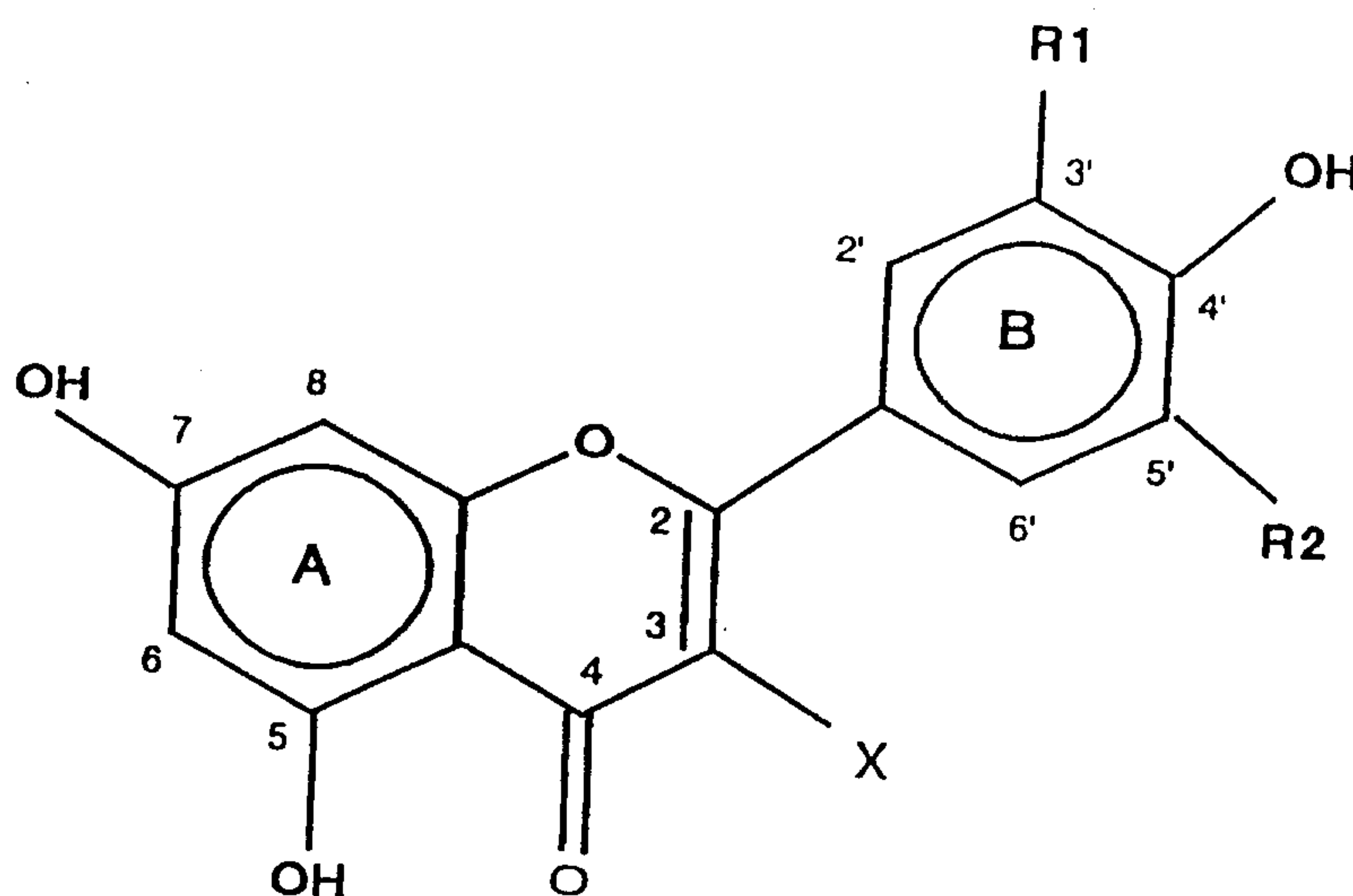
Platelet-blood vessel interactions are implicated in the development of thrombosis. Flavonoids inhibit platelet aggregation and adhesion (Frankel, E.N. et al., 1993, Lancet 341:1103-1104). Flavonoids antagonize thromboxane formation and increase platelet cyclic AMP levels. This is important because in addition, flavonoids scavenge free radicals and their antioxidant actions participate in their antithrombotic action (Gryglewski, R.J. et al., 1987, Biochem. Pharmacol. 36:317-322). Drug treatment is recommended for patients with thrombosis and ischemic heart disease. The medical therapy includes, but is not limited to aspirin and the combined use of beta-adrenergic blocking agents (e.g., propranolol, nadolol, timolol, etc.), nitrates (e.g., nitroglycerin) and calcium channel blockers (e.g., verapamil, nifedipine, diltiazem, etc.).

Four compounds were synthesized from the lemon flavonoid limocitrin 3',8-dimethoxy-3,5,7,4'-tetrahydroxyflavone in the present invention. Limocitrin occurs in the peel of lemon as limocitrin-3-O-glucoside, and can be produced from the 3-glucoside by enzymatic hydrolysis (Horwitz R.M., et al., 1960, J. Org. Chem. 25:21885-21887) or by a chemical synthesis procedure reported by Dryer D.L., et al., 1964, Tetrahedron 20:2977-2983. Two limocitrin analogues, 4,3,7-trimethoxylimocitrin and 3,5,6,4'-tetramethoxylimocitrin, also occur in orange peel (Tatum J.H., et al., 1972, Phytochemistry II: 2283-2288).

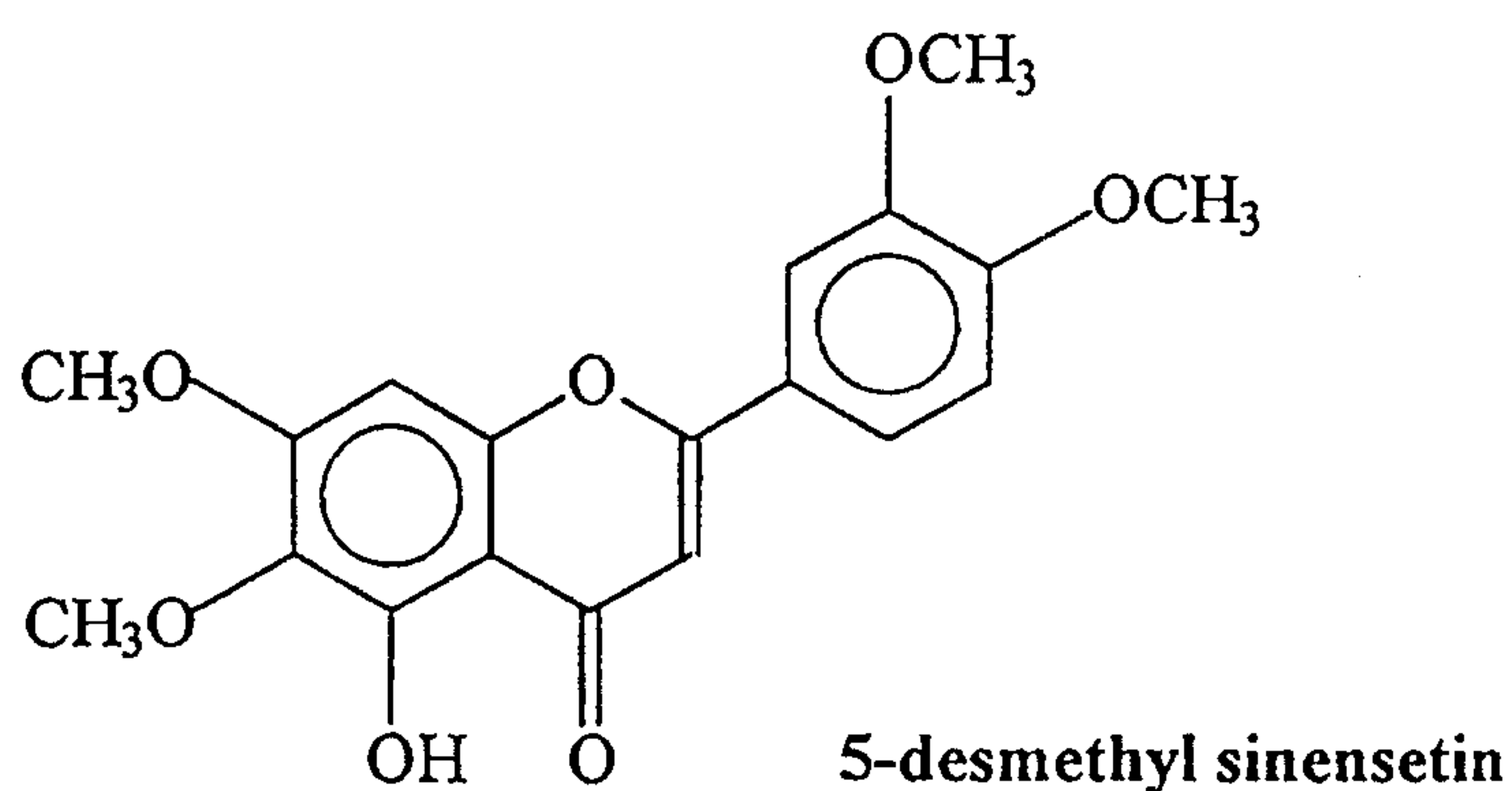
The present invention provides a number of different analogues of limocitrin including 3,5,7,4'-tetramethoxy limocitrin (3,5,7,8,3',4' hexamethoxyflavone or limocitrin-3,5,7,4'-tetramethyl ether), 3,7,4'-trimethoxylimocitrin (or limocitrin-3,7,4' trimethyl ether), 3,5,7,4'-tetraethoxylimocitrin (or limocitrin 3,5,7,4'-tetraethyl ether), limocitrin-3,7,4'

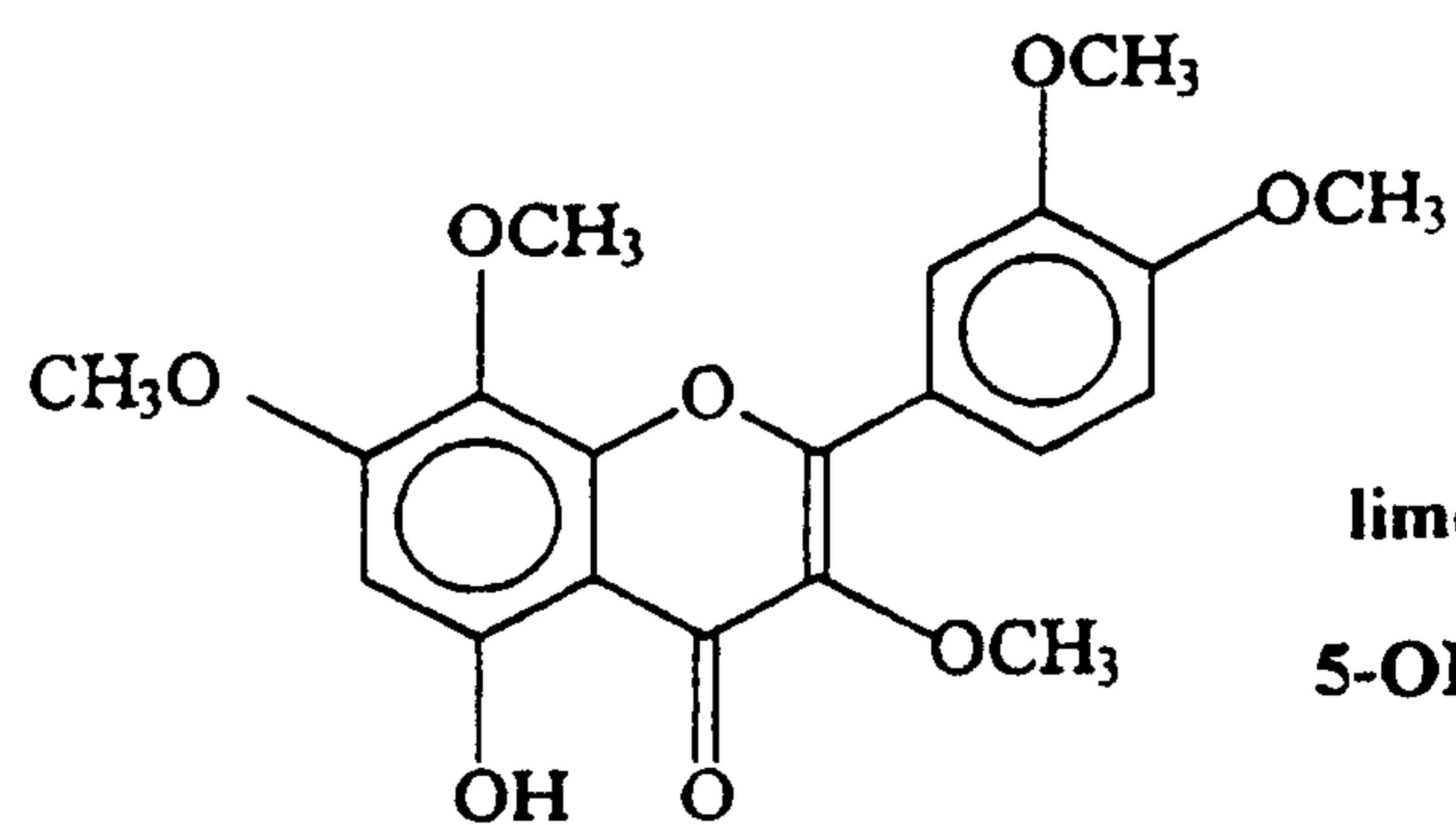
trimethyl ether 5-acetate, 3,7,4' trimethoxy limocitrin (or 5-hydro-3,7,8,3',4' pentamethoxy flavone); and 5-desmethyl sinensetin (or 5-hydroxy-6,7,3',4'-tetramethoxy flavone).

Flavonoids share the common skeleton of diphenylpyrans, e.g., two benzene rings A and B, linked through a heterocyclic pyran or pyrone ring C in the middle. This basic structure allows a multitude of substitution patterns and variations in the different rings, giving rise to flavonols, flavones, catechins, flavanones, anthocyanidins and isoflavonoids (Kühnau, J., 1976, World Rev. Nutr. Diet 24:117-191).

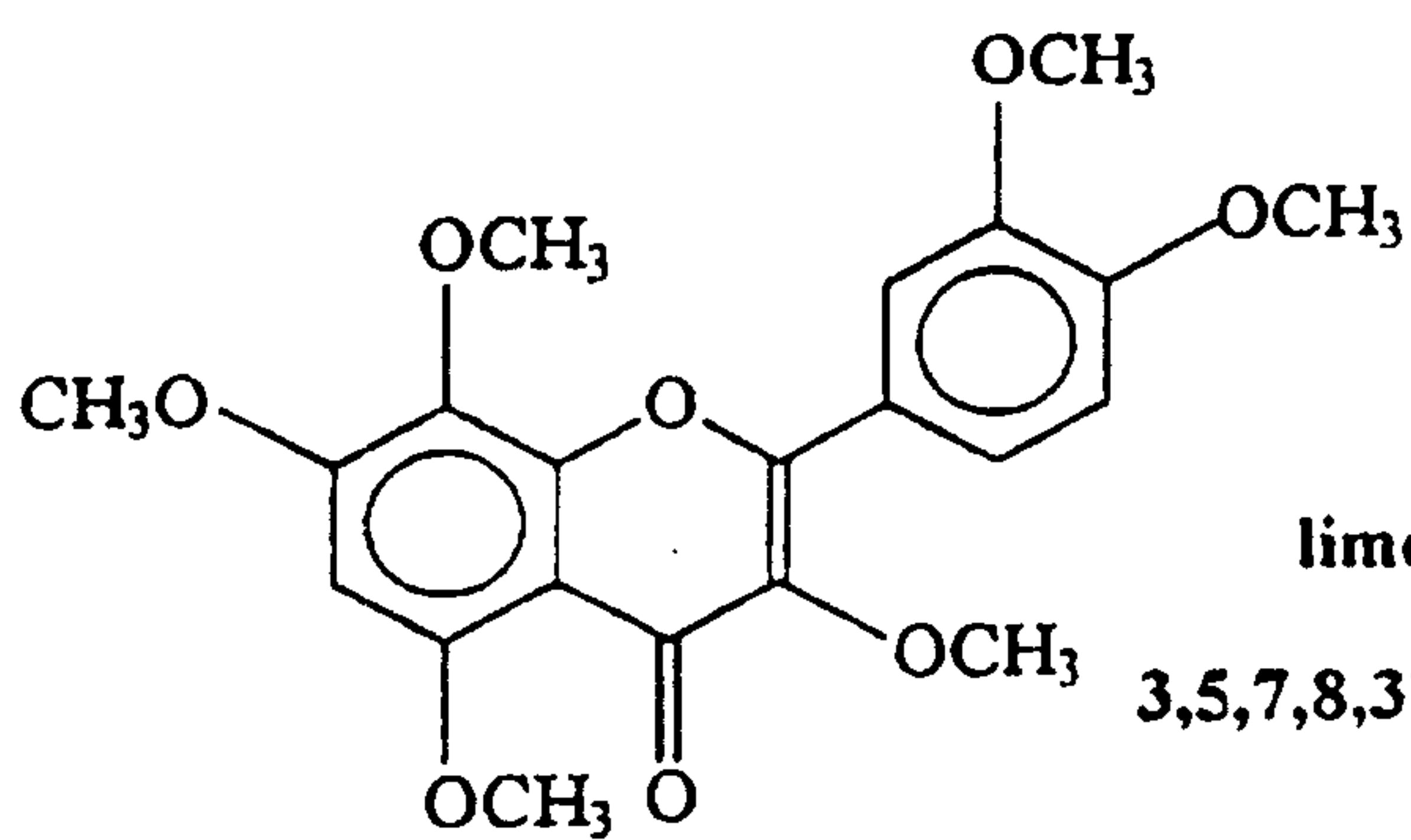


The basic structures of limocitrin analogues and 5-desmethylinensetin are similar to the above flavonoid structure. Specific analogues are represented below:

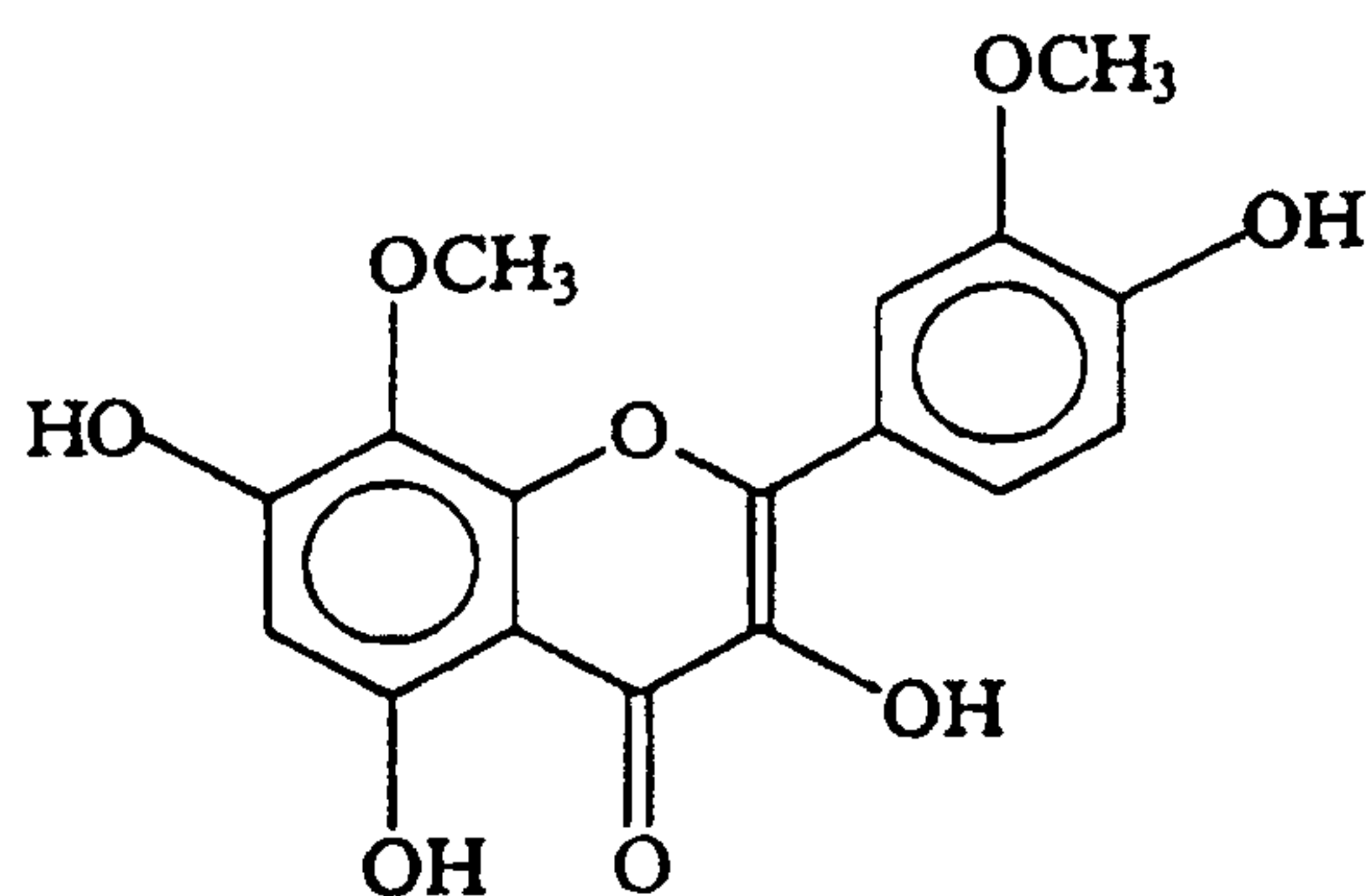




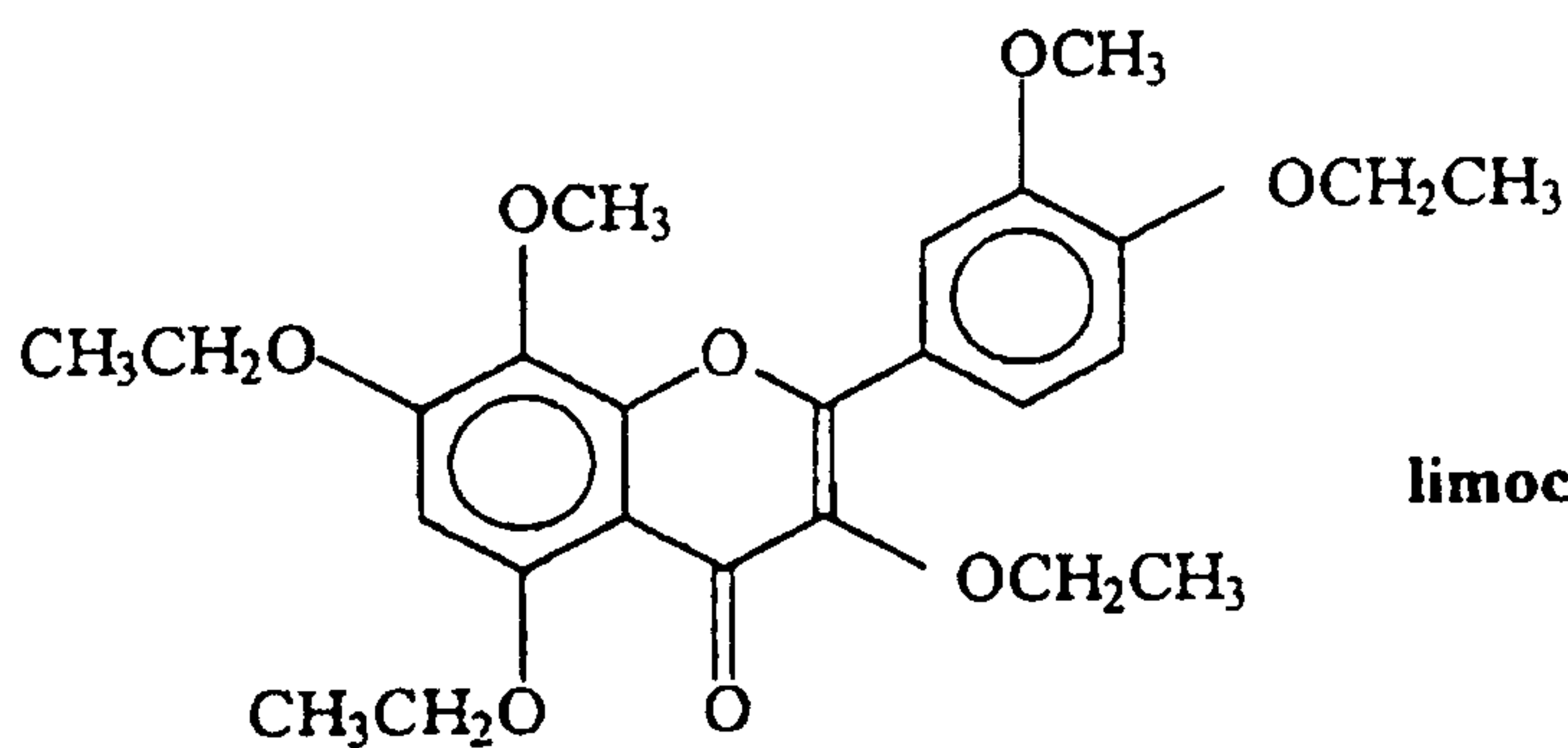
limocitrin 3,7,4'-trimethyl ether
5-OH-3,7,8,3',4'-pentamethoxyflavone



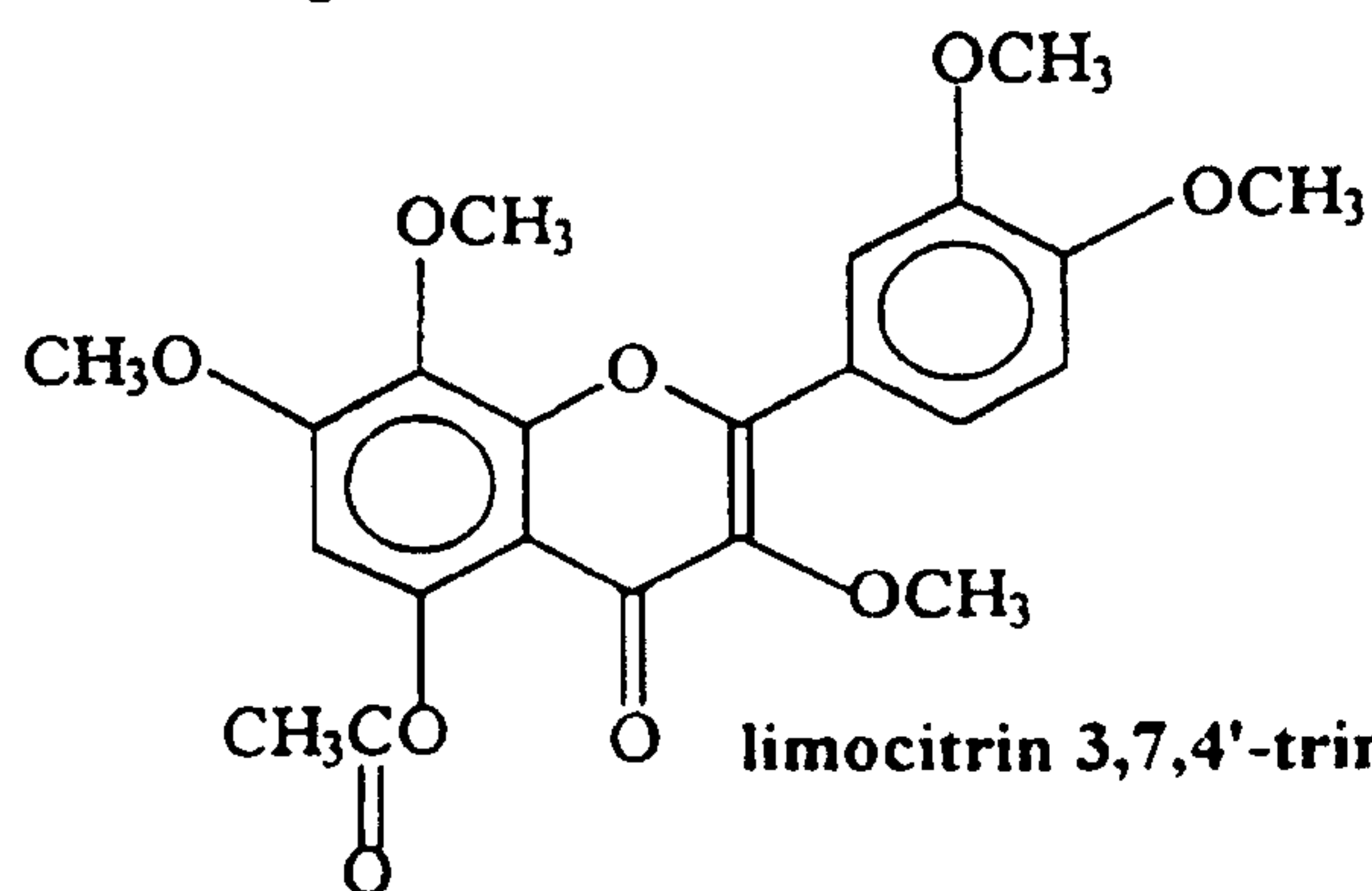
limocitrin 3,5,7,4'-tetramethyl ether
3,5,7,8,3',4'-hexamethoxyflavone



limocitrin
3,5,7,4'-tetrahydroxy-8,3'-dimethoxyflavone



limocitrin 3,5,7,4'-tetraethyl ether



limocitrin 3,7,4'-trimethyl ether-5-acetate

4.2 Dosage and Formulations.

Compounds structurally related to limocitrin and 5-desmethyl sinensetin may be formulated into pharmaceutical preparations for administration to humans for prevention and treatment of neoplastic diseases and/ or cardiovascular disease, atherosclerosis, thrombosis, myocardial infarction or stroke.

Many of the limocitrin and 5-desmethyl sinensetin compounds may be provided as compounds with pharmaceutically compatible counterions, a form in which they may be soluble.

The therapeutic compounds or pharmaceutical compositions may be administered intravenously, intraperitoneally, subcutaneously, intramuscularly, intrathecally, orally, rectally, topically or by aerosol.

Formulations suitable for oral administration include liquid solutions of the active compound dissolved in diluents such as saline, water or PEG 400; capsules or tablets, each containing a predetermined amount of the active agent as solid, granules or gelatin; suspensions in an approximate medium; and emulsions.

Formulations suitable for parenteral administration include aqueous and non-aqueous isotonic sterile solutions, which contain buffers, antioxidants and preservatives. The formulations may be in unit dose or multi-dose sealed containers.

Patient dosages for oral administration of limocitrins range from 1-1000 mg/day, commonly 1-500 mg/day, and typically from 1-100 mg/day. Stated in terms of patient with 70 kg body weight, usual dosages range from 0.01-15 mg/kg/day, commonly from 0.01-7.0 mg/kg/day, typically from 0.01 to 2.0 mg/kg/day.

Patient dosages for oral administration of synthetic limocitrin analogues range from 200-5000 mg/day, commonly 1000-2000 mg/day, and typically from 500-1500 mg/day.

Stated in terms of patient body weight, usual dosages range from 3-70 mg/kg/day, commonly from 14-30 mg/kg/day, typically from 7-21 mg/kg/day.

Dosage amount and interval may be adjusted individually to provide plasma levels of the active moiety which are sufficient to maintain the anti-proliferative, antioxidative and anti-metastatic effects.

Alternatively, one may administer the compound in a local, rather than oral manner, for example, via injection of the compound directly into a tumor, often in a depot or sustained release formulation.

A variety of delivery systems for the pharmacological compounds may be employed, including, but not limited to, liposomes and emulsions. The pharmaceutical compositions also may comprise suitable solid or gel phase carriers or excipients. Examples of such carriers or excipients include, but are not limited to, calcium carbonate, calcium phosphate, various sugars, starches, cellulose derivatives, gelatin, and polymers such as polyethylene glycols.

Furthermore, one may administer the agent in a targeted drug delivery system, for example, in a liposome coated with tumor-specific antibody. The liposomes will be targeted to and taken up selectively by the tumor.

In cases of local administration or selective uptake, the effective local concentration of the drug may not be related to plasma concentration.

5. EXAMPLE: EFFECT OF CITRUS LIMOCITRINS IN MDA-MB-435

CELLS AND MCF-7 ESTROGEN RECEPTOR (ER)-POSITIVE HUMAN BREAST CANCER CELLS

The effect of limocitrin analogues on the proliferation and growth of MDA-MB-435 estrogen receptor negative human breast cancer cells was studied in vitro, as measured by the incorporation of [³H] Thymidine.

Materials

The following compounds were synthesized from the lemon flavonoid limocitrin according to the procedures described by Horowitz, R.M. et al., J. Org. Chem.26:2899-2902: 4'3,7-trimethoxylimocitrin (5-hydroxy-3,7,8,3'4'-pentamethoxyflavone); 3,5,7,4'-tetramethoxylimocitrin(3,5,7,8,3',4'-hexamethoxyflavone);3,5,7,4'-tetra-O-ethyllimocitrin; and limocitrin-3,7,4'-trimethyl ether-5-acetate. A total of 23 compounds were tested (See Table 1) in the MDA-MB-435 ER-ve and MCF-7 ER+ve cells. Tissue culture medium and fetal calf serum were purchased from Gibco, Burlington, ON. [³H] Thymidine was purchased from ICN, Irvine, CA.

Cell Culture

MDA-MB-435 estrogen receptor-negative human breast cancer cells were maintained at 37°C in a minimum essential medium, supplemented with 10% (v/v) fetal bovine serum. The medium was equilibrated with a humidified atmosphere of 5% CO₂. Stock cultures were seeded at a density of 2 x 10⁴ cells/ml and allowed to multiply for 48 to 72 hours. MCF-7 estrogen receptor-positive human breast cancer cells were routinely maintained in minimum essential medium supplemented with 10% (v/v) fetal calf serum, 1 mM sodium pyruvate, 10µg/ml bovine insulin and 1% (v/v) antibiotic-antimycotic agents (10,000 units/ml penicillin G sodium, 10,000 µg/ml streptomycin sulfate and 25 µg/ml

amphotericin B in 0.85% saline). Cells were grown to confluence at 37°C in a humidified atmosphere containing 5% CO₂ in air and were passaged weekly, using 2% trypsin in citrate saline.

Incorporation of [³H] Thymidine into DNA

MDA-MB-435 cells were plated at 2×10^4 cells/well in 96-well, flat bottomed culture plates in a total volume of 200 μ l of medium and incubated at 37°C for 48 hours with or without test compounds. [³H] Thymidine (0.5 μ Ci/well) was then added and after 4 hours the cells were harvested onto a glass fiber filter paper using a semiautomatic 12-well cell harvester. Radioactivity on the filter paper was counted using Scintiverse in a liquid scintillation counter. The MCF-7 cells were seeded at a density of 2×10^4 cells/well in 96-well, flat bottomed tissue culture plates and were incubated at 37°C for 5 days. The compounds were then added at varying concentrations. The plates were incubated at 37°C for 2 days. Tritiated thymidine (0.5 μ Ci) was then added to each well to determine the number of dividing cells at each concentration. Four hours later, the medium and excess radioactive label were removed. Citrate saline with trypsin was added, the cells were harvested onto glass fiber filter paper and the radioactivity was counted. The percent of dividing cells was determined by comparing the number of cpm for the treated cells to that for the control cells.

Viability of Cells

Viability of cells was measured by MTT assay (Hansen, M.B. et al., J. Imm. Meth., 119, 203-210). In this assay a tetrazolium salt, MTT, is converted to a blue formazan product by dehydrogenases that are active in living cells. The intensity of the blue color developed is a measure of cell viability. MDA-MB-435 cells (8×10^4 /well) were seeded with various concentrations of the compounds in a 96-well plate in a total volume of 200 μ l of

medium. MTT (25 μ l of 5 mg/ml) was added to each well. After 3 hours, 100 μ l of extraction buffer consisting of 20% SDS dissolved in a DMF water (1/1) solution at pH 4.0 was added. The blue color formed was measured at 590 nm in a Dynatech MRX Microplate Reader. A similar assay technique is used for the MCF-7 cell line. The percentage of cells surviving was determined by comparing the absorbance of the treated cells with that of the control.

Results

Results are the average of 3 experiments. The two synthetic analogues, limocitrin 3,5,7,4'-tetraethyl ether and limocitrin 3,7,4'-trimethyl ether 5-acetate were the most effective. These compounds have IC_{50} of 0.5 and 0.9 ppm in estrogen receptor-negative (ER-) and 0.08 and 0.05 ppm in estrogen receptor-positive (ER+) human breast cancer cells respectively. The 3,5,7,4'-tetramethoxylimocitrin had comparable activity in the ER- cells but was less effective in ER+ cells with IC_{50} of 0.8 and 0.2 ppm respectively. The 3,7,4'-trimethoxylimocitrin was the least effective having an IC_{50} of 3.1 ppm in ER- cells and 0.5 in ER+ cells (Table 1). Tamoxifen, a drug widely used for the treatment of ER+ tumors has an IC_{50} of 0.04 ppm in ER+ cells but is not effective in ER- cells (IC_{50} 90 ppm). Tamoxifen is found to be toxic with serious side effects in some patients. These results indicate that the limocitrin compounds are potent inhibitors of both ER- and ER+ cells and are less likely to pose the problems of toxicity and side effects posed by tamoxifen.

TABLE 1

IC₅₀s in MDA-MB-435 estrogen receptor-negative and MCF-7 estrogen receptor-positive human breast cancer cells:

	ER-	ER+
	IC ₅₀	(μ g/mL)
1. Tangeretin	0.50	0.40
2. Heptamethoxyflavone	0.40	0.80
3. tetra-O-methylscutellarein	0.30	0.20
4. sinensetin	1.50	0.20
5. nobiletin	0.50	0.80
6. 5,6,7,8,4'-pentamethoxyflavone	0.50	0.40
7. 5,6,7,8,3',4'-tetramethoxyflavone	0.50	0.80
8. 5-hydroxy-6,7,8,3',4'-tetramethoxyflavone	0.30	0.09
9. 5,7,8,4'-tetramethoxyflavone	2.1	1.5
10. 5-hydroxy-6,7,8,3',4'-pentamethoxyflavone	0.90	0.50
11. gossypetin 3,7,8,3'4'-pentamethylether	0.80	0.20
12. 5-desmethyl sinensetin	0.02	0.01
13. quercetin tetramethylether	9.5	5.1
14. quercetin 3,5-dimethylether,7,3',4'-tribenzyl ether	2.3	1.9
15. quercetin tetramethyl ether	9.5	5.1
16. quercetin pentamethyl ether	7.4	7.0
17. quercetin 5,6,3',4'-tetramethylether 3-acetate	3.4	1.2
18. quercetin 5,7,3',4'-tetramethyl ether	9.5	5.1
19. 3,7,4'-trimethoxylimocitrin	3.1	0.5
20. 3,5,7,4'-tetramethoxylimocitrin	0.8	0.2
21. 3,5,7,4'-tetraethoxylimocitrin	0.5	0.08
22. limocitrin 3,7,4'-trimethyl ether 5-acetate	0.9	0.05
23. Tamoxifen	90	0.04

The present invention is not to be limited in scope by the embodiments disclosed in the examples which are intended as an illustration of one aspect of the invention and any methods which are functionally equivalent are within the scope of the invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims.

WO 00/19998

PCT/US99/23238

20

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

What is claimed is:

1. A method of preventing or inhibiting the growth of a cancer in an individual comprising administering to an individual in need thereof a therapeutically effective amount of a composition comprising a limocitrin.
2. The method according to claim 1 wherein the limocitrin comprises 3,5,7,4'-tetramethoxylimocitrin, limocitrin-3,5,7,4'-trimethyl ether-5 acetate, 3,5,7,4'-tetramethoxylimocitrin or 3,7,4'-trimethoxylimocitrin.
3. The method according to claim 1 further comprising one or more limocitrins.
4. The method according to claim 1 further comprising an effective amount of a chemotherapeutic agent.
5. The method according to claim 1 further comprising an effective amount of tamoxifen.
6. The method according to claim 3 further comprising an effective amount of a chemotherapeutic agent.
7. The method according to claim 1 wherein the cancer is selected from the group consisting of colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chondroma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung

carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma, retinoblastoma; leukemias, e.g., acute lymphocytic leukemia and acute myelocytic leukemia; chronic leukemia; polycythemia vera, lymphoma, multiple myeloma, Waldenstrom's macroglobulinemia, and heavy chain disease.

8. The method according to claim 1 wherein the composition is administered orally, intravenously, intraperitoneally, subcutaneously or intramuscularly.
9. A composition suitable for administering to a human subject for the prevention or treatment of cancer, said composition comprising at least two citrus limocitrins.
10. A method of preventing or inhibiting atherosclerosis in an individual comprising administering to an individual in need thereof a therapeutically effective amount of a composition comprising a limocitrin.
11. The method according to claim 10 wherein the limocitrin comprises limocitrin 3,5,7,4'-tetraethyl ether, limocitrin 3,7,4'-trimethyl ether-5 acetate, 3,5,7,4'-tetramethoxylimocitrin or 3,7,4'-trimethoxylimocitrin.
12. The method according to claim 10 further comprising administering a therapeutically effective amount of at least two limocitrins.
13. The method according to claim 10 further comprising administering a therapeutically effective amount of a cholesterol-lowering drug.
14. The method according to claim 12 further comprising administering a therapeutically effective amount of a cholesterol-lowering drug.
15. A method of preventing or inhibiting thrombosis in an individual comprising administering to an individual in need thereof a therapeutically effective amount of a composition comprising a limocitrin.

16. The method according to claim 15 wherein the citrus limocitrin comprises 3,5,7,4'-tetramethoxylimocitrin, limocitrin-3,5,7,4'-trimethyl ether-5 acetate, 3,5,7,4'-tetramethoxylimocitrin or 3,7,4'-trimethoxylimocitrin.
17. The method according to claim 15 further comprising administering a therapeutically effective amount of at least two limocitrins.
18. The method according to claim 15 further comprising administering a therapeutically effective amount of an antithrombotic drug.
19. The method according to claim 17 further comprising administering a therapeutically effective amount of an antithrombotic drug.
20. A composition suitable for administering to a human subject for the prevention or treatment of cancer, said composition comprising at least two limocitrins.
21. A method of preventing or inhibiting coronary heart disease in an individual comprising administering to an individual in need thereof a therapeutically effective amount of a composition comprising a limocitrin.
22. The method according to claim 21 wherein the citrus limocitrin comprises 3,5,7,4'-tetramethoxylimocitrin, limocitrin-3,5,7,4'-trimethyl ether-5 acetate, 3,5,7,4'-tetramethoxylimocitrin or 3,7,4'-trimethoxylimocitrin.
23. The method according to claim 21 further comprising administering a therapeutically effective amount of at least two limocitrins.
24. The method according to claim 21 further comprising administering a therapeutically effective amount of a cholesterol-lowering drug.
25. The method according to claim 23 further comprising administering a therapeutically effective amount of a cholesterol-lowering drug.

26. A composition suitable for administering to a human subject for the prevention or treatment of coronary heart disease, said composition comprising at least two limocitrins.
27. A composition suitable for administering to a human subject for the prevention or treatment of thrombosis, said composition comprising at least two limocitrins.
28. A method comprising administering an effective amount of a limocitrin compound to cancerous cells to reduce proliferation of said cells.
29. The method according to claim 28 wherein said limocitrin compounds are selected from the group consisting of 3,5,7,4'-tetramethoxylimocitrin, limocitrin-3,5,7,4'-trimethyl ether-5 acetate, 3,5,7,4'-tetramethoxylimocitrin or 3,7,4'-trimethoxylimocitrin, and mixtures thereof.
30. The method according to claim 28 wherein said administering is in vitro.
31. The method according to claim 1 further comprising 5 desmethylinensetin.
32. The method according to claim 31 further comprising an effective amount of a chemotherapeutic agent.
33. The method according to claim 31 further comprising an effective amount of tamoxifen.
34. The method according to claim 31 further comprising an effective amount of two or more limocitrins.
35. The method according to claim 31 wherein the cancer is selected from the group consisting of colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chondroma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma,

lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma, retinoblastoma; leukemias, e.g., acute lymphocytic leukemia and acute myelocytic leukemia; chronic leukemia; polycythemia vera, lymphoma, multiple myeloma, Waldenstrom's macroglobulinemia, and heavy chain disease.

36. The method according to claim 31 wherein the composition is administered orally, intravenously, intraperitoneally, subcutaneously or intramuscularly.

37. A method of preventing or inhibiting atherosclerosis in an individual comprising administering to an individual in need thereof a therapeutically effective amount of a composition comprising a limocitrin and 5-desmethyl sinensetin.

38. The method according to claim 38 further comprising administering a therapeutically effective amount of a cholesterol-lowering drug.

39. A method of preventing or inhibiting thrombosis in an individual comprising administering to an individual in need thereof a therapeutically effective amount of a composition comprising a limocitrin and 5 desmehtylsinensetin.

40. The method according to claim 39 further comprising administering a therapeutically effective amount of an antithrombotic drug.

41. A composition suitable for administering to a human subject for the prevention or treatment of cancer, said composition comprising at least a limocitrins and 5 desmethyl sinensetin.
42. A method of preventing or inhibiting coronary heart disease in an individual comprising administering to an individual in need thereof a therapeutically effective amount of a composition comprising a limocitrin and 5 desmethyl sinensetin.
43. The method according to claim 43 further comprising administering a therapeutically effective amount of a cholesterol-lowering drug.
44. A composition suitable for administering to a human subject for the prevention or treatment of coronary heart disease, said composition comprising a limocitrin and 5 desmethyl sinensetin.
45. A composition suitable for administering to a human subject for the prevention or treatment of thrombosis, said composition comprising a limocitrin and 5 desmethyl sinensetin.