This is to certify that the

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Synthesis and in Vitro Metabolism of Soybean Isoflavones

presented by

Yu-Chen Chang

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SYNTHESIS AND IN VITRO METABOLISM OF SOYBEAN ISOFLAVONES

By

Yu-Chen Chang

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ABSTRACT

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Soybean diets are known to decrease the number of tumors in rats induced by chemical carcinogens or by irradiation. Also, epidemiological studies indicated a negative correlation between the soybean consumption and breast cancer incidence. Published data showed that soybean isoflavones inhibited the growth of human breast cancer cells in vitro. However, in vivo studies of pure compounds were not available due to the unavailability of these compounds in substantial quantities. Several metabolites of genistein and daidzein were detected in human urine, nevertheless, their metabolic pathways remained unknown. It is our hypothesis that the isoflavone metabolites detected in the human urine are produced from the isoflavones in food by intestinal bacteria. Also, these metabolites may function as better anticancer agents than their parent isoflavones present in soy products.

A rapid two-step syntheses of isoflavones daidzein, genistein, formononetin and biochanin A, were accomplished. The intermediate ketones were first synthesized from commercially available and low cost starting materials. The ketones were then cyclized in a conventional microwave oven within 2 min to yield the respective isoflavones. The structures

of the ketones and isoflavones were confirmed by their ^{1}H- and ^{13}C-NMR spectra. This method provided a convenient and faster way to yield larger quantities of genistein and daidzein for biological studies.

Synthetic daidzein and genistein were incubated with human fecal bacteria under anaerobic conditions. Dihydrodaidzein, benzopyran-4,7-diol, 3-(4-hydroxyphenyl) and equol were isolated from the fermentation broth of daidzein. Only one metabolite, dihydrogenistein, was isolated and characterized from the fermentation broth of genistein. The metabolites isolated from this fermentation study were identical to the metabolites detected in human urine.

The metabolites isolated and characterized from the fermentation studies were synthesized in appreciable quantities for their efficacy studies. Equol, 5,7,4'-trihydroxyisoflavan, 4,7,4'-trihydroxyisoflavan, dihydrodaidzein, and dihydrogenistein were synthesized either from daidzein or genistein by hydrogenation. Antifungal, antibacterial, mosquitocidal, nematicidal and anticancer activities of these compounds were evaluated. Equol was the most active compound among all the metabolites assayed. Equol was also found to be anticarcinogenic in assays with mutant *Saccharomyces cerevisiae* stains.

To my husband, Pao-Chi

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I would like to thank my major advisor, Dr. Muraleedharan Nair, for his encouragement and help to fulfill my dream. He brought me into the research field of bioactive natural products and opened a magnificent view in front of me. I would also like to express my appreciation to my guidance committee members, Dr. Wayne Loescher, Jack Kelly, J. Ian Gray and William Helferich, for their advises and kindness.

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My husband, Pao-Chi, deserves my greatest thank. We have known each other for

more than ten years. He seems to know me more than I do. He always knows what is good for me and put that on top of his priority. Without his encouragement and understanding, I would never have the opportunity to write this dissertation. Thank you, thank everybody.

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ACN	Acetonitrile
$BF_3 \cdot Et_2O$	Boron trifluoride etherate
BHI	Brain heart infusion media
CFU	Colony forming unit
DMBA	7,12-dimethylbenz[α]anthrancene
DMF	Dimethyl formamide
DMSO	Dimethyl sulfoxide
EIMS	Electron impact ionization mass spectrometer
EGF	Epidermal growth factor
HPLC	High performance liquid chromatrography
$MeSO_2Cl$	Methanesulfonyl chloride
MNU	N-methyl-N-nitrosourea
NMR	Nuclear magnetic resonance
PDA	Photodiode array detector
PDA	Potato dextrose agar
PDGF	Platelet-derived growth factor
Pd/C	Palladium on active carbon
PTK	Protein tyrosine kinase
SHBG	Sex hormone binding globulin
THF	Tetrahydrofuran
TLC	Thin layer chromatography
VAM	Vesicular-arbuscular micorrhiza
VLC	Vacuum liquid chromatography
^{1}H-NMR	Proton nuclear magnetic resonance
^{13}C-NMR	^{13}Carbon nuclear magnetic resonance
δ	Chemical shifts
dd	doublet of doublet
J	Coupling constant
m.p.	Melting point
m/z	Mass-to-charge ratio
rel. int.	Relative intensity

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CHAPTER I

Literature Review

Breast cancer is one of the most prominent cancers among women in the United States. Approximately 1 in 8 women is diagnosed with breast cancer each year. Several epidemiological studies revealed that the incidence of breast cancer among women is higher in Western countries and lower in Asia (Haenszel and Kurihara, 1968; Armstrong and doll, 1975; Lee et al., 1991; Aldercreutz, 1990). When women with low risk of breast cancer immigrated to countries with a high incidence of breast cancer, their breast cancer risk became similar to the risk of host-country women (Haenszel and Kurihara, 1968; Staszewski et al., 1971; Aldercreutz, 1990; Locke and King, 1980; King and Locke, 1980; King et al., 1985; Dunn, 1977; Buell, 1973, Tominage, 1985). These observations suggest that breast cancer occurrence in these women is associated with environmental differences as well as with genetic differences (Barnes et al., 1990).

Dietary Fat and Breast Cancer

High fat and high calorie diets are correlated positively with breast cancer occurrence (Drasar and Irving, 1973; Armstrong and Doll, 1975). A positive correlation of fat intake and incidence of breast cancer was observed by several researchers (Talamini et al., 1984; Lubin

et al., 1986; Hislop et al., 1986). Also, several animal feeding studies confirmed the effects of dietary fat on the occurrence of breast cancer. In one study, rats were fed with diets containing high levels (20 %) of polyunsaturated fatty acids. The results showed a significant increase in the incidence and number of tumors induced by carcinogens and a decrease in tumor latency of these rats compared with rats fed with 5 % fat (Carrol et al., 1986; Ip and Sinha, 1981; Ip et al., 1985;). Similarly, a positive correlation was observed between the total fat intake and plasma estrone and estradiol levels among American and Oriental women (Goldin et al., 1986). It was found that the gut microbial β-glucuronidase activity was significantly increased with high fat diets and resulted in enhanced enterohepatic circulation of estrogens along with high plasma levels of androgens and estrogens (Goldin et al., 1982). However, the epidemiological studies of the relationship between dietary fat intake and breast cancer risk are controversial (Graham et al., 1982). Willett et al. (1987) reported that there is no positive correlation between dietary fat intake and the risk of breast cancer among U. S. women 34 to 59 years of age and having no history of cancers. Similarly, association between fat consumption and the occurrence of breast cancer was not observed among Japanese women (Hirohata et al., 1987).

Soybeans and Breast Cancer

One of the dietary factors which contribute to a lower breast cancer risk among Asian and the US women is the inclusion of soy products in their diet. It was reported that there is an inverse correlation between soy intake and the risk of breast cancer among 142,857 women in Japan observed for 17 years (Messina et al., 1994). Another study of the breast

cancer in Singapore among Chinese women with and without breast cancer revealed a negative correlation between high soy diet and the risk of breast cancer (Lee et al., 1991).

Aldercreutz et al.(1986[a,b], 1987, 1988) reported a strong positive correlation of the plasma sex-hormone binding globulin (SHBG) concentration, urinary phytoestrogen and lignan excretion with fiber intake in vegetarians and omnivores, and a negative correlation for these observations and breast cancer. The SHBG is an important transporter of estrogens and androgens. A higher SHBG concentration in plasma results in lower bioavailability of sex hormones in the circulatory system. The plasma levels of estrogens was 3-fold higher among American women than among Oriental women studied for breast cancer occurrence (Golding et al., 1986).

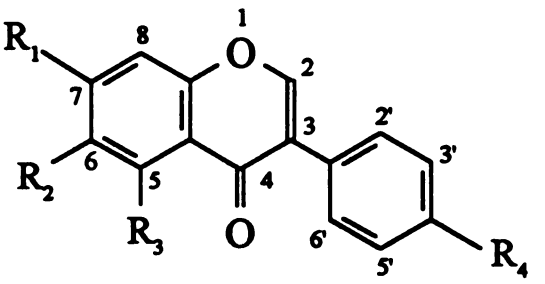
It was shown that chimpanzees are resistant to breast cancer and contain large amounts of phytoestrogens and their metabolites in the urine (Aldercreutz et al. 1986[b]). Similar excretion of phytoestrogens was observed from human vegetarians. Only small amounts of lignans and phytoestrogens were found in the urine from breast cancer patients. Therefore, it was suggested that the phytoestrogens present in vegetables and soybeans may play an important role in the prevention of breast cancer in chimpanzees and human vegetarians (Aldercreutz et al., 1986[b]).

In rats, soybean diets reduced mammary tumor occurrence induced by irradiation (Troll et al., 1980) and the carcinogen, N-methyl-N-nitrosourea (MNU) (Barnes et al., 1988). Troll et al. (1980) suggested that the protease-inhibitory activity of soy-rich diets may contribute to the reduction of cancer. However, Barnes et al. (1990) deactivated the protease inhibitors

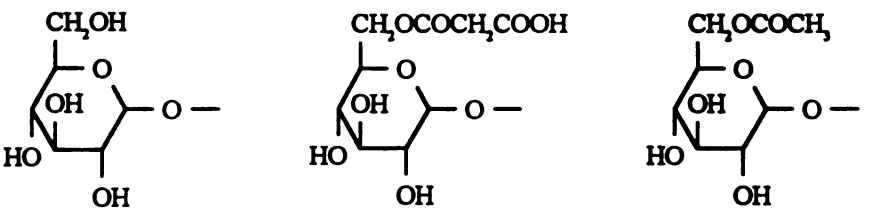
present in soybean by autoclaving the soybean chips for 40 min. When rats were fed these autoclaved soybean chips, the appearance of mammary tumors induced by the carcinogens, MNU or 7,12-dimethylbenz[α]anthrancene (DMBA), were not observed. There was no difference in the degree of inhibition between non-autoclaved and autoclaved soybean chips. Therefore, the protease inhibitors were not responsible for the tumor-reducing effect of soybean.

Isoflavone Contents of Soybeans and Soy Products

Soybeans contain high levels of isoflavonoids (Figure 1.1), various quantitative analysis of isoflavone contents in soybeans and soy foods have been reported. Naim et al. (1974) analyzed soy syrup obtained as a residue from the extraction of commercially available defatted soybean flakes. The results showed that all the isoflavones in soyflakes were present as glycosides with less than 1 % as of their aglycones. The percentage of isoflavones in soybean was about 0.25 %, of which 64 % was genistin, 23 % daidzin and 13 % glycitein 7-*O*-β-glycoside (Table 1.1). Eldridge and Kwolek (1983) also analyzed the isoflavonoid contents in many soybean varieties grown in different locations in Illinois, USA during 1980. Significant interactions among variety and location were observed for the total and individual isoflavonoid content. Anatomical distribution of soybean isoflavones in two variety of soybeans, Amsoy and Tiger, also was described by Eldridge and Kwolek (1983) (Table 1.2). It was found that hypocotyl and the hull contained 90 and 1 % of the total isoflavones, respectively. Therefore, dehulled soybean have little effect on the total isoflavonoid content. It was reported that the isoflavones predominantly exist as the 7-O-glucoside 6"-malonyl



R_1	R_2	R_3	R_4	
OH	H	H	OH	Daidzein
O-Glu	H	H	OH	Daidzin
OH	H	H	OMe	Formononetin
OH	H	OH	OH	Genistein
O-Glu	H	OH	OH	Genistin
OH	H	OH	OMe	Biochanin A
OH	OMe	H	OH	Glycitein
O-Glu	OMe	H	OH	Glycitin
O-6"-OMalGlu	H	H	OH	6"-O-Malonyldaidzin
O-6"-OMalGlu	H	OH	OH	6"-O-Malonylgenistin
O-6"-OMalGlu	OMe	H	OH	6"-O-Malonylglycitin
O-6"-OAcGlu	H	H	OH	6"-O-Acetyldaidzin
O-6"-OAcGlu	H	OH	OH	6"-O-Acetylgenistin
O-6"-OAcGlu	OMe	H	OH	6"-O-Acetylglycitin



O-Glu O-6"-OMalGlu O-6"-OAcGlu

Figure. 1.1 Soybean isoflavonoids

Table 1.1 Isoflavonoid contents of soybeans and soy flour (mg/100g)

Isoflavonoids	i	ii	iii	iv	v	vi	vii
Daidzein	0.6	nd	0.8-3.2	0.4-3.8	trace		4-48
Genistein	1.4	2.4-4	2.5-4.6	1.5-4.5	0.7-1.1		4-46
Glycitin	0.11		1.3-3.0	1.9-2.1	trace-2.2		trace-3
Daidzin	58.1	nd-11.7	30.5-84.9	14.8-78	3.7-11.5	40.6	48-77
Genistin	164.4	74.7-102.4	68-200.5	33.0-88.8	12.8-23.7	48.9	58-154
Glycitin	33.8		9.5-15.2	6.0-9.7	4.2-9.6	9.8	6-22
6"-OMalDaidzin				19.8-70.9	22.2-56.2	26	
6"-OMalGenistin				98.3-17.6	72.3-12.3	31.8	
6"-OMalGlycitin				7.2-11.8	6.0-18.3	8.5	
6"-OAcDaidzin				trace	trace-1.2	22.3	
6"-OAcGenistin				trace	trace-0.4	18.1	
6"-OAcGlycitin				trace-3.7	3.3-4.1	nd	
Data Sources	Naim 1974	Murphy 1982	Eldridge 1983	Wang 1994	Wang 1994	Barnes 1994	Eldridge 1982

i : An unknown variety of soybean

ii : Amsoy and Weber

iii : Harding, Amcor Sprite and Century

iv : Pioneer 9111, Pioneer 9202, Prize, HP204, LS301, XL72 and strayer

v : Keburi, Kuro diazu and Raiden

vi : Soy flour, unknown source

vii : Defatted Amsoy (1978 crop), Nutrisoy 7B, unflavored TVP, Textratein, Centex 300, Centex 300 SL, Centex 400, Centex 400 SL, Mira Tex, and Promote III SL varieties of soy flour

Table 1.2 Distribution of isoflavones in two varieties of soybeans (mg/100 g)

Isoflavones	Hull		Hypocotyl		Cotyledon	
	Amsoy	Tiger	Amsoy	Tiger	Amsoy	Tiger
Daidzin	6.6	8.6	1031.5	759.9	37.5	102.8
Glycitin-7-b-Glucoside			664.1	588.8	1.7	1.6
Genistin	2.8	7.4	5.3	9.1	113.9	205.8
Daidzein	0.7	1.0	19.0	14.0	1.4	2.8
Glycitein		1.5	11.8	9.3		
Genistein	0.5	1.5	24.7	24.2	2.8	5.9
Total	10.6	20.0	1756.5	1405.2	158.5	319.2

Eldridge, A. C. And Kwolek, W. F. (1983) *J. Agric. Food Chem.* 31, 394-396.

conjugates in soybean tissue (Graham, 1990; Kudou et al., 1991). Wang and Murphy (1994) analyzed the isoflavonoid content of several American and Japanese soybean varieties and confirmed that 6"-malonylgenistin, 6"-malonyldaidzin genistin and daidzin represented 83 to 93 % of the total isoflavone content. Among them, 6"-malonylgenistin was the major isoflavone constituent and represented 25 - 42 % of the total isoflavones. There was a significant difference in the total and individual isoflavone concentrations among different crop years. Also, the isoflavone contents were not greatly influenced by the growth locations.

The isoflavone contents of soy flour samples were reported by Eldridge (1983) and Barnes et al. (1994). Barnes et al. (1994) found that most of the isoflavones present in soy product were extracted with 80 % aqueous ACN or MeOH at room temperature for 2 h. At higher temperatures, the amounts of β-glycoside conjugates and aglycones were increased at the expense of 6"-malonylisoflavones. The conversion of isoflavone 6"-Malonylglycosides to β-glycosides and aglycones was greatly increased when temperature was elevated to 80°C. Eldridge (1983) analyzed the isoflavones in 10 commercial samples of soy flour after extracting them with 80 % MeOH, (Table 1.1). The conversion of isoflavone 6"-malonylglycosides to β-glycosides and aglycones upon heating explained the large variation shown in the results by Barnes et al (1994) and Eldridge (1983) (Table 1.1).

The isoflavone contents of various soy products were published by other researchers as well (Table 1.3). Murphy (1982) compared her results with the published data (Table 1.1) and concluded that there was a decrease in genistin content in processed soybean food products. Similar conclusions were described by Wang and Murphy (1994[b]). Generally, the

Table 1.3 Isoflavones in soy products (mg/100g)

Isoflavonoids	i	ii	iii	iv	v	vi	vii	viii	ix
Daidzein	8-21	5.7	0.9-2.6	3.9	1.5-3.0	0-13	4.6	13.7	3.4
Genistein	5-22	4.3	1.3-2.8	6.9	3.2-5.0	54-78	5.2	19.3	9.3
Glycitin	1-3	nd	nd	5.2	2.0-2.1		1.2	2.4	1.5
Daidzin	14-30	18.1	32.0-51.1	46	40.4-52.5	0-35	2.5	0.2	7.2
Genistin	55-80	31.4	50.1-84.1	55.1	67.4-77.5	51-104	8.4	0.5	12.3
Glycitin	3-6	5.8	3.2-4.5	6.8	6.8-7.8		0.8	1.4	1.8
6"-OMalDaidzin		18.1	nd	4.5	3.9-9.8		15.9	25.5	nd
6"-OMalGenistin		38.3	6.5-16.6	6.3	14.4-25.9		10.8	16.4	nd
6"-OMalGlycitin		3.8	nd	7.2	4.0-4.4		nd	nd	2.2
6"-OAcDaidzin		5.1	nd	39.7	0.5-1.2		0.8	1.1	0.1
6"-OAcGenistin		8.1	nd	74.3	2.2-2.7		0.1	nd	1.1
6"-OAcGlycitin			nd	10.2	3.3-3.3		2.9	nd	nd
Data Sources	Eldridge 1982	Barnes 1994	Barnes 1994	Wang 1994	Wang 1994	Murphy 1982	Wang 1994	Wang 1994	Wang 1994

i : Soy protein isolates from Ralston Purina Co., Edi Pro N, Edi Pro A, Supro 610, Supro 620, Supro 710

ii : Unknown brand of soy protein isolates

iii : Unknown brands of soy milk

iv : Unknown variety of roast soybeans

v : Unknown brand instant berverages, will be diluted by 4 before consumption

vi : Unknown brands of tofu

vii : Unknown brands of tofu

viii : Unknown brand of tempeh

ix : Unknown brand of miso

total isoflavone contents in soy food products are lower than soybean itself. 7-*O*-glucoside 6"-*O*-acetates were produced during heat treatment of soybeans (Farmakalidis and Murphy, 1985[a]). Therefore, the higher content of 6"-*O*-acetylisoflavones should be expected in extensively processed soy food.

Biological Activities of Isoflavones

The isoflavonoids daidzein, genistein, formononetin and biochanin A are estrogenic in sheep (Shutt et al., 1968), rats and guinea pigs (Farmakalidis and Murphy, 1984; Farmakalidis et al., 1985[b]). In sheep, the estrogenic activity of genistein was about 10^{-5} times than that of diethylstilbestrol administered intramuscularly (Shutt et al., 1968; Braden et al., 1967). Both genistein and biochanin A inhibited human stomach cancer cell proliferation in vitro (Yanagihara et al., 1993). It was suggested that the anticancer activity of genistein was estrogen-receptor independent. The growth of the human breast carcinoma cell lines, MDA-468 (estrogen-receptor negative), and MCF-7 and MCF-7-D-40 (estrogen-receptor positive) were inhibited in vitro by genistein (Peterson and Barnes, 1991). Biochanin A and daidzein are less inhibitory to the cancer cell growth when compared to genistein. Also, the isoflavones, genistin and daidzin, were not effective as anticarcinogens. It was shown that singlet oxygen played an important role in mutagenesis and carcinogenesis, particularly in tumor promotion. Genistein strongly inhibited H_2O_2 formation both in vivo and in vitro (Wei, et al., 1993)..

Genistein was reported as an inhibitor for protein tyrosine kinase (PTK) and DNA synthesis (Akiyama et al., 1987; Dean et al., 1989; Sit et al., 1991). Cell growth is controlled

by many growth factors, such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF). Non-proliferating cells treated with EGF will start proliferating again. The receptor protein for EGF was found to be associated with specific PTK activity. The phosphorylation of tyrosine is a rare event in normal cells. Phosphotyrosine accounts for only one in 2,000 of the phosphate molecules linked to proteins. However, in cancer cells, the amount of phosphotyrosine increased dramatically (Bishop, 1986). Also, several oncogene products were found to be the mutant forms of EGF receptor. Hence, the inhibition of PTK activity became an important property of compounds to be used as anticarcinogens. Recently, Uckun et al. (1995) published on the biotherapy of B-cell precursor leukemia by a complex of genistein with a monoclonal antibody of the B-cell-specific receptor, a member of the protein tyrosine kinases. This genistein-antibody immunoconjugate was over 1500 times more effective than unconjugated genistein at inhibiting the PTK activity of B-cell specific receptor and causing decreased tyrosine phosphorylation in leukemia cells. It was proposed that more genistein molecules were delivered into leukemia cells by the genistein-antibody immunoconjugate. Also, the immunoconjugates brought genistein molecule closer to the protein tyrosine kinase which enhanced the PTK inhibition of genistein.

Genistein also was reported as DNA topoisomerase II inhibitor (Markovits et al., 1989; Corbett et al., 1993). DNA topoisomerases catalyze the interconversion of topoisomers, the wound or unwound DNA strains. DNA strains are tightly wound to form chromosomes. The binding of topoisomerase on DNA untangles the DNA strains. This allows the DNA replication and transcription which are essential for cell growth. Drugs, acting as DNA topoisomerase poisons, can produce the cleavable DNA-topoisomerase-drug complex and

cause site-specific breakage of the chromosomal DNA. This event inhibits DNA replication, RNA synthesis and cell division, and eventually leads to cell death (Liu, 1990). Cancerous cells contain larger amount of topoisomerases which makes them good targets for topoisomerase inhibitors. Therefore, topoisomerase I (cleaves one strain of DNA) and II (cleaves two strains of DNA) attracted a great deal of interest in cancer research in the 1990s.

There is a homology in the sequence of PTK and human DNA topoisomerase II. Both PTK and DNA topoisomerase II are involved in the formation of phosphate ester between a phosphate group of a nucleotide and the hydroxyl group of tyrosine. This might be the reason that both enzymes, responding to the same inhibitor (Markovits et al., 1989).

The isoflavonoids, daidzein, formononetin, genistein and biochanin A, exhibit other important biological activities. Formononetin and biochanin A were reported as the signal molecules for vesicular-arbuscular mycorrhiza (VAM)-host plant symbiosis (Nair et al., 1991; Siqueira et al., 1991[a]; Safir et al., 1992) and as herbicide safeners (Siqueira et al., 1991[b]). Also, genistein and daidzein, isolated from the soybean (*Glycine max L.*) root extract, were responsible for the induction of *Brandyrhizobium japonicum nod* genes (Kosslak et al., 1987). Antifungal and antioxidant activities also were reported for soybean isoflavonoids (Ikehata et al., 1968; Naim et al., 1974; Murakami et al., 1984; Kramer et al., 1984).

Metabolism Studies of Soybeans and Isofavones

The lower gut is one of the major metabolic sites of the human body (Figure 1.2). In vitro metabolism studies revealed that the metabolisms accomplished by gut flora are mainly

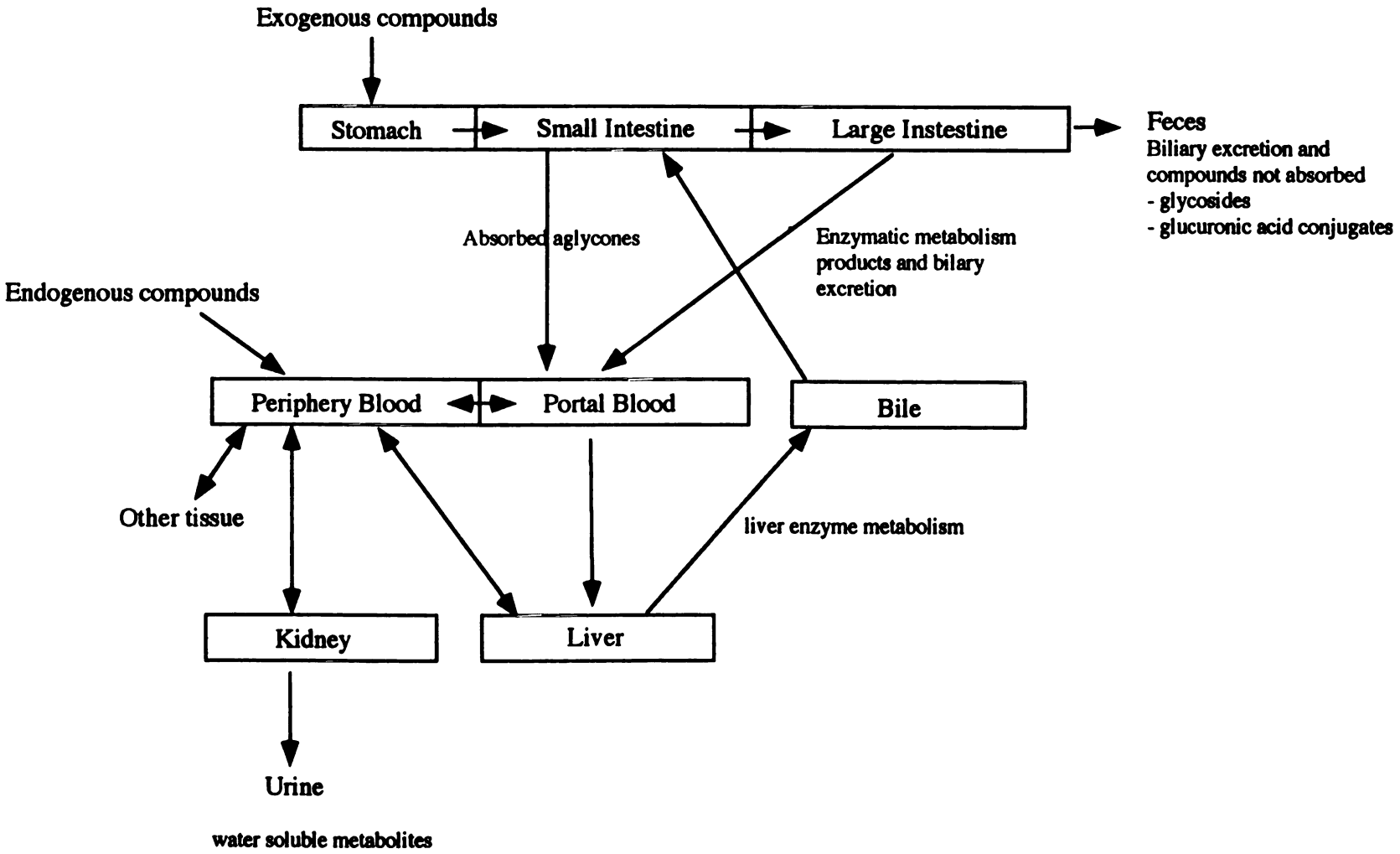
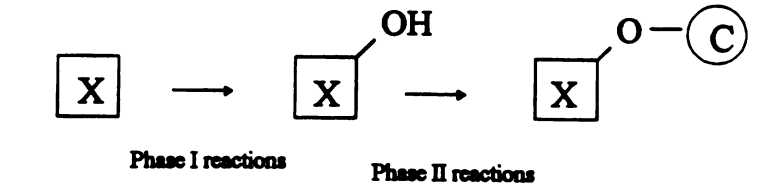


Figure 1.2 Human Metabolism of Endogenous and Exogenous Compounds

hydrolysis and reductions. Most oxidation and conjugation reactions are carried out by the liver (Gorrod, 1978). Endogenous or exogenous chemicals entering the human body are metabolized first by gut bacteria and then absorbed and transported to the liver through enterohepatic circulation for further transformation. The major role of liver enzymes is to transform these chemicals into more polar metabolites in order to excrete them into urine. The most common conjugates present in human and other mammalian species are glucuronides (Figure 1.3). There are several reasons for the formation of these conjugates in biological systems. They are: (i) the easy supply of carbohydrate as a conjugating agent; (ii) the high polarity of the polyhydroxy and carboxylic acid groups of the glucuronic acid; (iii) the lower toxicity and higher solubility of the glucuronide compounds; and (iv) the wide range of compounds that can be the substrates of UDP-glucuronyltransferase. Other conjugates include glycosides, sulfate esters, methylates, thiocyanate acetylates, amino acids and mercapturic acid conjugates (Figure 1.3). These polar metabolites are either excreted into the urine or into the gut with bile salts, where these compounds are metabolized further by intestinal microorganisms. The intestinal microorganisms contain important enzymes such as β-glucuronidase, β-galactosidase and β-glucosidase to metabolize chemicals or their conjugates. The chemical conjugates present in food must be hydrolyzed before they can be absorbed into the circulation system. Therefore, these glycosidases play an important role in the release and bioavailability of biologically active aglycones.

The incidence of colon cancer was significantly lower in germ-free rats than in conventional rats when both were treated with DMBA carcinogen. Dietary fat had no effect on tumor incidence in germ-free rats. However, conventional rats fed with high-fat diets were



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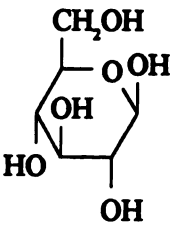
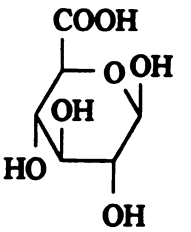


-D-Glucose

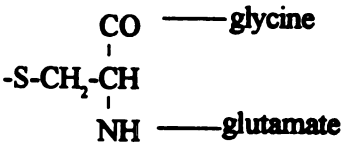


-D-Glucuronic acid

$-SO_3^-$
Sulfate

$-CH_3$
Methyl

$-SCN^-$
Thiocyanate



$-COCH_3$
Acetyl

Mercapturic acid

Figure 1.3 Types of conjugates of metabolites excreted to the urine

much more susceptible to the carcinogenic effect of DMBA (Gorbach and Goldin, 1990). These observations further emphasized the role of intestinal bacterial metabolism. Aldercreutz et al. (1984) reported that human subjects treated with antimicrobial drugs exhibit an increase in the excretion of conjugated steroid hormones in feces, and a decrease in the excretion of unconjugated steroid hormones in the urine. These results indicate that the intestinal bacteria play an important role in the metabolism of steroidal hormones. A number of reductive reactions of androgens and estrogens were observed when incubated with human fecal bacteria under anaerobic conditions (Lombardi et al., 1978; Jarvenpaa, et al., 1980).

Metabolic studies of soybean isoflavones showed that *p*-ethyl phenol was isolated as the only metabolite of genistein and biochanin A in the urine of sheep (Batterham et al., 1965; Braden et al., 1967; Shutt et al., 1970; Batterham et al., 1971) (Figure 1.4). Equol and *O*-desmethyl angolensin were identified as the metabolites in the urine when the sheep were fed with daidzein and formononetin (Batterham et al., 1965; Batterham et al., 1971; Shutt et al. 1971; Shutt and Braden, 1968). The ruminal fluid was capable of metabolizing biochanin A to genistein, and formononetin to daidzein and equol during fermentation (Dickinson et al., 1988).

Similar results were obtained with the urine and plasma of guinea-pigs and Sprague-Dawley rats fed with soy flour (Shutt and Braden, 1968; Axelson et al., 1984). In the urine of rats fed with soy flour, both equol and daidzein were characterized as monoglucuronide conjugates (Axelson et al., 1982[a]). Also, daidzin, the glucoside of daidzein, present in soy flour was confirmed as a precursor of equol (Axelson et al., 1984). Yasuda, et al. (1994)

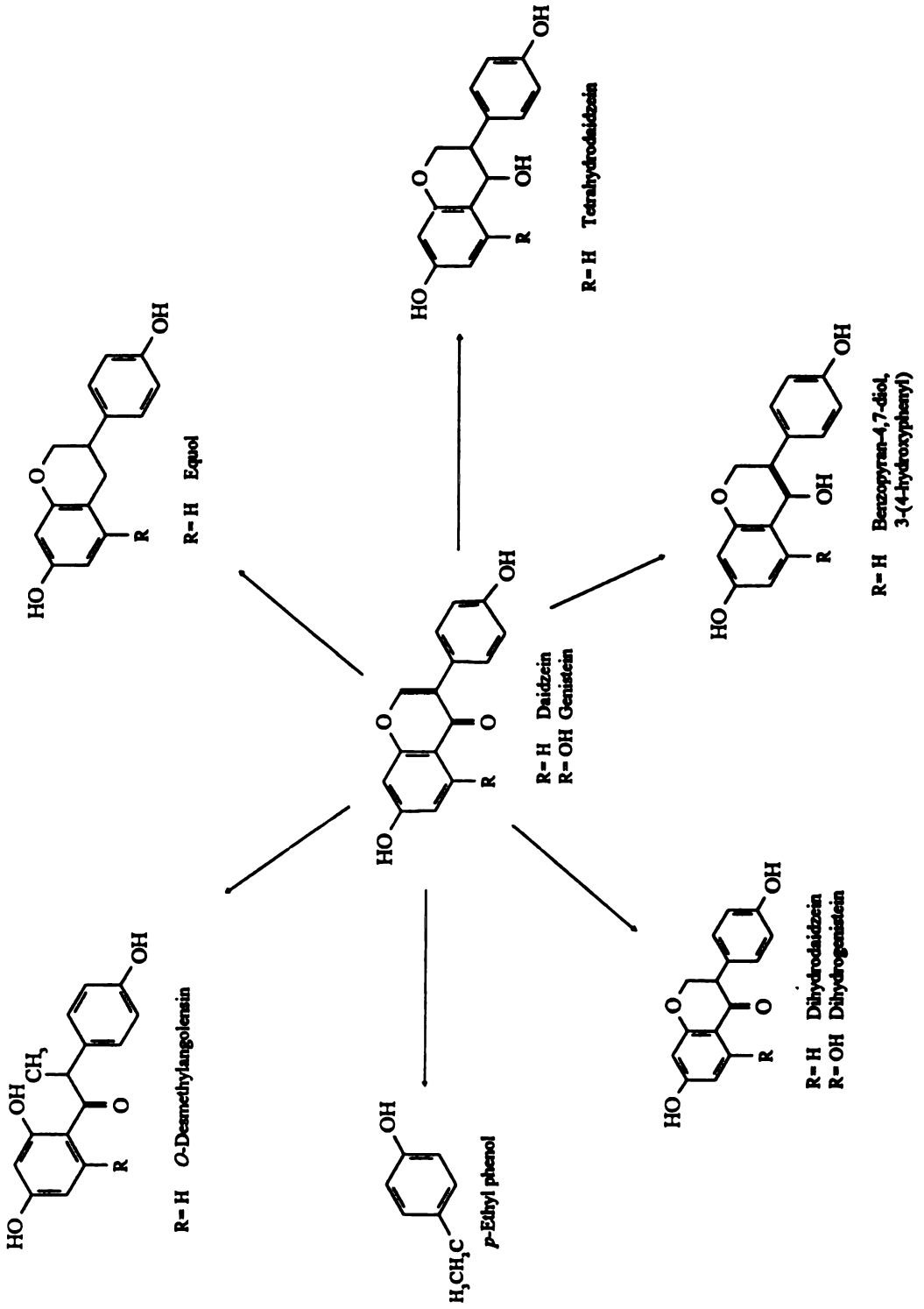


Figure 1.4 Metabolites of daidzein and genistein detected in the urine from rats and human

reported that the metabolites present in the urine and bile fluid of the rats fed with daidzin and daidzein were glucuronide and monosulfate and disulfate conjugates of daidzein. The excretion of equol was undectable in the urine of germ-free rats on soy diets, whereas the urine of conventional rats contained equol. This confirmed the bacterial transformation of daidzein to equol in rats (Axelson and Setchell, 1981).

Daidzein, equol and *O*-desmethyl angolensin were identified as the monoglucuronide conjugates in human urine (Axelson et al., 1982[b]; Bannwart et al., 1984[a,b]; Axelson et al., 1984). The majority of equol excreted in human urine was monoglucuronide conjugate and a small amount occurred as monosulfate or disulfate conjugates (Axelson et al, 1982[b]). Similar reslults were observed for daidzein excreted in human urine (Axelson et al, 1984). Kelly et al (1993) confirmed the presence of equol, *O*-desmethyl angolensin, dihydrodaidzein, 6'-hydroxy-*O*-desmethyl angolensin, dehydro-*O*-desmethyl angolensin, benzopyran-4,7-diol, 3-(4-hydroxyphenyl) and tetrahydrodaidzein as the metabolites of daidzein (Figure 1.4). Dihydrogenistein was the only metabolite of genistein from human urine. *p*-Ethyl phenol was detected as the metabolite of genistein in the urine of rumens (Batterham et al., 1965; Batterham et al., 1971; Shutt et al. 1971; Shutt and Braden, 1968). However, it was not detected in human urine. The metabolism studies of daidzein and genistein in humans showed a large variation in the production of metabolites. Also, quantitative analysis of the metabolites revealed that an inverse relationship was observed between the production of equol and *O*-desmethyl angolensin.

Synthesis of Isoflavones

GENISTEIN.— Genistein was synthesized from phloroglucinol and *p*-methoxyphenyl acetonitrile as following the method of Shriner and Hull (1945) (Figure 1.5). A stream of dry HCl gas was passed through an ethereal solution of phloroglucinol and *p*-methoxyphenyl acetonitrile until saturation for two days. The ether layer then was decanted and the resulting precipitate was refluxed with 2 % HCl for 4 h. The resulting ketone was crystallized upon cooling. The ketone was condensed with ethyl formate and metallic sodium followed by acidification. Biochanin A then was hydrolyzed with HI to yield genistein.

A modified procedure was reported later by Yoder et al. (1954) (Figure 1.6). The same step was employed for the production of the ketone as above, using phloroglucinol and *p*-hydroxyphenyl acetonitrile. The ketone then was treated with ethyl oxalyl chloride in pyridine followed by alkalic hydrolysis. The 2-carboxylic acid of genistein thus afforded was decarboxylated to produce genistein was achieved by pyrolysis.

BIOCHANIN A.— The total synthesis of biochanin A was reported by Shriner and Hull (1945) (Figure 1.5). The method reported by Yoder et al. (1954) for the synthesis of genistein was used also for the synthesis of biochanin A by substituting *p*-methoxyphenyl acetonitrile.

The cyclization of ketone also was accomplished by heating it with dimethyl formamide (DMF), boron trifluoride etherate (BF_3-Et_2O) and methanesulfonyl chloride ($MeSO_2Cl$) (Bass, 1976) (Figure 1.7). This method provided a convenient cyclization of the ketone with

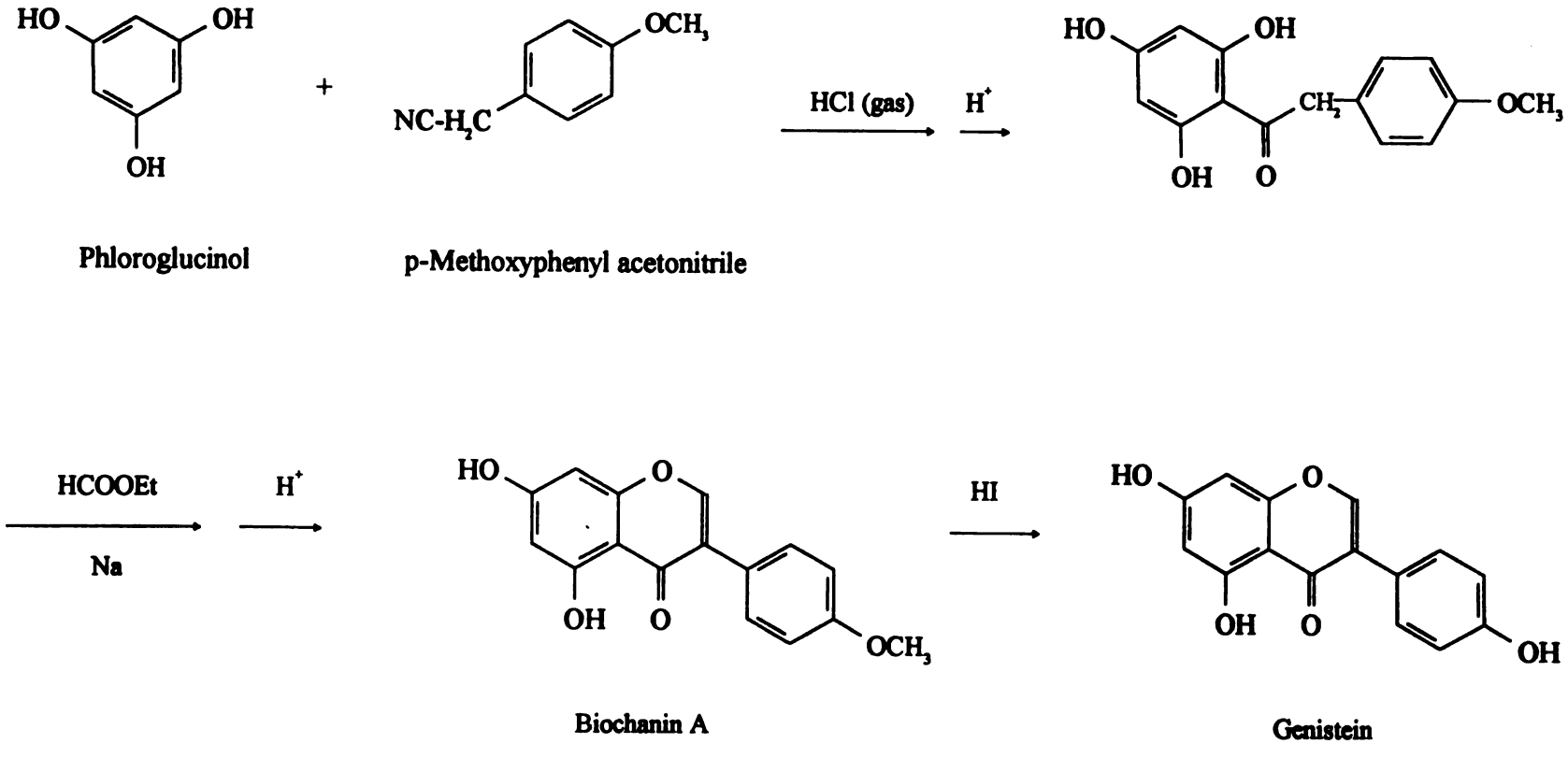


Figure 1.5 The synthesis of genistein as reported by R. C. Shriner and C. J. Hull (1945) *J. Org. Chem.* **10**, 288-291.

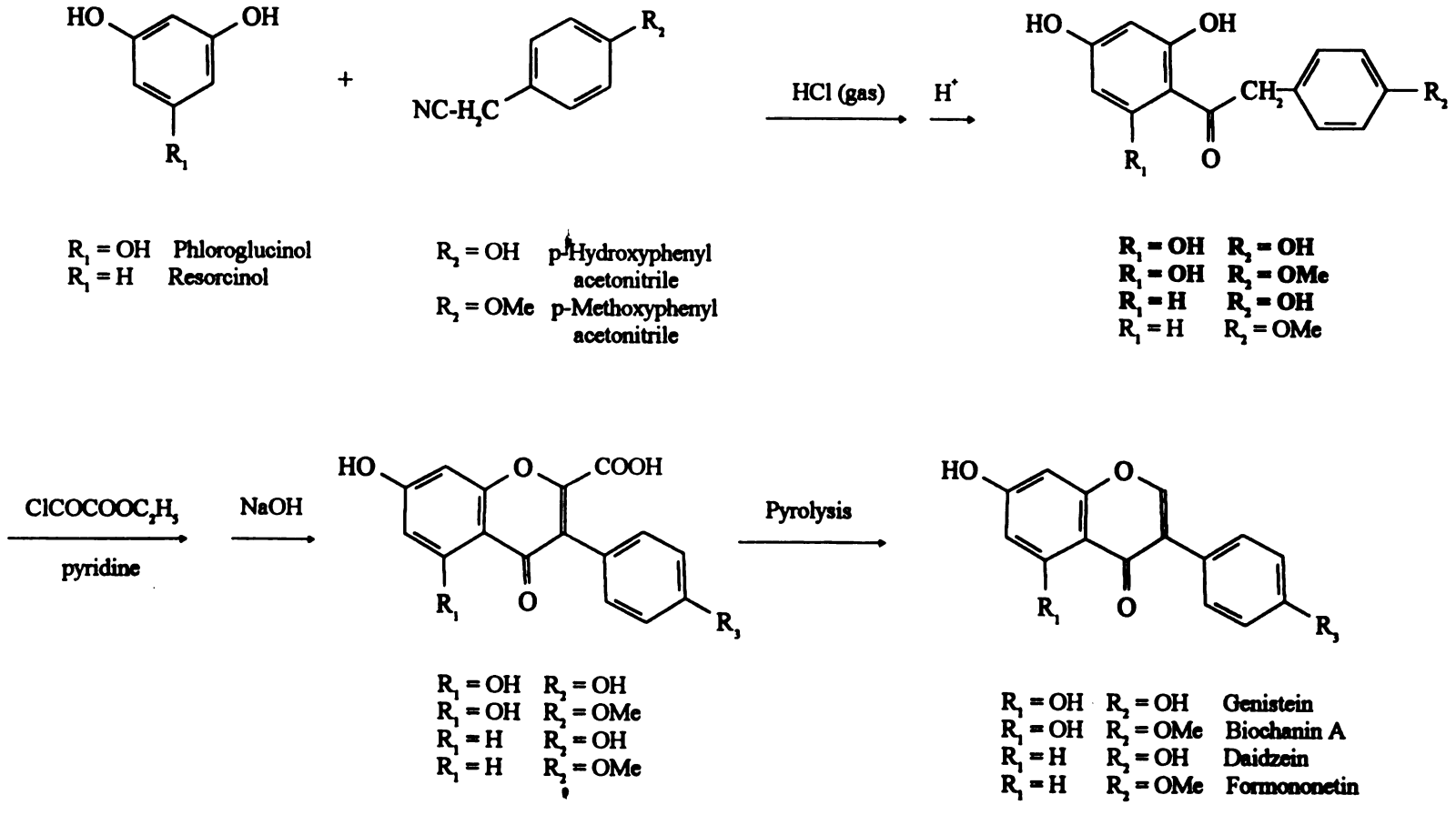
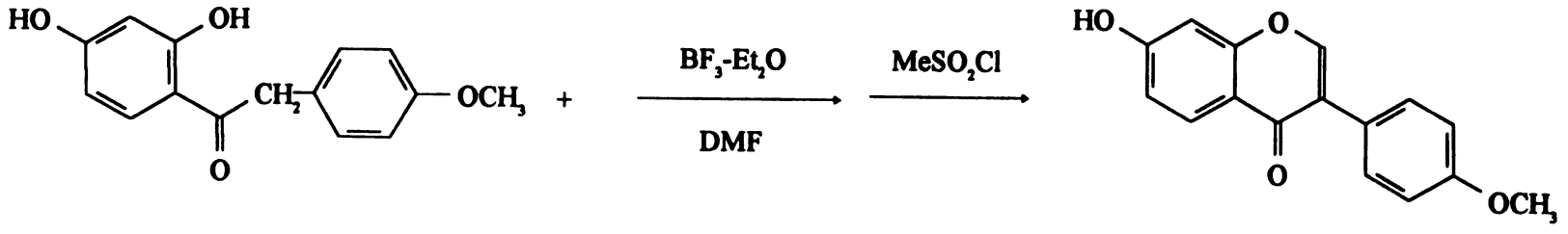


Figure 1.6 The synthesis of isoflavones as reported by L. Yoder, E. Cheng, and W. Burroughs (1954) *Iowa Acad. Sci.* **61**, 271-277.



Biochanin A

Figure 1.7 The synthesis of biochanin A as reported by R. J. Bass, (1976) *J. C. S. Chem. Comm.*, 78-79.

different substituents to produce corresponding isoflavones.

DAIDZEIN.— Daidzein was synthesized from resorcinol by replacing phloroglucinol in the synthesis of genistein (Yoder et al. 1954) (Figure 1.6). An alternative cyclization of the ketone was achieved by the cyclization of the intermediate ketone with dimethoxy-dimethylaminomethane in DMF for 3 h (Pelter and Foot, 1976) (Figure 1.8). Modified Vilsmeier-Haack reaction using $POCl_3$ and DMF as reagent also was used to cyclize the corresponding ketones to form daidzein and formononetin (Kagal et al., 1962).

Sekizaki et al. (1993) reported a new procedure for synthesis of isoflavones using the condensation of corresponding acetophenone and aldehyde to produce the key intermediate tetrahydropyranyl-chalcone (Figure 1.9). These chalcones were oxidized and cyclized with thallium (III) nitrate trihydrate in methanol. Using this method, 19 isoflavones were synthesized, including daidzein and formononetin, but not genistein and biochanin A.

FORMONONETIN.— The methods used for the synthesis of daidzein described earlier were utilized also for the synthesis of formononetin (Kagal et al., 1962; Pelter and Foot, 1976; Sekizaki et al. 1993) (Figure 1.6, 1.8, and 1.9).

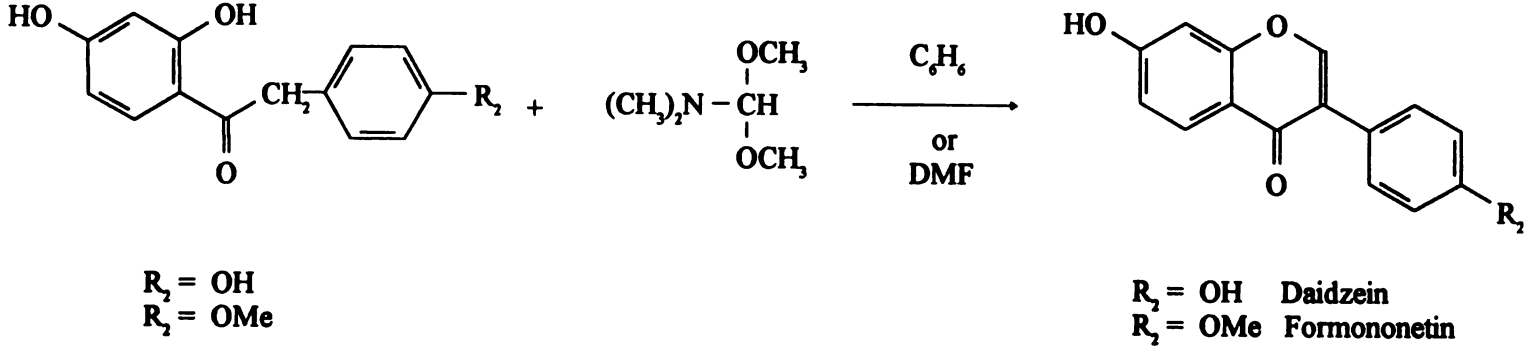


Figure 1.8 The synthesis of daidzein and formononetin as reported by A. Pelter and S. Foot (1976) *Synthesis*, 326.

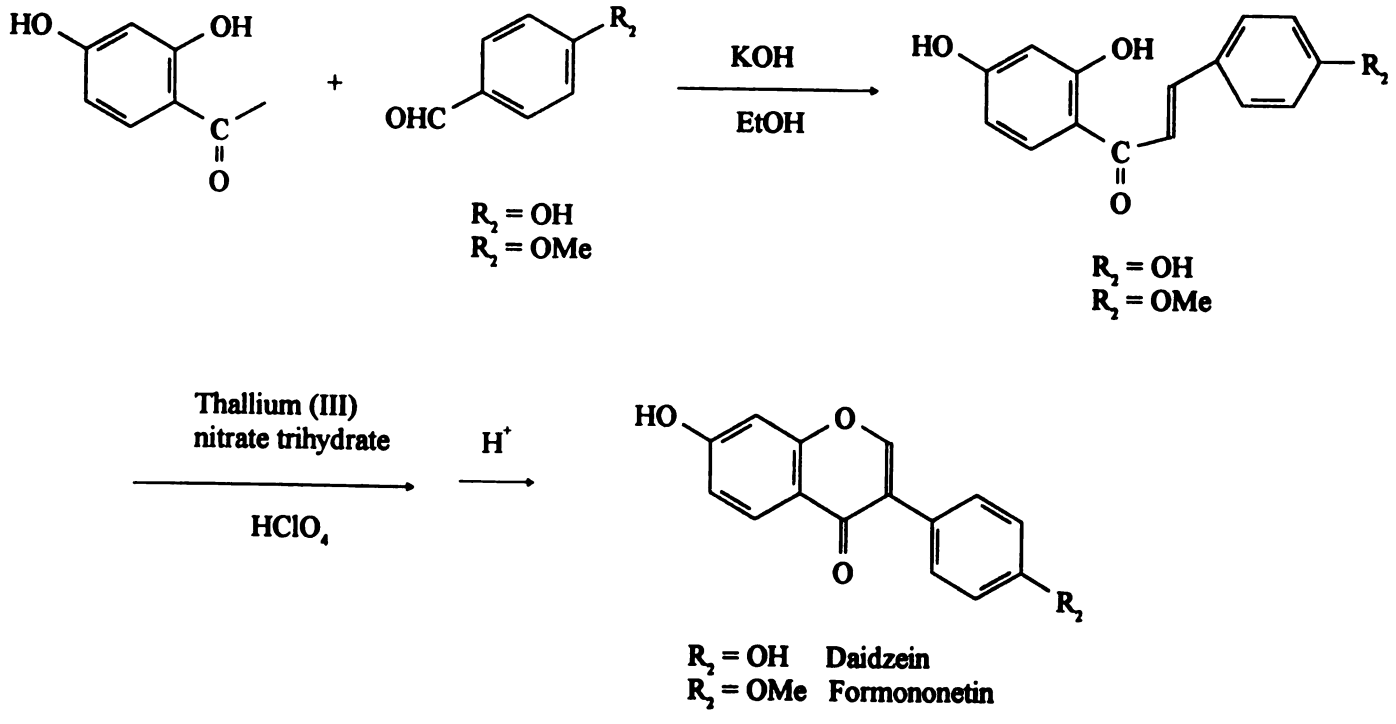


Figure 1.9 The synthesis of daidzein and formononetin as reported by H. Seikizaki et al. (1993) *Biol. Pharm. Bull.* **16(7)**, 698-701.

CHAPTER II

Introduction

Approximately 1 in 8 women is diagnosed with breast cancer in the United States during their life time. Epidemiological studies found that when women living in countries with low breast cancer risk immigrate to the countries where women have higher breast cancer incidence, the breast cancer incidence of the immigrants became similar to the women in the host countries. This suggests that breast cancer is associated with environmental as well as genetic factors.

In many instances, diets are correlated with the incidence of breast cancer. Among all the dietary factors, most researchers believed that fat is the most important factor. Also, many animal feeding experiments confirmed the detrimental effect of dietary fat. However, some epidemiological studies did not observe correlation between the fat intake and breast cancer incidence.

Another dietary factor that may be related to the breast cancer incidence is soybean intake. Epidemiological studies found that Japanese women have much lower breast cancer rate than the women in the United States. Other studies in Asian countries such as Japan,

Taiwan, China and Singapore indicated a negative correlation between the soybean intake and breast cancer occurrence. In Taiwan, it was estimated that the daily consumption of soybean was 35 g/person and provided 35 mg or more of soybean isoflavones, such as genistein and daidzein. However, in the United States, the soybean intake is less than 2 g/day per person.

In animal experiments, rats were fed with soybean diet and carcinogens. It was observed that less tumors were induced by chemical carcinogens or irradiation in soybean fed rats. This study suggested that the lower breast cancer risk may be associated with the higher soybean consumption by both humans and laboratory animals.

It was reported that the isoflavones present in soybean such as genistein and daidzein, the aglycones of genistin and daidzin, inhibited the growth of breast cancer cell both in vitro and in vivo. However, most of the soybean isoflavones are very expensive or not commercially available. This seriously limited the evaluation of these isoflavones as anticarcinogens. Therefore, the first objective of our research was focussed on the synthesis of soybean isoflavones in large quantities. The availability of these isoflavones will allow further efficacy tests including animal feeding studies.

Many bioactive metabolites are produced in the large intestine by bacterial metabolism. Various metabolites of genistein and daidzein have been detected in the urine from sheeps, rats and humans. However, the metabolic pathways of these compounds have not been evaluated. Since most of the metabolites of soybean isoflavones detected in biological systems are their reduction products of isoflavones, it is possible that these isoflavones are metabolized initially by the intestinal bacteria. Therefore, the second part of this dissertation

contained the study of the metabolic pathways of genistein and daidzein. Daidzein and genistein were incubated with human fecal bacteria under anaerobic conditions. The metabolites produced in the fermentation broth were isolated and characterized by spectral methods.

It is possible that the metabolites produced in vivo and in vitro feeding experiments with isoflavones are more biologically active than their parent compounds. These metabolites were detected in small quantities in the biological systems and their biological activities were not reported so far. To determine the biological activities of these metabolites of daidzein and genistein, we have synthesized the metabolites of daidzein and genistein detected in the biological systems by the hydrogenation of either genistein or daidzein. The chapter V of this dissertation describes the antibacterial, antifungal, mosquitocidal, nematicidal and anticancer activities of the hydrogenation products of daidzein and genistein.

CHAPTER III *

Microwave-Mediated Synthesis of Anticarcinogenic Isoflavones from Soybeans

Abstract

Soybean isoflavonoids, 7,4'-dihydroxyisoflavone (daidzein), 7-hydroxy-4'-methoxyisoflavone (formononetin), 5,7,4'-trihydroxyisoflavone (genistein) and 5,7-dihydroxy-4'-methoxyisoflavone (biochanin A), were synthesized with high yields by cyclization of their corresponding ketones in a conventional microwave oven.

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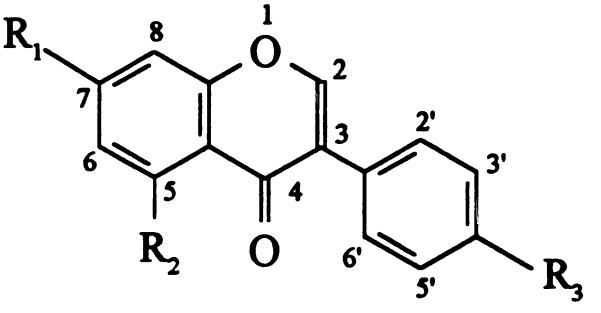
Introduction

Soybean isoflavonoids, daidzein, formononetin, genistein and biochanin A (Figure 3.1), are reported to have important biological activities, such as being the signal molecules for vesicular-arbuscular mycorrhiza infection on host plants (Nair et al., 1991; Siqueira et al., 1991[a]; Safir et al., 1992); herbicide safening effect (Siqueira et al., 1991[b]); estrogenic and anticarcinogenic activities in sheep and rats (Braden et al., 1967; Troll et al., 1980) and they have been implicated in the inhibition of growth of human breast cancer cells (Peterson and Barnes, 1991). Epidemiological studies showed that the incidence of breast cancer is high in North America and North West Europe (Drasar and Irving, 1973). Diets normally are considered an important factor; breast cancer is highly correlated with a high fat and animal protein diets (Drasar and Irving, 1973; Lee et al., 1991). It was reported that women consuming high levels of isoflavonoid-containing diets have lower breast cancer incidence than the women with low intake of such diets (Aldercreutz et al., 1988; Aldercreutz, 1990; Lee et al., 1991; Messina and Messina, 1991; Messina and Barnes, 1991; Setchell et al., 1984). However, pre-clinical studies of these important compounds have not been evaluated adequately due to the limited quantities of isoflavonoids available. Most of the soybean isoflavonoids are very expensive which seriously limits their evaluation as potential anticarcinogens. Therefore, the source of these isoflavones became our first priority when investigating them as anticarcinogens.

The reported syntheses of many soybean isoflavonoids including genistein, biochanin A, daidzein and formononetin are very time-consuming (Bass, 1976; Baker et al., 1953;



R_1= OH,	R_2= H,	R_3= OH	Daidzein
R_1= O-Glu,	R_2= H,	R_3= OMe	Daidein
R_1= OH,	R_2= H,	R_3= OMe,	Formononetin
R_1=OH,	R_2=OH,	R_3=OH	Genistein
R_1= O-Glu,	R_2= OH,	R_3=OH	Genistin
R_1= OH,	R_2= OH,	R_3= Ome,	Biochanin A

Figure 3.1. Soybean isoflavones

Farkas et al., 1971; Pelter and Foot, 1976; Shriner and Hull, 1945; Yoder et al., 1954). The use of the microwave energy in organic syntheses are now popular (Abramovitch, 1991). It was shown that commercial microwave-oven treatment dramatically reduced the reaction times of many organic reactions such as the Diels-Alder and Claisen reactions (Giguere et al., 1986) and α-vinyl ß-lactam synthesis (Banik et al., 1992). In this paper, we describe effective and rapid synthses of daidzein, formononetin, genistein and biochanin A by cyclizing their corresponding ketones using an unmodified microwave oven.

Experimental

INSTRUMENTS.—^{1}H and ^{13}C NMR spectra were recorded on Varian VXR 300 and 500 MHz spectrometers, respectively, in CD_3OD or d_6-DMSO solution at ambient temperature. The noncorrected melting points were recorded on a Thomas Model 40 micro hot-stage apparatus.

CHEMICALS.—Resorcinol, 4-hydroxyphenylacetic acid, BF_3 etherate, N,N-dimethyl-formamide dimethyl acetal, 4-methoxyphenylacetic acid, phloroglucinol, 4-hydroxyphenyl acetonitrile, and methanesulfonyl chloride were purchased from Aldrich Chemical Company (Milwaukee, WI, USA);

4-HYDROXYBENZYL 2,4-DIHYDROXYPHENYL KETONE, 1.—Resorcinol (2.9 g) was added to a mixture containing 4-hydroxyphenylacetic acid (2 g) and BF_3 etherate (4.5 ml). The reaction mixture was refluxed for 10 min, cooled and treated with saturated aqueous NaOAc (30 ml) and $NaHCO_3$ (15 ml), respectively. The precipitate formed was filtered off,

washed with water, dried, and then washed with $CHCl_3$ to give yellow needle-like crystals (2.8 g, 88%); m.p. 188-190°C; ^{1}H-NMR (CD_3OD) δ 7.82 (1H, d, J= 8.7 Hz, H-6), 7.08 (2H, dd, J= 6.6, 2.1 Hz, H-2', 6'), 6.72 (1H, dd, J= 6.6, 2.1 Hz, H-3', 5'), 6.35 (1H, dd, J= 8.7, 2.4 Hz, H-5), 6.24 (1H, d, J=2.1 Hz, H-3), 4.09 (2H, s, -CH_2-); ^{13}C-NMR (CD_3OD) δ 202.66 (CO), 164.80 (C-2), 164.52 (C-4), 155.26 (C-4'), 132.65 (C-1'), 129.51 (C-2', C-6'), 125.38 (C-6), 114.58 (C-3', C-5'), 111.72 (C-1), 107.43 (C-5), 101.87 (C-3), 42.83 (-CH_2-).

DAIDZEIN.—N,N-dimethylformamide dimethyl acetal (0.5 ml) and THF (0.5 ml) was added to a pressure-resistant vial containing compound 1 (40.9 mg). The reaction mixture was heated in a microwave for 2 min at medium energy, yielding a red solution. Methanol (2 ml) was added to the reaction product and evaporated to dryness *in vacuo*. The crude product thus obtained was purified by preparative TLC ($CHCl_3$/MeOH 9:1) and recrystallized from aqueous methanol to give daidzein (27.8 mg, 71 %); m.p. 290°C (decomposed); ^{1}H-NMR (d_6-DMSO) δ 8.27 (1H, s, H-2), 7.95 (1H, d, J= 9.0 Hz, H-5), 7.36 (2H, d, J= 6.6, 1.8 Hz, H-2', 6'), 6.92 (1H, dd, J= 8.7, 2.4 Hz, H-6), 6.84 (1H, d, J= 2.1 Hz, H-8), 6.79 (1H, d, J= 6.6, 1.8 Hz, H-3', 5'); ^{13}C-NMR (d_6-DMSO) δ 174.64 (C-4), 162.44 (C-4'), 157.38 (C-8a), 157.11 (C-7), 130.00 (C-2', C-6'), 127.22 (C-5), 123.46 (C-3), 122.52 (C-1'), 116.62 (C-4a), 115.05 (C-6), 114.90 (C-3', C-5'), 102.04 (C-8).

4-METHOXYBENZYL 2,4-DIHYDROXYPHENYL KETONE, 2.—The same synthetic procedure was employed as in compound 1 by substituting 4-methoxyphenylacetic acid (2.2 g) instead of 4-hydroxyphenylacetic acid. The corresponding ketone was yellow needle-like crystals (2.47 g, 51%); m.p. 159-163°C; ^{1}H-NMR (CD_3OD) δ 7.94 (1H, d, J= 9 Hz, H-6),

7.20 (2H, d, J= 8.4 Hz, H-2', 6'), 6.87 (2H, d, J= 8.7 Hz, H-3', 5'), 6.33 (2H, dd, J= 8.8, 2.7 Hz), 6.25 (1H, d, J= 2.1 Hz, H-3), 4.20 (2H, s,-CH_2-), 3.71 (3H, s, -OMe); ^{13}C-NMR (CD_3OD) δ 202.40 (CO), 164.89 (C-2), 164.64 (C-4), 157.99 (C-4'), 133.49 (C-1), 130.43 (C-2', C-6'), 126.93 (C-6), 113.79 (C-3', C-5'), 112.40 (C-1), 108.20 (C-5), 102.44 (C-3), 54.94 (-OMe), 43.16 (-CH_2-).

FORMONONETIN.—Using the same procedure as for daidzein, compound **2** (40 mg) was converted to formononetin. The crude precipitate was recrystallized from aqueous methanol to give formononetin (32.3 mg, 91%); ^{1}H-NMR (d_6-DMSO) δ 8.30 (1H, s, H-2), 7.95 (1H, d, J= 9 Hz, H-5), 7.49 (2H, d, J= 8.4 Hz, H-2', H-6'), 6.97 (2H, d, J= 8.7 Hz, H-3', H-5'), 6.92 (1H, dd, J= 8.7, 2.1 Hz, H-6), 6.84 (1H, d, J= 1.8 Hz, H-8), 3.76 (3H, s, -OMe); ^{13}C-NMR (d_6-DMSO) δ 174.50 (C-4), 163.67 (C-4'), 158.89 (C-8a), 157.57 (C-7), 152.87 (C-2), 130.00 (C-2', C-6'), 127.09 (C-5), 124.34 (C-3), 123.04 (C-1'), 116.02 (C-4a), 115.56 (C-6), 113.54 (C-3', C-5'), 102.00 (C-8), 55.10 (-OMe).

4-HYDROXYBENZYL 2,4,6-TRIHYDROXYPHENYL KETONE, **3**.—Phloroglucinol (1.0 g) and 4-hydroxyphenyl acetonitrile (1.1 g) in ether (10 ml) was cooled in an ice bath and saturated with a stream of HCl gas (HCl was produced by reacting NaCl and conc. H_2SO_4). The reaction mixture was refrigerated for 12 h, saturated again with HCl gas and refrigerated for another 12 h. Aafter decanting the ether, the precipitate was washed further with ether. The white precipitate thus obtained was refluxed with 2% aqueous HCl (20 ml) for 3 h and cooled. The solution was extracted twice with ether (50 ml each), and the organic layer was neutralized with saturated $NaHCO_3$ solution. The ether was removed *in vacuo* to give yellow

needle-like crystals (1.0 g, 46.5%); m.p. 258-262°C; ^{1}H-NMR (CD_3OD) δ 7.05 (2H, d, J= 8.1 Hz, H-3', H-5'), 6.69 (2H, d, J=8.1, H-2', H-6'), 5.80 (2H, s, H-3, H-5), 4.26 (2H, s, -CH2-); ^{13}C-NMR (CD_3OD) δ 205.08 (CO), 166.24 (C-2, C-6), 164.80 (C-4), 156.90 (C-4'), 131.66 (C-2', C-6'), 128.20 (C-1'), 115.98 (C-3'. C-5'), 95.83 (C-3, C-5), 49.54 (-CH_2-).

GENISTEIN.—BF_3 etherate (1 ml) was added to a solution containing DMF (2 ml) and compound **3** (50 mg) in a beaker. The reaction mixture was heated in a microwave for 15 sec using low energy, followed by the addition of methanesulfonyl chloride (CH_3SO_2Cl, 1 ml). The resulting product was heated in a microwave again for 1 min at low energy. A ight yellowish precipitate, obtained by the addition of water (100 ml) into the reaction mixture, was centrifuged, washed with water (10 ml x 3) and recrystallized from aqueous methanol to give genistein (43 mg, 80 %); m.p. 291-296°C; ^{1}H-NMR (d_6-DMSO) δ 8.30 (1H, s, H-2), 7.35 (2H, dd, J= 6.6, 1.8 Hz, H-2', H-6'), 6.80 (2H, dd, J= 6.6, 1.8 Hz, H-8), 6.21 (1H, d, J= 1.8 Hz, H-6); ^{13}C-NMR (d_6-DMSO) δ 180.68 (C-4), 164.77 (C-4'), 162.47 (C-5), 158.06 (C-8a), 157.86 (C-7), 154.44 (C-2), 130.64 (C-2', C-6'), 122.75 (C-3), 121.69 (C-1'), 115.51 (C-3', 5'), 104.93 (C-4a), 99.43 (C-8), 94.13 (C-6).

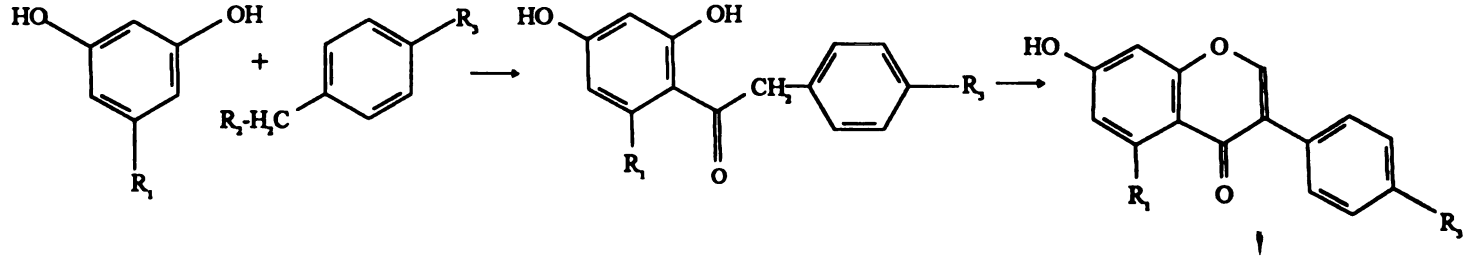
4-METHOXYBENZYL 2,4,6-TRIHYDROXYPHENYL KETONE, **4**.—Using 4-hydroxyphenyl acetonitrile (1 g) and phloroglucinol (1 g), a synthetic procedure similar to that used with compound **3** was employed to synthesize Compound **4**, yellow plate-like crystals (0.88 g, 47 %); m.p. 195-197°C; ^{1}H-NMR (CD_3OD) δ 7.15 (2H, d, J= 8.7 Hz, H-2',H-6'), 6.83 (2H, d, J= 8.4 Hz, H-3', H-5'), 5.80 (2H, s, H-3, H-5), 4.89 (2H, s, -CH_2-), 3.76 (3H, s, -OMe); ^{13}C-NMR (CD_3OD) δ 205.0 (CO), 165.80 (C-2, C-6), 165.75 (C-4), 159.77 (C-4'), 131.67 (C-2',

C-6'), 130.22 (C-1'), 114.71 (C-3', C-5'), 105.29 (C-1), 95.87 (C-3, C-5), 49.84 (-CH_2-).

BIOCHANIN A.—Biochanin A (45.2 mg, 86%) was synthesized from compound **4** (50.5 mg) using the same procedure as for genistein. m.p. 180-184°C; ^{1}H-NMR (d_6-DMSO, 300MHz) δ 8.36 (1H, s, H-2), 7.48 (2H, d, J= 8.4 Hz, H-2', H-6'), 6.99 (2H, d, J= 8.7 Hz, H-3', H-5'), 6.38 (1H, d, J=2.4 Hz, H-8), 6.22 (1H, d, J= 2.1 Hz, H-6), 3.77 (3H, -OMe); ^{13}C-NMR (d_6-DMSO, 500MHz) δ 180.07 (C-4), 164.29 (C-4'),161.97 (C-5), 159.15 (C-8a), 157.56 (C-7), 154.17 (C-2), 130.15 (C-2', C-6'), 122.90 (C-3), 121.95 (C-1'), 113.69 (C-3', C-5'), 104.45 (C-4a), 98.99 (C-8), 93.67 (C-6), 55.135 (-OMe).

Results and Discussion

4-Hydroxybenzyl 2,4-dihydroxyphenyl ketone (**1**), as needle-like crystals (88%), was synthesized by refluxing resorcinol and 4-hydroxyphenylacetic acid with boron trifluoride etherate (BF_3.Et_2O) (Figure 3.2). The ABX pattern in A ring of **1** and a singlet of 2 protons of the benzylic methylene group at 4.09 ppm were confirmed by the ^{1}H-NMR spectrum. The synthesis of this ketone was reported by Shriner and Hull (1945) and Yoder et al. (1954) by saturating a solution of resorcinol and 4-hydroxyphenyl acetonitrile in ether with dry HCl over a period of three days. Pelter and Foot (1976) reported that the cyclization of **1** to daidzein can be achieved by refluxing **1** with N,N-dimethylformamide dimethyl acetal in DMF for 3 h, yielding 76 % daidzein. Using N,N-dimethylformamide dimethyl acetal and THF as the solvent, daidzein was obtained (71 %) from **1** under medium microwave energy for 2 min, which gave an overall yield of 57 %. To prevent the evaporation of N,N-dimethylformamide dimethyl acetal during the cyclization of **1**, the reaction was carried out in a sealed vial. The



R_1	R_2	R_3	Ketones	Isoflavones
H	COOH	OH	1	Daidzein
H	COOH	OMe	2	Formononetin
OH	CN	OH	3	Genistein
OH	CN	OMe	4	Biochanin A

Figure 3.2 Synthesis of isoflavones

resulting product, after recrystallization, did not show the -CH_2- signal appeared at 4.09 ppm in ketone **1**. The H-2 signal was at δ 8.2, as a singlet, in its ^{1}H-NMR spectrum. Both ^{1}H- and ^{13}C-NMR spectra of the product were identical to an authentic sample of daidzein.

Similarly, 4-methoxybenzyl 2,4-dihydroxyphenyl ketone (**2**) was synthesized (51 %), as in the case of compound **1**, by replacing the starting material ,4-hydroxyphenylacetic acid, with 4-methoxyphenylacetic acid. Using N,N-dimethylformamide dimethyl acetal and THF as the solvent, formononetin was produced (91%, overall 45 %) by the cyclization of **2** under medium microwave energy for 1 min, as in for daidzein. The ^{1}H- and ^{13}C-NMR spectra of **2** were similar to **1** and contained the signal for a methoxy group. The ^{1}H- and ^{13}C-NMR spectra of formononetin were identical to the published data (Nair et al., 1991).

Syntheses of ketones **3** and **4**, for genistein and biochanin A synthesis, respectively, were conducted by modification of the procedure reported by Yoder et al. (1954). The 4-hydroxybenzyl 2,4,6-trihydroxyphenyl ketone (**3**) was produced by bubbling dry HCl into a solution of phloroglucinol and p-hydroxyphenyl acetonitrile in dry ether, with 46 % yield. Compound **3** gave distinct singlets of two protons each at δ 6.69 and δ 4.26 for H-2' and H-6' and the methylene protons, respectively, in the ^{1}H-NMR spectrum. The cyclization of **3** with BF_3 etherate in DMF and methanesulfonyl chloride (Bass, 1976) also was accomplished in a microwave oven for 2 min at low energy and afforded genistein in high purity (80 %, overall 36 %). The structure of genistein was confirmed by ^{1}H- and ^{13}C-NMR spectra.

Using identical procedures used for the synthesis of **3**, 4-methoxybenzyl 2,4,6-trihydroxyphenyl ketone (**4**), for biochanin A synthesis, was prepared (47 %) from p-

methoxyphenyl acetonitrile and phloroglucinol as the starting materials. Similarly, the cyclization of **4** to biochanin A (86 %, overall 40 %) was carried out in a microwave-oven using the same conditions as used in the genistein synthesis. The structures of **4** and biochanin A were confirmed by their ^{1}H- and ^{13}C-NMR spectra.

Microwave conversion of ketones **2, 3, 4,** to formononetin, genistein and biochanin A afforded superior yields than the reported values of 85 % (Pelter and Foot, 1976), 74 % (Yoder et al., 1954) and 65 % (Bass, 1976), respectively. The microwave conversion of the ketone **1** to daidzein with 71 % yield is comparable to the yield reported by Pelter and Foot (1976). The overall yields for the isoflavones are not available from published reports. However, the overall yield of isoflavones daidzein, formononetin, genistein and biochanin A under microwave conditions were 57, 40, 36 and 40 %, respectively. Our synthesis of these isoflavones has the advantages of reduced cost and time consumption. For example, the 4-hydroxyphenylacetic acid used for the synthesis of **1** is considerably cheaper than its corresponding nitrile. The syntheses of these soybean isoflavonoids, especially genistein and daidzein, in substantial quantities using less expensive reagents in a very short time facilitate their in vivo evaluation as anticarcinogens for human medicine.

CHAPTER IV*

Metabolism of Daidzein And Genistein Anticarcinogens by Intestinal Bacteria

Abstract

Isoflavones, daidzein {1} and genistein {2}, were fermented with human fecal bacteria under anaerobic conditions. Dihydrodaidzein {3}, benzopyran-4,7-diol, 3-(4-hydroxyphenyl) {4} and equol {5} were isolated from the fermentation broth of daidzein. Only one metabolite, dihydrogenistein {6}, was isolated and characterized from the fermentation broth of genistein. The metabolites **3 - 6** were identified by spectral methods.

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Introduction

Endogenous and exogenous chemicals such as estrogens (Jarvenpaa et al., 1980), androgens (Lambardi et al., 1978), and safflower yellow B (Meselhy et al., 1993) are metabolized by intestinal bacteria. The biological activities of these compounds can be altered dramatically by the metabolism by intestinal bacteria. Often these metabolites are mutagenic (Shu et al., 1991; Gorbach and Goldin, 1990). Soybean products are an integral part of human diet in Asian countries. It was implicated that soybean, especially the isoflavones daidzein and genistein present in it, may provide protection against breast cancer and function as anticarcinogens (Aldercreutz, 1988). The animal metabolism studies of daidzein and genistein present in soybean diets were carried out in sheep, rats and humans. However, the metabolism of pure daidzein and genistein in humans is not reported yet.

Several isoflavone metabolites have been detected in urine collected from human subjects on soy diets. They were identified as dihydrodaidzein *O*-desmethyl angolensin, glycitein, 6'-hydroxy-*O*-desmethyl angolensin, equol dihydrogenistein, and dehydro-*O*-desmethyl angolensin ((Braden et al., 1967; Axelson and Setchell, 1981; Axelson et al., 1982[a,b]; Bannwart er al., 1984; Bannwart et al., 1984; Kelly et al., 1993; Yasuda er al., 1994) as glucuronide or sulfate conjugates (Axelson et al., 1982[b]; Yasuda er al., 1994). Equol was the major metabolite identified in the urine of sheep, guinea-pigs (Shutt and Branden, 1968) and humans (Axelson et al., 1982[b]). Equol also was detected in the fermentation broth of soy protein incubated with human fecal bacteria (Setchell et al., 1984). In another study, the germ-free rats did not excrete equol upon feeding soy diets, where as

conventional rats' urine contained equol (Axelson and Setchell, 1981). This suggested that intestinal bacteria metabolized the isoflavone daidzein in soy diets to equol.

Flavonoids quercetin, kaempferol and naringenin underwent C-ring cleavage at C-3 and C-4 upon the incubation with *Clostridium* strains isolated from human intestinal bacteria (Winter et al., 1991). A similar fragmentation of isoflavonoids by intestinal bacteria is not known. *p*-Ethyl phenol was the major metabolite of genistein in the urine of ruminants (Batterham er al., 1965), but not in human urine (Kelly et al., 1993). Using gc-ms, Kelly et al. (1993) confirmed the presence of dihydrodaidzein and tetrahydrodaidzein in human urine. Dihydrodaidzein was detected in the urine from all human subjects studied, whereas tetrahydrodaidzein was found only in the urine from a single subject. Also, only one in twelve subjects excreted dihydrogenistein in a trace amounts. These data suggested that the metabolism of daidzein and genistein vary in humans.

It is important to note that there are no published data on the intestinal metabolism of isoflavones, an important class of antioxidants present in many foods. Therefore, we have conducted an *in vitro* metabolism study of daidzein and genistein using human fecal bacteria.

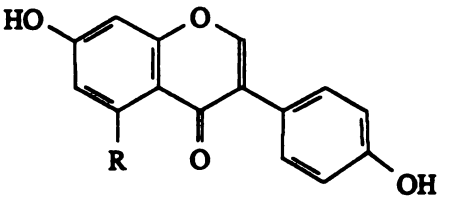
Experimental

GENERAL.—^{1}H and ^{13}C-NMR spectra were recorded on a Varian VXR 500 MH_Z spectrometers in CD_3OD solution at ambient temperature. Mass spectra were acquired on a JEOL HX-110 double-focusing mass spectrometer (JEOL,Tokyo, Japan). Sep-Pak cartridges (C-18) were purchased from Waters (Milford, Massachusetts, USA). HPLC

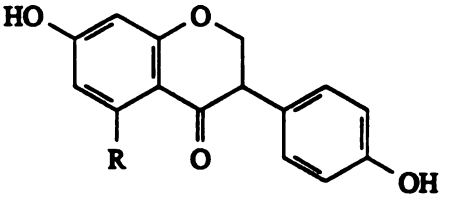
analyses were performed with an HPLC system equipped with automatic gradient controller, autosampler and photodiode array detector (PDA) (Waters, Milford, Massachusetts, USA). Recycling Preparative HPLC (LC-20) and C-18 reverse phase column (Jaigel, S-343-15; 15 μm, 250 x 20 mm) were purchased from Japan Analytical Industrial Ltd., (Tokyo, Japan). C-18 reverse phase capcell pak columns (AG-120 S-5 μm, 30 % carbon loading, 5 μm, 250 x 10 mm and AG-120 S-5 μm, 30 % carbon loading, 5 μm, 250 x 4.6 mm) were purchased from Shiseido Co. Ltd. (Tokyo, Japan).

CHEMICALS AND FERMENTATION MEDIA.—Daidzein and genistein were synthesized in our laboratory (Chang et al., 1994). Compounds **3**, **5** and **6** were isolated from the fermentation of both **1** and **2** and used as standards for the quantification of these compounds in the fermentation media. BHI dehydrated media was purchased from Difco Lab (Detroit, MI, USA) and vitamin K, heme, cystine chloride and resazurine were purchased from Aldrich Chemical Company (Milwaukee, WI, USA);

IN VITRO ANAEROBIC FERMENTATION.—Commercially available BHI media (3.7 g / 25 ml) was supplemented with vitamin K (20 μl / 100 ml) and heme solution (1 ml / 100 ml media from 50 mg / 100 ml stock solution) and mixed with 5 mg of the isoflavone, daidzein or genistein. Cystine chloride (50 mg / 100 ml) and resazurine (0.4 ml / 100 ml media from 25 mg / 100 ml stock solution) were used as reducing agent and O_2 indicator, respectively (Holdmen et al., 1977). The pH of the media was adjusted to 7 with 1N NaOH solution and autoclaved for 15 min under anaerobic conditions. Fresh human feces (1 g) was suspended



1 R= H
2 R= OH



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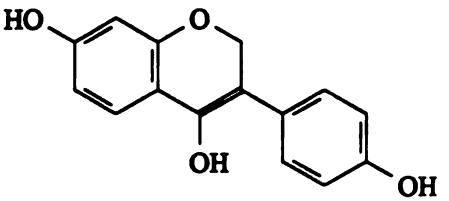
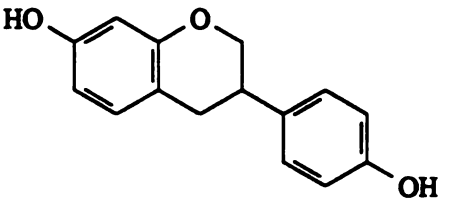
6 R= OH



4 R= H



5 R= H

Figure 4.1 Metabolites isolated from fermentation broth of daidzein and genistein incubated with human fecal bacteria

in pre-reduced BHI media (10 ml). Pre-reduced supplemented BHI media (25 ml) was inoculated with the fecal suspension (0.5 ml) and incubated for 3 days at 37°C under anaerobic conditions. After 3 days of incubation, 1 ml of the fermentation broth was sampled and analyzed by HPLC. The remaining fermentation broth was lyophilized for the isolation and purification of metabolites.

PURIFICATION OF FERMENTATION PRODUCTS.—Fermentation broth of daidzein (125 ml) was lyophilized, and the resulting solid was extracted with hexane/$CHCl_3$ (1:1, 25 ml x 2). The hexane/$CHCl_3$ extract was discarded. The residue then was extracted with MeOH (25 ml x 2). The MeOH extract was evaporated to dryness under vacuum and the residue was purified on a recycling preparative HPLC using C-18 reverse phase column. The solvent system, MeOH/H_2O, 60:40, was used as the mobile phase under isocratic condition at a flow rate of 3 ml/min. The metabolites were detected under uv at 210 nm. The fractions containing isoflavone and its metabolites were purified further by HPLC on a C-18 reverse phase capcell pak column using MeOH/H_2O, 40:60, as the mobile phase at a flow rate of 1 ml/min. The compounds were monitored by a photodiode array detector (PDA) at 210 nm.

Lyophilized fermentation broth of genistein (100 ml) was purified by vacuum liquid chromatography with $CHCl_3$,MeOH, 4:1, as the solvent system. Fractions containing isoflavones and their metabolites were combined and purified by recycling preparative HPLC on a C-18 reverse phase column using MeOH/H_2O, 60:40, as the mobile phase at a flow rate of 3 ml/min. The metabolites were monitored under uv at 210 nm.

HPLC ANALYSIS OF ISOFLAVONES AND THEIR METABOLITES.—The fermentation broth

(1 ml) was passed through a C-18 Sep-Pak cartridge which was pre-conditioned with MeOH (5 ml) and H_2O (10 ml). The cartridge then was washed successively with water (5 ml), ACN/H_2O, 30:70, (1 ml), and finally with ACN/H_2O, 90:10, (2 ml). The ACN/H_2O, 90:10, eluate was analyzed for isoflavones and their metabolites on a C-18 reverse phase capcell pak column. The mobile phase was ACN and H_2O under a linear gradient of ACN/H_2O 30:70 to 100 % ACN (final) in 15 min at a flow rate of 0.5 ml/min. The column was eluted with 100 % ACN for an additional 10 min. The compounds were monitored using a PDA detector and the data were collected at 200-360 nm and processed to obtain the results at 210 nm. The isoflavones have comparable absorption maxima at 210 and 262 nm. The HPLC analysis of the isoflavones and their metabolites were carried out at 210 nm, since uv spectra of the metabolites showed the absorption maxima at 210 nm. The metabolites formed during the fermentation of compound **1** and **2** were monitored by withdrawing samples (1 ml) from the fermentation broth at 24, 48 and 72 h, respectively.

Calibration curves for compound **1** - **3**, **5** and **6** were created by analyzing the respective solution by HPLC as mentioned above. The solutions were prepared by the serial dilution of respective stock solutions to afford 0.39, 0.78, 1.56, 3.12, 6.25, 12.5 and 25.0 μg/ml concentration, respectively. Calibration curves were generated by Millenium 2010 chromatograph manager by the following equation: $y = A + Bx$, where y = response calculated for the standard peak at 210 nm; A = intercept of calibration curve; B = slope of calibration curve; x = the amount of standard, respectively.

DIHYDRODAIDZEIN, **3**.— ^{1}H-NMR: δ 7.74 (1H, d, J=8.5 Hz, H-5), 7.08 (2H, d, J=8.5

Hz, H-3', 5'), 6.74 (2H, d, J=8.5 Hz, H-2', 6'), 6.48 (1H, dd, J=8.75, 2.0 Hz, H-6), 6.31 (1H, d, J=2.5 Hz, H-8), 4.57 (1H, dd, J=11.5, 5.5 Hz, H-2a), 4.53 (1H, dd, J=11.25, 8.0 Hz, H-2b), 3.83 (1H, dd, J=8.0, 5.5 Hz, H-3); ^{13}C-NMR: δ 194.07 (C-4), 166.89(C-4'), 165.54 (C-8a), 157.96 (C-7), 130.64 (C-2', 6'), 130.30 (C-5), 128.10 (C-1'), 116.56 (C-4a), 115.11 (C-6), 103.66 (C-3', 5'), 103.48 (C-8), 73.18 (C-2), 52.56 (C-3).

BENZOPYRAN-4,7-DIOL, 3-(4-HYDROXYPHENYL), **4**.— ^{1}H-NMR: δ 7.82 (1H, d, J=8.5 Hz, H-5), 7.09 (2H, d, J=8.5 Hz, H-2', 6'), 6.72 (2H, J=8.5 Hz, H-3', 5'), 6.33 (1H, dd, J=9.0, 2.5 Hz, H-6), 6.22 (1H, d, J=2.0 Hz, H-8), 4.10 (2H, s, H-2).

EQUOL, **5**.—EIMS m/z (rel. int.): 242 (82), 120 (100); ^{1}H-NMR: δ 7.18 (2H, d, J=9 Hz, H-2',6'), 6.88 (1H, d, J=8.0 Hz, H-5), 6.76 (2H, d, J=8.5 Hz, H-3', 5'), 6.33 (1H, dd, J=8.5, 2.5 Hz, H-6), 6.24 (1H, d, J=2.5 Hz, H-8), 4.19 (1H, ddd, J=10.5, 3.5, 2.0 Hz, H-2a), 3.91 (1H, dd, J=10.5, 10.5 Hz, H-2b), 3.05 (1H, mm, H-3), 2.87 (1H, dd, J=15.8, 10.0 Hz, H-4a), 2.82 (1H, ddd, J=16.0, 6.0, 1.5 Hz, H-4b); ^{13}C-NMR: δ 155.70 (C-4' or C-7), 155.42 (C-4' or C-7), 154.39 (C-8a), 131.97 (C-1'), 129.25 (C-5), 127.41 (C-2', 6'), 114.52 (C-3', 5'), 112.69 (C-4a), 107.20 (C-6), 101.90 (C-8), 70.29 (C-2), 37.53 (C-3), 31.12 (C-4).

DIHYDROGENISTEIN, **6**.—EIMS m/z (rel. int.): 272 (25), 153 (100); ^{1}H-NMR: δ 7.10 (2H, d, J=9.0 Hz, H-2", 6"), 6.75 (2H, d, J=9.0 Hz, H-3", 5"), 5.83 (2H, s, H-3', 5'), 4.51 (1H, dd, J=11.5, 5.0 Hz, H-2a), 4.44 (1H, dd, J=11.5, 7.5 Hz, H-2b), 3.83 (1H, dd, J=7.5, 4.5 Hz, H-3); ^{13}C-NMR: δ 196.50 (C-4), 166.71 (C-4'), 164.00 (C-5), 162.92 (C-8a), 156.16 (C-7), 128.84 (C-2', 6'), 125.88 (C-1'), 114.65 (C-3', 5'), 101.48 (C-4a), 95.33 (C-8), 94.10 (C-6), 70.77 (C-2), 49.74 (C-3).

Results and Discussion

Fermentation studies of endogenous and exogenous chemicals with fecal bacteria have provided valuable information to elucidate their metabolic pathways. The incubation of daidzein with human feces afforded compounds **3** - **5** (Figure 4.1). After 72 h of incubation, the fermentation broth of compound **1** was purified. The band with a higher R_f value than daidzein was collected and further purified by HPLC to afford **3**. ^{1}H-NMR spectra of this compound showed the presence of two aromatic rings with an ABX substitution pattern in one ring and para-substitution in the other. The dd signals appearing at 4.57 and 4.53 ppm in **3** were assigned to the H-2 protons. A one proton, dd, at 3.83 ppm confirmed the presence of H-3 in compound **3**. The absence of an olefinic proton in **3** , appeared at 8.02 ppm in **1**, indicating that the double bond between C-2 and C-3 in compound **1** was reduced. Therefore, the ^{1}H-NMR data of compound **3** indicated that it is a metabolite of daidzein.

The ^{1}H-NMR spectra of compound **4** showed that the protons of ring A and B in **4** gave similar multiplicity and chemical shifts to the ring A and B protons in daidzein. The H-2 singlet appearing at 8.02 ppm in daidzein was absent in **4**. Therefore, the olefinic bond between C-2 and C-3 in daidzein was reduced to yield **4**. A two-proton singlet at 4.10 ppm in compound **4** was assigned to H-2 protons. This confirmed the presence of a double bond between C-3 and C-4 in **4** and the structure of **4** as benzopyran-4,7-diol, 3-(4-hydroxyphenyl). A third metabolite, isolated from the fermentation broth of daidzein and purified by recycling preparative HPLC, was compound **5**. The ^{1}H-NMR spectral data of compound **5** was identical to the published values for equol (Aldercreutz, et al., 1986[b]).

The only metabolite isolated from the fermentation broth of genistein was compound **6**. ^{1}H-NMR signals of **6** in the aromatic region was similar to that of genistein. The absence of a singlet at 8.30 ppm in **6** indicated that the olefinic bond between C-2 and C-3 in genistein was reduced. Three dd signals appeared at 4.51, 4.44 and 3.83 ppm in **6** were assigned to H-2a, H-2b and H-3 protons, respectively.

The production of metabolites during the fermentation of isoflavones **1** and **2** with human fecal bacteria was monitored by HPLC (Appendices III, IV and V). Compound **3** was the major metabolite during the 72-h fermentation of compound **1** (Figure 4.2). Compound **5** was the major metabolite reported for daidzein in human urine from those whose consumed the soy diet. In our studies, the fecal bacterial metabolism of daidzein afforded compound **3** in higher yield than **5**. This indicated that compound **3** was metabolized further to compound **5** prior to excretion, as evidenced by previous *in vitro* metabolism studies (Setchell et al., 1984). Since compound **4** was isolated in a very small quantity, the quantification of this compound was not carried out.

The estrogenic activity of genistein in ruminants was considerably lower when given intraruminally than intramuscularly (Braden et al., 1967). This implied that the metabolism of genistein by rumen fluid may be responsible for the lack of estrogenic activity. *p*-Ethyl phenol, the reported end-product of genistein metabolism, was detected in the urine of sheep fed with soy diets. This compound was not detected in our fermentation studies when genistein was incubated with human feces. The HPLC analysis of the fermentation products of genistein with fecal bacteria showed that the amount of genistein declined rapidly during

24 h. However, a corresponding increase in the amount of compound **6** was not observed (Figure 4.2). This indicated that genistein was metabolized to several other compounds that were not detected in our study. We did not isolate any other metabolite from the fermentation broth of genistein.

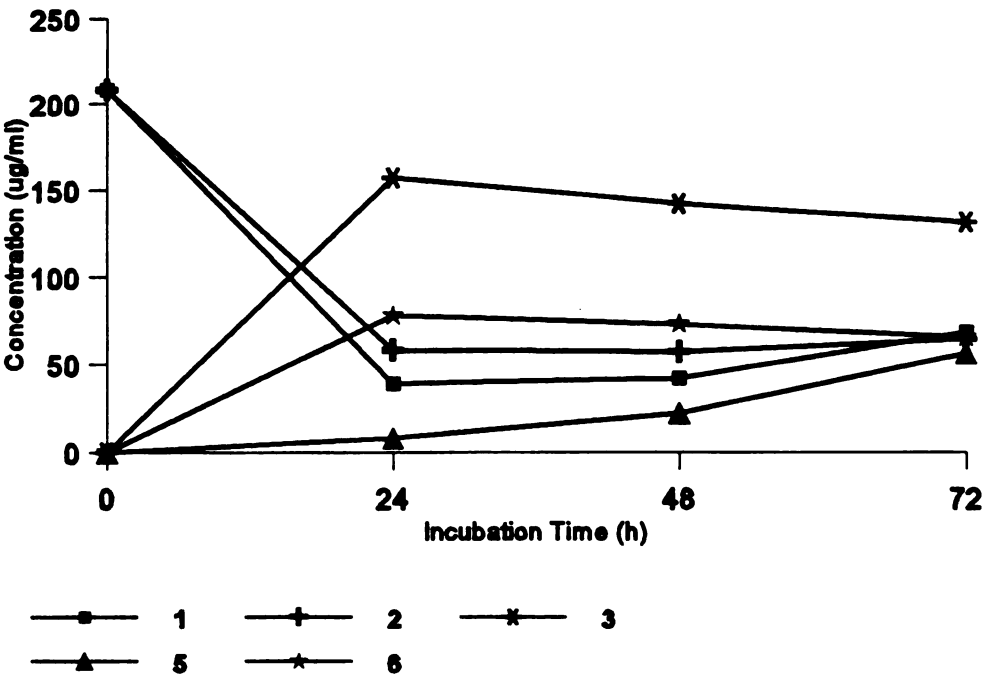


Figure 4.2 Production of metabolites of daidzein and genistein by human fecal bacteria at 72 h.

1: Daidzein; 2: Genistein; 3: Dihydrodaidzein;

5: Equol; 6: Dihydrogenistein

CHAPTER V*

Metabolites of Anticancer Daidzein and Genistein And Their Biological Activities

Abstract

Daidzein and genistein, the isoflavones present in soybean, were synthesized earlier to study their metabolism in humans. The metabolites detected were mostly reduction products of daidzein or genistein. We have synthesized these metabolites to evaluate their efficacy. Equol {**3**}, 5,7,4'-trihydroxyisoflavan {**5**}, 4,7,4'-trihydroxyisoflavan {**6**}, dihydrodaidzein {**8**}, and dihydrogenistein {**9**}, were synthesized either from daidzein {**1**} or genistein {**2**} by hydrogenation and characterized by spectral methods. During acetylation and NMR experiments, compound **9** was converted to an intermediate enol form, a novel compound **10**. Antifungal, antibacterial, mosquitocidal, nematicidal and inhibition of topoisomerase activities of these compounds were evaluated. Equol {**3**} was the most active compound among the five metabolites assayed.

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Introduction

Several epidemiological studies has shown that soybean products reduced the incidence of breast cancer in women (Messina et al., 1994; Lee et al., 1991). Animal studies also revealed that soybean isoflavonoids daidzein {**1**} and genistein {**2**} are responsible for this protective effect Troll et al., 1980; Barnes et al., 1988; Messina et al., 1994). Singlet oxygen species play an important role in mutagenesis and carcinogenesis, particularly in tumor promotion (Wei et al., 1993). Tyrosine-kinase activity is associated with growth factors which are involved in the uncontrolled growth of cancer cells (Chang and Geahlen, 1992). Genistein has been implicated by inhibiting H_2O_2 formation both *in vivo* and *in vitro* studies (Wei etal., 1993). Also, it acts as an inhibitor for tyrosine kinase and DNA synthesis (Dean et al., 1989; Sit et al., 1991). Soybean diets were able to lower mammary tumor in rats induced by radiation (Troll et al., 1980) or by the carcinogen, N-methyl-N-nitrosourea (MNU) (Barnes et al., 1988). Isoflavonoids present in soybean are metabolized in the digestive track (Setchell et al., 1984) and are considered to reduce the incidence of breast cancer (Messina et al., 1994).

Several metabolites are detected in the urine from human subjects on soybean diet (Kelly et al., 1993). The major metabolites of daidzein {**1**} and genistein {**2**} are their reduction products, equol {**3**}, 7,4'-dihydroxyisoflavanone {**8**}, tetrahydrodaidzein {**6**} and *O*-desmethyl angolensin and 5,7,4'-trihydroxyisoflavanone {**9**}. The biological activities of these metabolites, isolated from physiological samples in trace quantities, have not been evaluated. We have now synthesized compounds **3**, **6**, **8** and **9** to evaluate their biological

activities.

The synthesis of isoflavanones and isoflavans from daidzein and genistein were reported earlier (Inoue, 1964; Szabo, 1973; Lamberton et al., 1978). Hydrogenation reactions of isoflavones were non-selective and often resulted in complex mixtures (Inoue, 1964; Szabo, 1973; Lamberton et al., 1978). A selective catalytic hydrogenation of several isoflavones to their corresponding isoflavanones was published (Krishnamurty and Sathanarayana, 1986). Daidzein {1} and genistein {2} were not used to produce isoflavanones by previous researchers (Krishnamurty and Sathanarayana, 1986). In this paper, we report the synthesis of several hydrogenation products of daidzein and genistein and their biological activities.

Experimental

GENERAL.—^{1}H- and ^{13}C-NMR spectra were recorded on a Varian VXR 300 and 500 MH_Z spectrometers (Varian, California, USA), respectively, at ambient temperature. The melting points were recorded on a Thomas model 40 micro hot-stage apparatus and were not corrected. Mass spectra were acquired on a JEOL HX-110 double focusing mass spectrometer (JEOL, Tokyo, Japan). Preparative silica gel TLC plates were purchased from Analtech Inc. (Newark, Delaware, USA). Recycling preparative HPLC LC-20 and C-18 reverse phase column (Jaigel, S-343-15; 15 μm, 250 x 20 mm) were purchased from Dychrom (Santa Clara, California, USA)

CHEMICALS AND CELL CULTURE MEDIA.——Daidzein {1} and genistein {2} were synthesized in our laboratory (Chang et al., 1994). Pd/C and $Pd/BaSO_4$ (5%) were purchased

from Aldrich Chemical Company (Milwaukee, Wisconsin, USA). YMG (yeast extract 4 g/L, maltose 10 g/L, glucose 4 g/L and agar 12 g/L), PDA (potato dextrose agar) and Emmons (neopeptone 10 g/L, glucose 20 g/L and agar 15 g/L) media were prepared as published (Nair et al., 1989) and the ingredients were purchased from Difco Lab (Detroit, Michigan, USA). NG medium (NaCl 3.0 g/L, bacto peptone 2.5 g/L, cholesterol 1 ml/L from 5 mg/ml stock solution, $CaCl_2$ 1 ml/L from 1 M stock solution, $MgSO_4$ 1 ml from 1 M stock solution, and potassium phosphate buffer 25 ml/L of stock solution containing KH_2PO_4 11.97 g/100ml and K_2HPO_4 2.09 g/100 ml). YPDA medium (yeast extract 20 g/L, peptone 10 g/L and dextrose 20 g/L, and adenine sulfate 2 ml/L from 0.5% stock solution) were purchased from Difco Lab (Detroit, Michigan, USA). Adenine sulfate and camptothecin were purchased from Aldrich Chemical Company (Milwaukee, Wisconsin, USA).

HYDROGENATION OF ISOFLAVONES.—A solution of daidzein {**1**} or genistein {**2**} in EtOH or glacial acetic acid was flushed with H_2 for 15 min and then added to a pre-reduced ethanolic or glacial acetic acid solution containing 5% Pd/C. The reaction mixture was stirred at room temperature under H_2 atmosphere until the sioflavone was not detectable on silica gel TLC. The reaction mixture was filtered through a celite bed and the resulting solution was dried under vacuum.

EQUOL {**3**}.—The product from the hydrogenation of daidzein {**1**} in glacial acetic acid was recrystallized from $MeOH/H_2O$ to yield **3** (46.7 %); m.p. 150-152°; EIMS m/z (rel. int.): 242 (82), 120 (100); ^{1}H-NMR (CD_3OD) δ 7.18 (2H, d, J=9 Hz, H-2',6'), 6.88 (1H, d, J=8.0 Hz, H-5), 6.76 (2H, d, J=8.5 Hz), 6.33 (1H, dd, J=8.5, 2.5 Hz, H-6), 6.24 (1H, d, J=2.5 Hz,

H-8), 4.19 (1H, ddd, J=10.5, 3.5, 2.0 Hz, H-2a), 3.91 (1H, dd, J=10.5, 10.5 Hz, H-2b), 3.05 (1H, m, H-3), 2.87 (1H, dd, J=15.8, 10.0 Hz, H-4a), 2.82 (1H, ddd, J=16.0, 6.0, 1.5 Hz, H-4b); ^{13}C-NMR (CD_3OD) δ 155.70 (C-4' or C-7), 155.42 (C-4' or C-7), 154.39 (C-8a), 131.97 (C-1'), 129.25 (C-5), 127.41 (C-2', 6'), 114.52 (C-3', 5'), 112.69 (C-4a), 107.20 (C-6), 101.90 (C-8), 70.29 (C-2), 37.53 (C-3), 31.12 (C-4).

7,4'-DIMETHOXYEQUOL {**4**}.—A solution of compound **3** (20.4 mg) in acetone (15 ml) was stirred with K_2CO_3 (5 g) for 15 min and refluxed with dimethyl sulfate (50 μl) for 8 h. The reaction mixture was cooled to room temperature, the resulting solution then filtered and the resulting solution was dried under vacuum. The product was dissolved in $CHCl_3$ (50 ml) and washed with H_2O (20 ml x 2) followed by washing with saturated $NaHCO_3$ solution (25 ml x 1) and H_2O (25 ml x 2). The resulting $CHCl_3$ solution was evaporated under vacuum and the white precipitate was recrystallized from MeOH to give needle-like crystals (16 mg); m.p. 112-113°; ^{1}H-NMR ($CDCl_3$) δ 7.17 (2H, d, J=8.4 Hz, H-2', 6'), 6.99 (1H, d, J=7.8 Hz, H-5), 6.90 (2H, d, J=8.7 Hz, H-3', 5'), 6.49 (1H, dd, J=8.4, 2.7 Hz, H-6), 6.43 (1H, d, J=2.7 Hz, H-8), 4.31 (1H, ddd, J=10.5, 3.0, 1.5 Hz, H-2a), 3.98 (1H, dd, J=10.2, 10.2 Hz, H-2b), 3.81 (3H, s, OMe), 3.78 (3H, s, OMe), 3.17 (1H, m, H-3), 2.95 (2H, d, J=8.4 Hz, H-4).

4,7,4'-TRIHYDROXYISOFLAVAN {**6**}.—Amorphous powder, produced from the hydrogenation of daidzein {**1**} using ethanol as the solvent (60 %); m.p. 204-208°; EIMS m/z (rel. int.): 240 (100); ^{1}H-NMR (CD_3OD) δ 7.21 (1H, d, J=8.5 Hz, H-5), 7.07 (2H, d, J=8.5 Hz, H-2',6'), 6.72 (2H, d, J=8.5 Hz, H-3', 5'), 6.40 (1H, dd, J=8.5, 2.0 Hz, H-6), 6.22 (1H, d, J=2.0 Hz, H-8), 4.74 (1H, d, J=7.5 Hz, H-4), 4.25 (1H, dd, J=11, 3.5 Hz, H-2a), 4.16 (1H,

dd, J=10.75, 8.0 Hz, H-2b), 2.99 (1H, ddd, J=7.5, 7.5, 3.5 Hz, H-3); ^{13}C-NMR (CD_3OD) δ 157.42 (C-8a), 155.62, 155.20 (C-4' and C-7), 130.39 (C-1'), 129.59 (C-5), 128.56 (C-2', 6'), 114.86 (C-3',5'), 114.72 (C-4a), 108.19 (C-6), 101.88 (C-8), 67.70 (C-4), 68.23 (C-2), 45.96 (C-3).

4,7,4'-TRIACETYLISOFLAVAN {7}.—To a solution of compound 6 (9.8 mg) in pyridine (1 ml), acetic anhydride (200 μl) was added and left in the dark at room temperature for 24 h. The reaction mixture was dried under vacuum and purified by preparative TLC using $CHCl_3$/MeOH, 12:1, as the mobile phase to give amorphous white solid (14.6 mg) ; m.p. 115-117°; ^{1}H-NMR ($CDCl_3$) δ 7.34 (2H, dd, J=8.5, 2.5, H-2',6'), 7.22 (1H, dd, J=8.5, 2.5 Hz, H-5), 7.04 (2H, dd, J=8.75, 2.5 Hz, H-3',5'), 6.68 (1H, ddd, J=8.5, 2.5, 2.0 Hz, H-6), 6.65 (1H, d, J=2.5 Hz, H-8), 6.13 (1H, dd, J=5.25, 2.5 Hz, H-4), 4.45 (2H, m, H-2), 3.39 (1H, m, H-3), 2.26 (6H, s, CH_3- of C-7, 4'), 2.05 (3H, s, CH_3- of C-4)

DIHYDRODAIDZEIN {8}.—The reaction mixture from the hydrogenation of daidzein {1} in ethanol and $Pd/BaSO_4$ was purified on a recycling preparative HPLC (C-18 column, mobile phase H_2O:MeOH 30:70 at a flow rate of 2 ml/min, detected at 210 nm) and yielding compound 8 (41.4 %) as the major product; m.p. 198-200°; ^{1}H-NMR (CD_3OD) δ 7.74 (1H, d, J=9.0 Hz, H-5), 7.07 (2H, d, J=8.5 Hz, H-3', 5'), 6.74 (2H, d, J=9.0 Hz, H-2', 6'), 6.50 (1H, dd, J=8.75, 2.0 Hz, H-6), 6.33 (1H, d, J=2.5 Hz, H-8), 4.56 (1H, dd, J=11.5, 5.5 Hz, H-2a), 4.52 (1H, dd, J=11.25, 8.0 Hz, H-2b), 3.82 (1H, dd, J=8.0, 5.5 Hz, H-3); ^{13}C-NMR (CD_3OD) δ 194.07 (C-4), 166.89(C-4'), 165.54 (C-8a), 157.96 (C-7), 130.90 C-3', 5'), 130.64 (C-2', 6'), 130.30 (C-5), 128.10 (C-1'), 116.56 (C-4a), 115.11 (C-6), 103.66 (C-8),

73.18 (C-2), 52.56 (C-3).

DIHYDROGENISTEIN {9}.—The reaction product from the hydrogenation of genistein in {2} ethanol and Pd/C was purified by preparative TLC using solvent system (10:1) $CHCl_3$/MeOH. The major component was recrystallized from MeOH/H_2O and gave colorless needle-like crystals, compound **9** (67 %); m.p. 196-198°; EIMS m/z (rel. int.): 272 (25), 153 (100); ^{1}H-NMR (CD_3OD) δ 7.09 (2H, d, J=8.5 Hz, H-2", 6"), 6.75 (2H, d, J=8.5 Hz, H-3", 5"), 5.88 (2H, s, H-3', 5'), 4.51 (1H, dd, J=11.5, 5.0 Hz, H-2a), 4.47 (1H, dd, J=11.5, 7.75 Hz, H-2b), 3.86 (1H, dd, J=7.75, 5.5 Hz, H-3); ^{13}C-NMR (CD_3OD) δ 196.50 (C-4), 166.71 (C-4'), 164.00 (C-5), 162.92 (C-8a), 156.16 (C-7), 128.84 (C-2', 6'), 125.88 (C-1'), 114.65 (C-3', 5'), 101.48 (C-4a), 95.33 (C-8), 94.10 (C-6), 70.77 (C-2), 49.74 (C-3).

5,7,4'-TRIHYDROXYISOFLAVAN {5}.—The hydrogenation of genistein {2} with Pd/C in glacial acetic acid yielded a mixture of two compounds. The reaction mixture was purified by preparative TLC developed with (10:1) $CHCl_3$/MeOH. The compound with the similar R_f value to genistein was identified as compound **9** (17.8 %). The second compound with a lower R_f value was recrystallized from MeOH/H_2O and gave needle-like crystals, compound **5** (27.0 %); m.p. 209-211°; EIMS m/z (rel. int.): 258 (58), 139 (100); ^{1}H-NMR (CD_3OD) δ 7.12 (2H, d, J=8.5 Hz, H-2', 6'), 6.79 (2H, d, J=9.0 Hz, H-3', 5'), 5.97 (1H, d, J=2.5 Hz, H-6), 5.86 (1H, d, J=2.5 Hz, H-8), 4.18 (1H, ddd, J=10.5, 3.5, 2.0 Hz, H-2a), 3.89 (1H, dd, J=10.0, 10.0 Hz, H-2b), 3.02 (1H, m, H-3), 2.88 (1H, ddd, J=16.0, 5.5, 2.0 Hz, H-4a), 2.61 (1H, dd, J=16.0, 10.75 Hz, H-4b); ^{13}C-NMR (CD_3OD) δ 155.43 (C-4'), 155.36 (C-5), 155.13 (C-8a), 155.10 (C-7), 132.45 (C-1'), 127.49 (C-2', 6'), 114.59 (C-3', 5'), 101.09 (C-

4a), 94.37 (C-8), 93.95 (C-6), 70.08 (C-2), 37.10 (C-3), 25.82 (C-4).

4,5,7,4'-TETRAHYDROXYISOFLAVANONE {**10**}.—Compound **9** (12 mg) in d_5-pyridine (0.75 ml) was treated with d_4-acetic acid (100μl) in an NMR tube and its ^{1}H-NMR spectrum was recorded; ^{1}H-NMR (d_5-pyridine and 100 μl of d_4-acetic acid) δ 7.20 (2H, d, J=8.7 Hz, H-2', 6'), 7.05 (2H, d, J=8.7 Hz, H-3', 5'), 6.36 (1H, d, J=1.8 Hz, H=6, 8), 4.48 (1H, d, J=12.0 Hz, H-2a), 4.43 (1H, d, J=12.0 Hz, H-2b).

4,5,7,4'-TETRAACETATEISOFLAVANONE {**11**}.—Acetic anhydride (200 µl) was added to a solution of compound **9** (20 mg) in pyridine (1 ml) and stored in the dark at room temperature for 24 h. Crushed ice was added into the reaction mixture and the white precipitate formed was isolated by centrifugation. This precipitate was then recrystallized from MeOH to give colorless needle-like crystals, compound **11** (22 mg); m.p. 191-192°; ^{1}H-NMR ($CDCl_3$) δ 7.36 (2H, d, J=8.7 Hz, H-2', 6'), 7.11 (2H, d, J=8.7 Hz, H-3', 5'), 6.66 (1H, d, J=2.1 Hz, H-6), 6.47 (1H, d, J=2.4 Hz, H-8), 5.03 (2H, s, H-2), 2.31 (3H, s, CH_3), 2.29 (3H, s, CH_3), 2.27 (3H, s, CH_3), 2.10 (3H, s, CH_3); ^{13}C-NMR ($CDCl_3$) δ 168.77 (C=O), 167.97 (C=O), 167.92 (C=O), 167.29 (C=O), 156.13 (C-8a), 150.72 (C-4'), 149.83 (C-5), 145.38 (C-7), 136.14 (C-4), 130.74 (C-1'), 128.58 (C-2', 6'), 121.31 (C-3', 5'), 111.29 (C-3), 109.88 (C-8), 107.54 (C-6), 68.55 (C-2), 20.62 (2 x CH_3), 20.49 (CH_3), 20.08 (CH_3).

Antimicrobial assay.—Antifungal and antibacterial assays of compounds **1**, **2**, **3**, **5**, **6**, **8**, **9** and **11** were carried out according to the procedure reported earlier (Nair et al., 1989). Cultures of *Fusarium oxysporum* (MSU-SM-1322), *Fusarium moniliforme* (MSU-SM-1323), *Gleosporum spp.* and *Rhizoctonia spp.* were grown on potato dextrose agar (PDA)

medium. Cultures of *Candida albicans* and *Aspergillus flavus* (MSU strains) were grown on YMG medium, and cultures of *Staphylococcus epidermidis* (ATCC 25923), *Streptococcus aureus* (MSU strain), and *Escherichia coli* (ATCC 25922) were grown on Emmons medium.

Cell or spore suspension of the test organisms were made by adding 10 ml of sterile saline solution to a fully grown culture in a Petri dish and stirring with a glass rod gently. The cell concentration was adjusted to 10^6 colony forming units per milliliter (CFU/ml). Bioassay plates were made by spreading evenly of the cell suspension (100 µl) in Petri dishes containing appropriate medium (20 ml). The test compound (250 µg/25 µl of DMSO) was spotted carefully in the center of the bioassay plates and the plates were incubated at 27°C for 72 h. The zone of inhibition, characterized by the absence of microorganism growth, was measured.

MOSQUITOCIDAL AND NEMATICIDAL ASSAYS.—The mosquito larvae, *Aedes aegyptii*, (Michigan State University, courtesy of Dr. Raikhel) were used to test the insecticidal property of compounds **1**, **2**, **3**, **5**, **6**, **8**, **9** and **11** using the procedure reported earlier (Nair et al., 1989). About 200 mosquito eggs, *(Aedes aegyptii)*, were placed in 500 ml distilled and sonicated water and hatched. Fifteen mosquito larvae (4 days old, 4th instar) were placed in 980 µl of water in a test tube. DMSO (20 µl) solutions containing various concentration of test compounds were added into each test tube. Control received pure DMSO (20 µl). The test tubes were covered and left at room temperature. The number of dead larvae were recorded at 2-, 4-, 6-, 24-, and 48-h intervals. Each treatment was repeated in triplicate.

Nematicidal activity was carried out on *Panagrellus redivivus* and *Caenorhabditis elegans* using the procedure reported earlier (Nair et al., 1993). The nematode suspension

(NG medium) (48 µl) containing 20 - 30 nematodes at various developmental stages were transferred into each well of a 96-well tissue culture plate. Test compounds **1, 2, 3, 5, 6, 8, 9** and **11** in DMSO (2 µl) were added to each well and mixed gently. Each treatment was repeated in triplicate. The inoculated plates were held in a humid chamber and mortality was recorded at 2-, 4-, 6-, 24-, and 48-h intervals.

TOPOISOMERASE ASSAYS.—*Saccharomyces cerevisiae* mutant cell cultures, JN394, JN394 t_1 and JN394 $t_{2\text{-}5}$, were supplied by Dr. John Nitiss of St. Jude Children's Research Hospital (Jannatipour et al., 1993; Nitiss et al., 1993). JN394 is hypersensitive to topoisomerase I poisons due to the mutations that destroyed the *RAD*52 repair pathway. JN394 t_1 is isogenic to JN394 except that *top1* gene is deleted. The deletion of *top1* gene results in the lack of the response to topoisomerase I poisons. JN394 $t_{2\text{-}5}$, the cell culture that carries the *top2-5* gene, is resistant to the topoisomerase II poisons but responds to the topoisomerase I poisons.

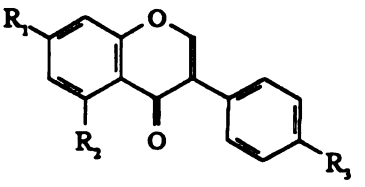
The organisms were cultured in Petri dishes containing YPDA medium (20 ml). The cells from a fully grown plate were suspended in saline solution (10 ml). The cell suspension was diluted to obtain 5 x 10^6 CFU/ml. YPDA liquid media (1.95 ml) were inoculated with 25 µl of the cell suspension (5 x 10^6 CFU/ml) from JN394, JN394 t_1 and JN394 $t_{2\text{-}5}$, respectively. Test compounds, daidzein {**1**}, genistein {**2**}, dihydrodaidzein {**8**} and dihydrogenistein {**9**}, were dissolved in DMSO and were added to the test tubes (25 µl) to give the final concentration at 250 ppm. Each treatment was repeated in triplicates (data not shown). The positive control, a *top-I* poison, camptothecin, was tested at 10 ppm. Since

equol {3} showed an excellent activity in the preliminary plate assay, it was tested at concentrations of 100, 50, 25, and 10 ppm. The test tubes containing cell cultures and compounds were incubated at 27° for 24 h. At the end of incubation period, a serial dilution of each cell suspension was prepared. An aliquot (100 µl) from each dilution was spread evenly on a Petri dish containing YPDA media and incubated at 27° for 72 h. The number of colonies were counted at the end of the incubation period and evaluated for the activity of test compounds (Figure 5.1).

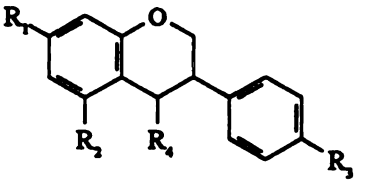
Results and Discussion

Equol {3} (Figure 5.1) was produced by the hydrogenation of daidzein {1} in glacial acetic acid with Pd/C as the catalyst (Appendix VI). The disappearance of C-ring olefinic proton chemical shift at δ 8.27 indicated that the olefinic bond between C-2 and C-3 of **1** (Chang et al., 1994) was reduced. Also, the hydrogenation product gave ^{13}C-NMR chemical shifts at δ 70.27, 37.53 and 31.12 which were assigned to C-2, C-3 and C-4, respectively. The absence of C=O signal in the ^{13}C-NMR spectrum of **3** confirmed it as equol. Methylation of **3** afforded a dimethoxy product, **4**. Compound **3** was previously synthesized by the reduction of *O,O*-diacetyl-daidzein followed by the hydrolysis of the resulting product in ethanolic NaOH (Lamberton et al., 1978).

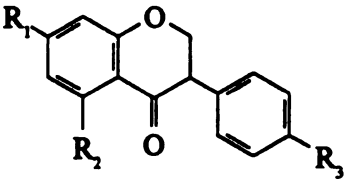
Aldercreutz et al. (1986[b]) reported the synthesis of equol {3} by the hydrogenation of daidzein {1} in EtOH. In our laboratory, this procedure yielded only 4,7,4'-trihydroxy-isoflavan {6} (Appendix VI). The H-2 proton of **6** in its ^{1}H-NMR spectrum appeared as two dd at 4.25 and 4.16 ppm, respectively. The ddd at 2.99 ppm in **6** was assigned to the H-3



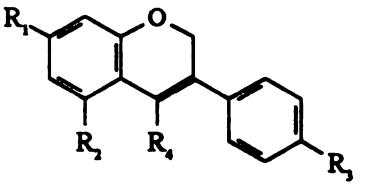
	R_1	R_2	R_3
1	OH	H	OH
2	OH	OH	OH



	R_1	R_2	R_3	R_4
3	OH	H	OH	H
4	OMe	H	OMe	H
5	OH	OH	OH	H
6	OH	H	OH	OH
7	OAc	H	OAc	OAc



	R_1	R_2	R_3
8	OH	H	OH
9	OH	OH	OH



	R_1	R_2	R_3	R_4
10	OH	OH	OH	OH
11	OAc	OAc	OAc	OAc

Figure 5.1 Reduction products of daidzein and genistein

proton. The ^{13}C-NMR spectrum of **6** gave a signal at δ 67.70 and was assigned to C-4 which confirmed that the C=O group in daidzein {**1**} was partially reduced. Acetylation of **6** gave a triacetate, **7**, and provided an additional evidence for the existence of C-4 -OH group in **6**.

The hydrogenation of daidzein {**1**} over Pd/$BaSO_4$ in EtOH yielded **8** as the major product (Appendix VI). ^{13}C-NMR of compound **8** showed a signal at δ 194.07 which indicate that the C=O group at C-4 was not reduced. Also, ^{1}H-NMR of **8** did not give the olefinic proton signal at δ 8.02. The H-2 protons appeared as dd at 4.56 and 4.52 ppm, respectively, and H-3 proton as dd at 3.82 ppm. Therefore, the NMR data confirmed the existence of a C=O group at C-4 and the reduction of the olefinic bond between C-2 and C-3 in compound **8**.

Compound **9** was produced by the hydrogenation of genistein {**2**} in ethanol over Pd/C (Appendix VII). This compound gave R_f value similar to genistein {**2**} on silica gel TLC and its structure was confirmed by ^{1}H and ^{13} C-NMR spectra. However, hydrogenation of genistein {**2**} in glacial acetic acid over Pd/C yielded a mixture of compounds **5** and **9** due to partial hydrogenation (Appendix VII). Compound **5** had a lower R_f on TLC than **9**. The structure of compound **5** was identified as 5,7,4'-trihydroxyisoflavan.

Acetylation of compound **9** in pyridine and acetic anhydride gave interesting results. Purification and characterization of the acetylated product **11** confirmed the presence of four hydroxyl groups in compound **11** even though compound **9** had only three -OH groups. The H-2 proton of **11** in its ^{1}H-NMR spectrum appeared as a 2H singlet at δ 5.03. The signals at 136.14 and 111.29 ppm were assigned to the olefinic carbons C-3 and C-4 formed by the

enolization of the C-4 carbonyl group.

The tetraacetate **11** was yielded from the enol **10**. This indicated that during acetylation condition the entire keto form was converted to the enol, compound **10**. The enolization of **9** was also observed during NMR experiment with d_5-pyridine as the solvent. The addition of 100 µl of d_4-acetic acid to a d_5-pyridine solution of **9** caused an instant enolization of **9** to **10**. The H-2a and H-2b signals of **10** appeared as doublets at 4.48 and 4.43 ppm, respectively. However, the enol form was unstable and converted completely to the keto form during isolation and purification. The same was true when the tetraacetate, **11**, was hydrolyzed to produce compound **10**.

The enolization of compound **9** was absent in CD_3OD/d_4-acetic acid and observed only in d_5-pyridine as the solvent. The corresponding isoflavanone **8** did not enolize in d_5-pyridine and d_4-acetic acid. The enolization of **9** in pyridine/acetic acid may be induced by the 5-OH group in the A-ring complexing with the pyridine solvent. It is possible that **10** can exist in biological systems as a metabolite of genistein {**2**}.

Compound **3** showed growth inhibition for *Fusarium oxysporum*, *Fusarium moniliforme*, *Gleosporum spp.*, *Rhizoctonia spp.* and *Aspergillus flavus* (all fungi), *Candida albicans* (yeast) and bacteria *Staphylococcus epidermidis*, *Streptococcus aureus*, and *Escherichia coli* at 250 µg in plate assays. Mosquitocidal (*Aedes aegyptii* larve) and nematicidal (*Panagrellus redivivus* and *Caenorhabditis elegans*) assays with **3** showed 100% mortality at 250 ppm within 24 h. Compound **9** inhibited the growth of all microorganisms tested at 250 µg concentration, excluding *A. flavus* and *E. coli*. Mosquitocidal or nematicidal

activities were not observed for **9**. Compounds **1, 2, 5, 6, 8,** and **11** were not active against bacteria, fungi, yeast, mosquito larvae or nematodes tested.

Genistein {**2**} is reported to have topoisomerase II inhibitory activity (Markovitis et al., 1989; Corbett et al., 1993). Therefore, we have evaluated both topoisomerase I and II activities for compound **1 - 9** using mutant yeast strains JN394, JN394 t_1 and JN394 $t_{2\text{-}5}$ (Jannatipour et al., 1993; Nitiss et al., 1993). In our experiments, the inhibitory concentration (IC_{50}) for genestein {**2**} was 250 ppm for JN394. Also, it inhibited the growth of JN394t_1 and JN394$t_{2\text{-}5}$ by 30%. Compounds **4 - 7** did not show topoisomerase inhibition in preliminary plate assay. Camptothecin, a *topo-I* poison, was used as the positive control against JN394 and JN394 $t_{2\text{-}5}$ at 10 ppm. At this concentration it showed 97% inhibition for the growth of JN394 and JN394$t_{2\text{-}5}$. Camptothecin did not inhibit the growth of JN394 t_1 (Figure 5.2). Compounds **8** and **9** inhibited the growth of JN394 $t_{2\text{-}5}$, but had no effect on JN394. Compound **3** inhibited the growth of these yeast strains with IC_{50} at 50ppm (Figure 5.3).

Various compounds are identified as topoisomerase inhibitors (Constantinou et al., 1995; Kawada et al., 1995). These include camptothecin and etopocide, the topoisomerase I and II inhibitors, respectively. Drugs, acting as DNA topoisomerase inhibitors, will produce cleavable DNA-topoisomerase-drug complex and cause site-specific cleavage of chromosomal DNA (Liu, 1990). This event will inhibit DNA replication, RNA synthesis and cell division which eventually leads to cell death (Liu, 1990). Cancerous cells contain larger quantities of topoisomerases, therefore, the inhibitory activities of certain chemicals on topoisomerases

have attracted a great deal of interest in cancer research recently (Liu, 1990). Our results revealed that equol {3} inhibited the growth of JN394 and JN394 $t_{2\text{-}5}$ at 50 ppm concentration and indicated that it is similar to other topoisomerase I drugs (Jannatipour et al., 1993; Nitiss et al., 1993).

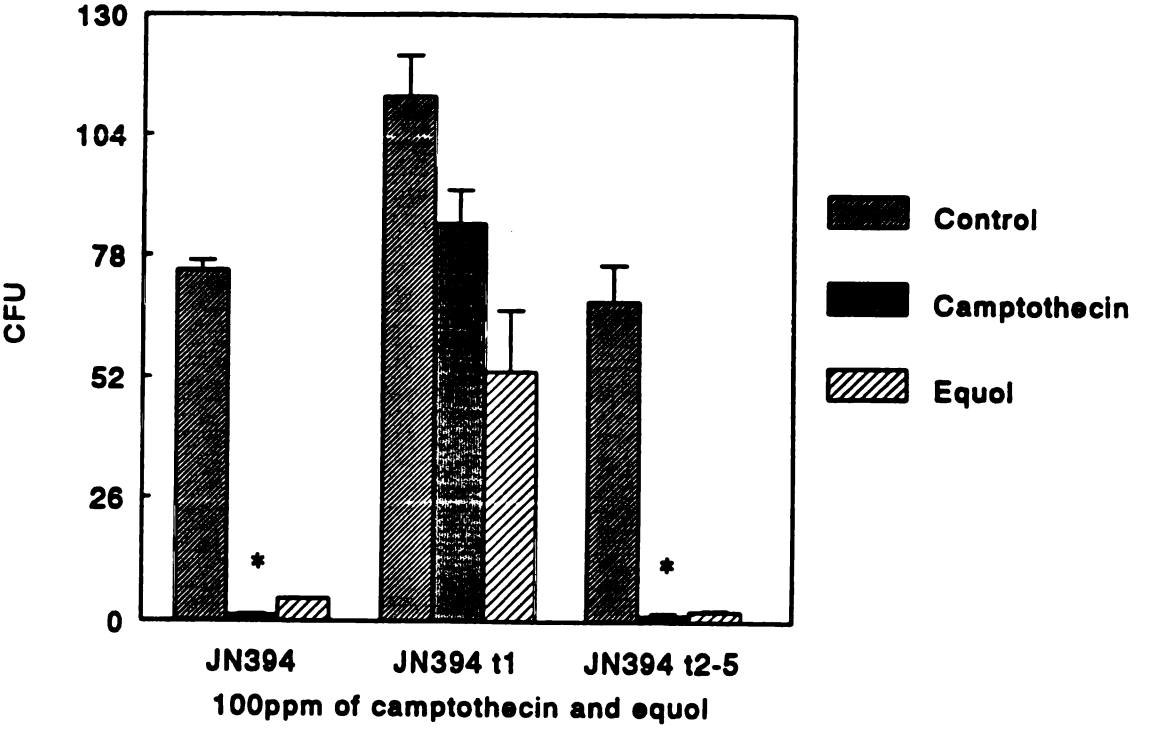


Figure 5.2 Effect of camptothecin and equol at 10 and 100 ppm, respectively, on the cell growth of yeast strains JN394, JN394 t_1 and JN394 t_{2-5}

* Cell number x 100

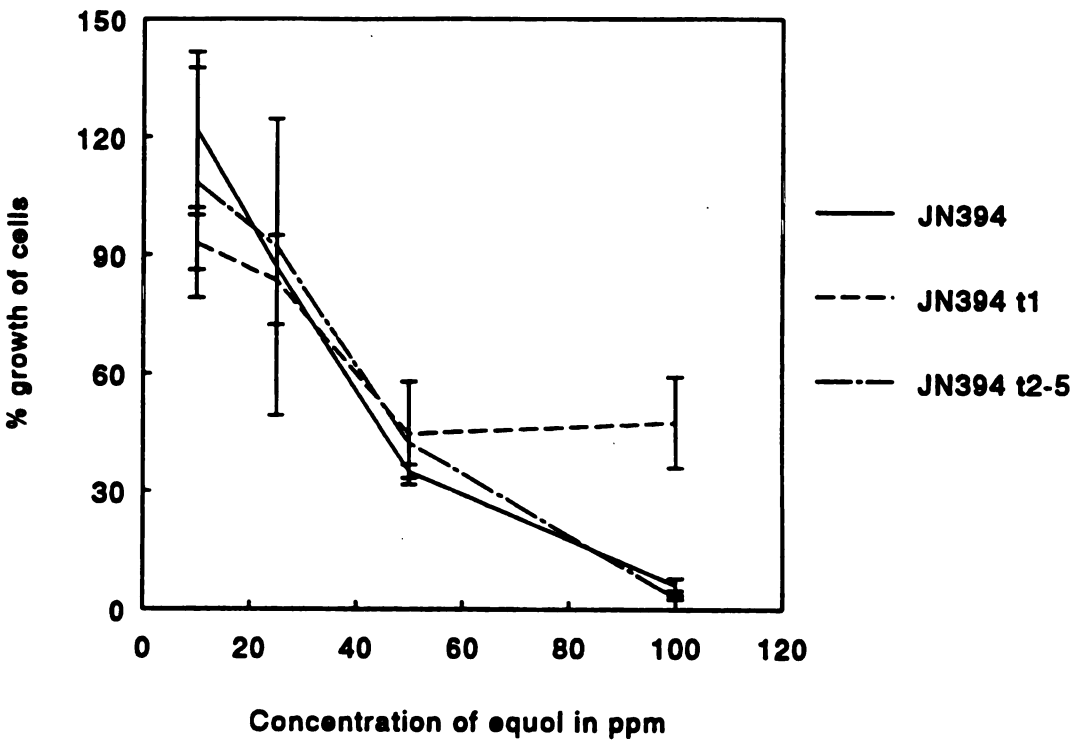


Figure 5.3 Effect of equol at various concentration on the cell growth of yeast strains JN394, JN394 t_1 and JN394 t_{2-5} .....

CHAPTER VI

Summary and Conclusions

The third chapter of this dissertation describes the synthesis of anticarcinogenic isoflavones, genistein, daidzein and their 4-methoxy derivatives, biochanin A and formononetin. These compounds were previously synthesized by other researchers, however, the synthetic procedures were time consuming and costly. We have developed a two-step synthetic route for the production of daidzein and genistein which reduced both time and expenses dramatically. In the first step, the corresponding ketones for daidzein and genistein were produced by Friedal-Craft acylation reactions using commercially available starting materials, resorcinol, phloroglucinol, 4-hydroxyphenyl acetic acid and 4-hydroxyphenyl acetonitrile, respectively. The second step involved the cyclization of the ketone by heating it with N, N-dimethylformamide dimethyl acetal and THF in a conventional microwave oven for 2 min to yield daidzein at 71 % yield. Similarly, genistein was synthesized by the cyclization of its corresponding ketone by heating with DMF, methanesulfonyl chloride and BF_3 etherate in a conventional microwave oven for 2 min. The yield of genistein was 80 %.

Several isoflavone metabolites such as equol, *O*-desmethyl angolensin, dihydrodaidzein, 6'-hydroxy-*O*-desmethyl angolensin, dehydro-*O*-desmethyl angolensin, benzopyran-4,7-diol-3-(4-hydroxyphenyl) and tetrahydrodaidzein and dihydrogenistein were detected in the urine from human subjects on soy diet. The pathways for the formation of isoflavone metabolites have not been studied. In order to confirm the hypothesis that the isoflavone metabolites detected in human urine were the results of intestinal bacterial metabolism, genistein and daidzein were incubated, respectively, with human feces under anaerobic conditions for 72

h. The metabolites produced were isolated and characterized by ^{1}H-NMR spectra. Three metabolites, dihydrodaidzein, benzopyran-4,7-diol-3-(4-hydroxyphenyl) and equol, were isolated from the fermentation broth of daidzein. These metabolites were previously detected in human urine by other researchers. Dihydrogenistein was the only metabolite of genistein reported in the urine from human subjects on soy diets. We have isolated dihydrogenistein from the fermentation broth of genistein with human feces. Another metabolite of genistein, *p*-ethylphenol, found in the urine of sheeps was not detected in the urine from humans. Similarly, we did not isolate this compound from the fermentation broth of genistein with human feces. This indicated that metabolism of genistein were not similar in humans and sheeps.

The metabolites formed during the fermentation of daidzein and genistein with human feces during 72 h period were monitored. The results indicated that the amount of daidzein decreased considerably in 24 h. Dihydrodaidzein, the major metabolite of daidzein, was detected within 24 h. Equol was detected only in smaller quantities at 72 h. Therefore, it is possible that equol was derived from dihydrodaidzein. However, additional experiments are required to confirm this hypothesis. Even though the level of genistein declined rapidly in 24 h, a corresponding increase in dihydrogenistein was not observed. This suggests that genistein was metabolized to other compound(s) which were not identified in our studies.

The metabolites isolated from fermentation broth of daidzein or genistein with human feces were in small quantities and were not sufficient to carry out biological studies. Therefore, they were synthesized from either genistein or daidzein by hydrogenation using Pd/C as the catalyst in glacial acetic acid or ethanol as the solvent. The ^{1}H-NMR spectra of these synthetic products were identical to the compounds isolated from fermentation broth of daidzein and genistein. Also, antibacterial, antifungal, mosquitocidal, nematicidal and anticancer activities of these synthetic metabolites were determined.

Antibacterial activity of isoflavone metabolites were carried out on *Staphylococcus epidermidis*, *Streptococcus aureus* and *Escherichia coli*. Equol inhibited the growth of all the bacteria tested at 250 μg. Dihydrogenistein inhibited the growth of *Staphylococcus* and

Streptococcus but not on *E. coli* at 250 μg. Other metabolites did not show antibacterial activity.

Test fungi, *Fusarium oxysporum, Fusarium moniliforme, Gleosporum spp. Rhizoctonia spp.* and *Asperigillus flavus* were used to determine antifungal activity of the isoflavone metabolites. Equol inhibited the growth of all the fungi tested at 250 μg. Except on *A. flavus*, dihydrogenistein inhibited the growth of fungi tested at 250 μg. Other metabolites did not show antibacterial activity. Equol was also active in *Candida albicans* at 250 μg.

Equol gave 100 % mortality when tested on mosquito larvae, *Aedes aegyptii*, and nematodes, *Panagrellu redivivus* and *Caenorhabditis elegans* within 24 h at 250 ppm. Other isoflavone metabolites did not show mosquitocidal or nematicidal activities.

Mutant *Saccharomyces cerevisiae* strains were used to determine the anticancer activity of the isoflavone metabolites. Topoisomerases are the enzymes that catalyze the interconversion of topoisomers. This interconversion is a crucial step in living organisms for DNA replication, RNA and protein synthesis. The interference of topoisomerase activity will eventually lead to cell death. Cancerous cells proliferate continuously and hence, contain more topoisomerases. Therefore, topoisomerase poisons are potential anticancer drugs. In the anticancer activity assay, three mutant *S. cerevisiae* strains, JN394, JN394t_1 and JN394t_{2-5}, that carry different genotype and respond to *top-I* or *top-II* poisons were used. All the DNA repair genes have been deleted from the mutant *S. cerevisiae* strains which makes them sensitive to DNA damage caused by UV light or chemicals. JN394t_1 was derived from JN394 and with the *top-I* gene deleted. Therefore, the growth of JN394t_1 is not inhibited by *top-I* drugs. JN394t_{2-5} was similar to JN394 and carrying the mutant *top-II* gene. Therefore, JN394t_{2-5} is resistant to *top-II* drugs. The known *top-I* poison camptothecin was used as the positive control. Equol showed a similar inhibition pattern as camptothecin in the assays. All other metabolites showed weak or no activity in this assay and hence additional efficacy studies were not conducted for these compounds.

The synthesis of genistein, daidzein, biochanin A and formononetin in large quantities allowed us to conduct further research on these isoflavones as anticarcinogens and

antioxidants. Compounds present in ingested foods can be metabolized at various metabolic sites such as liver and intestine. Our fermentation studies of genistein and daidzein with human feces confirmed the hypothesis that the isoflavone metabolites of daidzein and genistein present in soybean detected in human urine were derived by intestinal bacteria. Various biological activities of the metabolite equol have been reported by other researchers. Anticancer assays with the isoflavone metabolites in our laboratory showed that equol can function as a *top-I* drug and may be responsible for the anticancer activity observed in animals and humans for soybean and soy foods. Also, equol gave a broad spectrum of biological activities in our experiments and could also contribute to the control of pathogens in humans. Additional experiments are required to explain these biological activities observed for equol and other soy isoflavone metabolites.

BIBLIOGRAPHY

Abramovitch, R. A. (1991) Applications of Microwave Energy in Organic Chemistry. A Review. *Org. Prep. Proc. Int.* **23**, 683-711.

Akiyama, T., Ishida, J., Nakagawa, S., Ogawara, H., Watanabe, S. I., Itoh, N., Shibuya, M., and Fukami, Y. (1987) Genistein, a Specific Inhibitor of Tyrosine-Specific Protein Kinases. *J. Biol. Chem.* **262**, 5592-5595.

Aldercreutz, H., Fotsis, T., Bannwart, C., Wahala, K., Makela, T., Brunow, G., and Hase, T. (1986[a]) Determination of Urinary Lignans and Phytoestrogen Metabolites, Potential Antiestrogens and Anticarcinogens, in Urine of Women on Various Habitual Diets. *J. Steroid Biochem.* **25**, 791-797.

Aldercreutz, H., Musey, P. I., Fotsis, T., Bannwart, C., Wahala, K., Wakela, T., Brunow, G., and Hase, T. (1986[b]) Identification of Lignans and Phytoestrogens in Urine of Chimpanzees. *Clinica Chimica Acta* **158**, 147-154.

Aldercreutz, H. (1990) Western Diet and Western Diseases: Some Hormonal and Biochemical Mechanisms and Associations. *Scand. J. Clin. Lab. Invest.* **50, suppl 201**, 3-23.

Aldercreutz, H., Hockerstedt, K., Bannwart, C., Bloigu, S., Hamalainen, E., Fotsis, T., and Ollus, A. (1987) Effect of Dietary Components, Including Lignans and Phytoestrogens, on Enterohepatic Circulation and Liver Metabolism of Estrogens and on Sex Hormone Binding Globulin (SHBG). *J. Steroid Biochem* **27**, 1135-1144.

Aldercreutz, H., Hockerstedt, K., Bannwart, C., Hamalainen, E., Fotsis, T., and Bloigu, S. (1988) Association Between Dietary Fiber, Urinary Excretion of Lignans and Isoflavonic Phytoestrogens and Plasma Non-Protein Bound Sex Hormones in Relation to Breast Cancer. *Prog. in Cancer Res. Therapy* **35**, 409- 412.

Aldercreutz, H., Pulkkinen, M. O., Hamalainen, E., and Korpela, J. T. (1984) Studies on the Role of Intestinal Bacteria in Metabolism of Synthetic and Natural Steroid Hormones. *J. Steroid Biochem* **20**, 217-229.

Armstrong, B., and Doll, R. (1975) Environmental Factors and Cancer Incidence and Mortality in Different Countries, with Special Reference to Dietary Practices. *J. Cancer* **15**, 617-631.

Axelson, M., Sjovall, J., Gustafsson, B. E., and Setchell, K. D. R. (1982[a]) Origin of Lignans in Mammals and Identification of a Precursor from Plants. *Nature* **298**, 659-660.

Axelson, M., Kirt, D. N., Farrant, R. D., Cooley, G., Lawson, A. M., and Setchell, K. D. R. (1982[b]) The Identification of the Weak Oestrogen Equol [7-hydroxy-3-(4'-hydroxyphenyl) chroman] in Human Urine. *Biochem. J.* **201**, 353-357.

Axelson, M., and Setchell, K. D. R. (1981) The Excretion of Lignans in Rats-Evidence for an Intestinal Bacterial Source for This New Group of Compounds. *FEBS Letters* **123**, 337-342.

Axelson, M., Sjovall, J., Gustafsson, B. E., and Setchell, K. D. R. (1984) Soya Dietary Source of the Non-steroidal Oestrogen Equol in Man and Animal. *Endocrinology* **102**, 49-56.

Baker, W., Chadderton, J., Harborne, J. B., and Ollis, W. D. (1953) A New Synthesis of Isoflavones. Part I. *J. Chem. Soc.* 1852-1860.

Banik, B. K., Manhas, M. S., Kaluza, Z., Barakat, K. J., and Bose, A. K. (1992) Microwave-Induced Organic Reaction Enhancement Chemistry. Convenient Synthesis of Enantiopure α-Hydroxy-β- Lactams. *Tetrahedron Lett.* **33**, 3603-3606.

Bannwart, C., Aldercreutz, H., Fotsis, T., K. Wahala, Hase, T., and Brunow, G. (1984[a]) Identification of O-Desmethyl Angolensin, a Metabolite of Daidzein, and of Matairesinol, One Likely Plant Precursor of the Animal Lignan Enterolactone, in Human Urine. *Finn. Chem. Lett.* **4-5**, 120-125.

Bannwart, C., Fotsis, T., Heikkinen, R., and Aldercreutz, H. (1984[b]) Identification of the Isoflavonic Phytoestrogen Daidzein in Human Urine. *Clin. Chim. Acta* **136**, 165-172.

Barnes, S., Grubbs, C., and Setchell, K. D. R. (1988) Chemoprevention by Powdered Soybean Chips (PSC) of Mammary Tumors in Rats. *Breast Cancer Res. Treat.* **12**, 128.

Barnes, S., Grubbs, C., Setchell, K. D. R., and Carlson, J. (1990) Soybeans Inhibit Mammary Tumors in Models of Breast Cancer. In Mutagens and Carcinogens in the diet. Ed. by Pariza, M., Wiley-Liss, New York, pp 239-253.

Barnes, S., Kirk, M., and Coward, L. (1994) Isoflavones and Their Conjugates in Soy Foods: Extraction Conditions and Analysis by HPLC-Mass Spectrometry. *J. Agric. Food Chem.* **42**, 2466-2474.

Bass, R. T. (1976) Synthesis of Chromones by Cyclization of 2- Hydroxyphenyl Ketone with Boron Trifluoride-Diethyl Ether and Methanesulphonyl Chloride. *J. C. S. Chem. Comm.* 78-79.

Batterham, T. J., Shutt, D. A., Hart, N. K., Braden, A. W. H., and Tweeddale, H. J. (1971) Metabolism of Intraruminally Administered [4-^{14}C]Formononetin and [4-^{14}C]Biochanin A in Sheep. *Aust. J. Agric. Res.* **22**, 131-138.

Bishop, J. M. In "Cancer Biology" Ed. by Friedberg, C., W. H. Freeman and Company, New York, 1986, pp. 66-76.

Braden, A. W. H., Hart, N. K., and Lamberton, J. A. (1967) The Oestrogenic Activity and Metabolism of Certain Isoflavones in Sheep. *Aust. J. Agric. Res.* **18**, 335-348.

Buell, P. (1973) Changing Incidence of Breast Cancer in Japanese-American Women. *J. Natl. Cancer Inst.* **51**, 1479-1483.

Carroll, K. K., Braden, L. M., Bell, J. A., and Kalamegham, R. (1986) Fat and Cancer. *Cancer* **58**, 1818-1823.

Chang, C. J., and Geahlen, R. L. (1992) Protein-Tyrosine Kinase Inhibition: Mechanism-Based Discovery of Antitumor Agents. *J. Nat. Prod.* **55**, 1529-1560.

Chang, Y. C., Nair, M. G., Santell, R. C., and William Helferich (1994) Microwave-Mediated Synthesis of Anticarcinogenic Isoflavones from Soybeans. *J. Agric. Food Chem.* **42**, 1869-1871.

Constantinou, A., Mehta, R., Runyan, C., Rao, K., Vaughan, A. and Moon, R. (1995) Flavonoids as DNA Topoisomerase antagonists and Poisons: Structure-Activity Relationships. *J. Nat. Prod.* **58**, 217-225.

Corbett, A. H., Hong, D., and Osheroff, N. (1993) Exploiting Mechanistic Differences Between Drug Classed to Define Functional Drug Interaction Domains on Topoisomerase II. *J. Biol. Chem.* **268**, 14394-14398.

Dean, N. M., Kanemitsu, M., and Boynton, A. L. (1989) Effects of the Tyrosine-kinase Inhibitor Genistein on DNA Synthesis and Phospholipid-derived Second Messenger Generation in Mouse 10T1/2 Fibroblasts and Rat Liver T51B Cells. *Biochem. Biophy. Res. Comm.* **165**, 795-801.

Dickinson, J. M., Smith, G. R., Randel, R. D., and Pemberton, I. J. (1988) In Vitro Metabolism of Formononetin and Biochanin A in Bovine Rumen Fluid. *J. Anim. Sci.* **66**, 1969-1973.

Drasar, B. S., and Irving, D. (1973) Environmental Factors and Cancer of the Colon and Breast. *Br. J. Cancer* **27**, 167-172.

Dunn, J. E. (1977) Breast Cancer Among American Japanese in the San Francisco Bay Area. *Natl. Cancer Inst. Monogr.* **47**, 157-160.

Eldridge, A. C. (1982) Determination of Isoflavones in Soybean Flours, Protein Concentrates, and Isolates. *J. Agric. Food Chem.* **30**, 353-355.

Eldridge, A. C., and Kwokek, W. F. (1983) Soybean Isoflavones: Effect of Environment and Variety on Composition. *J. Agric. Food Chem.* **31**, 394-396.

Farkas, L., Gottsegen, A., Nogradi, M., and Antus, S. (1971) Synthesis of the Natural Isoflavanones Ferreirin, Dalbergioidin, and Ougenen. *J. C. S.*, 1994-2000.

Farmakalidis, E., Hathcock, J. N., and Murphy, P. A. (1985[b]) Oestrogenic Potency of Genistein and Daidzein in Mice. *Fd Chem. Toxic.* **23**, 741-745.

Farmakalidis, E., and Murphy, P. A. (1984) Oestrogenic Response of the CD-1 Mouse to the Soya-Bean Isoflavones Genistein, Genistin and Daidzin. *Fd Chem. Toxic.* **22**, 237-239.

Farmakalidis, E., and Murphy, P. A. (1985[a]) Isolation of 6"-*O*-Acetylgenistin and 6"-*O*-Acetyldaidzin from Toasted Defatted Soyflakes. *J. Agric. Food Chem.* **33**, 385-389.

Giguere, R. J., Bray, T. L., and Duncan, S. M. (1986) Application of Commercial Microwave Ovens to Organic Synthesis. *Tetrahedron Lett.* **27**, 4945-4948.

Goldin, B. R., Aldercreutz, H., Gorbach, S. L., Warram, J. H., Dwyer, J. T., Swenson, L., and Woods, M. N. (1982) Estrogen Excretion Patterns and Plasma Levels in Vegetarian and Omnivorous Women. *N. Engl. J. Med.* **307**, 1542-1547.

Goldin, B. R., Aldercreutz, H., Gorbach, S. L., Woods, M. N., Dwyer, J. T., Conlon, T., Bohn, E., and Gershoff, S. N. (1986) The Relationship Between Estrogen Levels and Diets of Caucasian American and Oriental Immigrant Women. *Am. J. Clin. Nutr.* **44**, 945-953.

Gorbach, S. L., and Goldin, B. R. (1990) The Intestinal Microflora and the Colon Cancer Connection. *Rev. Infect. Diseases* **12**, S252-S261.

Graham, S., Marshall, J., Mettlin, C., Rzepka, T., Nemoto, T., and Byers, T. (1982) Diet in the Epidemiology of Breast Cancer. *Am. J. Epidemiol.* **116**, 68-75.

Graham, T. L. (1990) A Rapid, High Resolution High Performance Liquid Chromatography Profiling Procedure for Plant and Microbial Aromatic Secondary Metabolites. *Plant Physiol.* **95**, 584-593.

Gorrod, J. W., in "Drug Metabolism in Man" Ed. by J. W. Gorrod and A. H. Beckett, Taylor and Francis Ltd., London, 1978, Chapter 10, pp. 157-174.

Haenszel, W., and Kurihara, M. (1968) Studies of Japanese Migrants. I. Mortality from Cancer and Other Diseases Among Japanese in the United States. *J. Nat. Cancer Inst.* **40**, 43-68.

Hirohata, T., Nomura, A. M. Y., Hankin, J. H., Kolonel, L. N., and Lee, J. (1987) An Epidemiologic Study on the Association Between Diet and Breast Cancer. *J. Natl. Cancer Insti.* **78**, 595- 600.

Hislop, T. G., Coldman, A. J., Elwood, J. M., Brauer, G., and Kan, L. (1986) Childhood and Recent Eating Patterns and Risk of Breast Cancer. *Cancer Detect. Prevent.* **9**, 47-58.

Holdmen, L. V., Cato, E. P., and Moore, W. E. C. (ed.), An Anaerobic Laboratory Manual, 4th ed. Department of Anaerobic Microbiology, Virginia Polytechnic Institute and State University, Blackburg, 1977.

Ikehata, H., Wakaizumi, M., and Murata, K. (1968) Antioxidant and Antichemolytic Activity of a New Isoflavone, "Factor 2" Isolated from Tempeh. *Agric. Biol. Chem.* **32**, 740-746.

Inoue, N. (1964) Studies of Synthetic Isoflavanones. V. The Reduction of Isoflavanones. *Bull. Chem. Soc. Japan* **37**, 601-605.

Ip, D., Carter, C. A., and Ip, M. M. (1985) Requirement of Essential Fatty Acid for Mammary Tumorigenesis in the Rat. *Cancer Res.* **45**, 1997-2003.

Ip, C., and Sinha, D. K. (1981) Enhancement of Mammary Tumorigenesis by Dietary Selenium Deficiency in Rats with a High Polyunsaturated Fat Intake. *Cancer Res.* **41**, 31-38.

Jannatipour, M., Liu, Y.-X., and Nitiss, J. L. (1993) The *top*2-5 Mutant of Yeast Topoisomerase II Encodes and Enzyme Resistant to Etoposide and Amsarcrine. *J. Biol. Chem.* **268**, 18586-18592.

Jarvenpaa, P., Kosunen, T., Fotsis, T., and Aldercreutz, H. (1980) In Vitro Meatbolism of

Estrogens by Isolated Intestinal Micro-organisms and by Human Faecal Microflora. *J. Steroid Biochem* **13**, 345-349.

Kagal, S. A., Nair, P. M., and Venketaraman, K. (1962) A Synthesis of Isoflavones by a Modified Vilsmeier-Haack Reaction. *Tetrahedron Lett.* 593-597.

Kawada, S.-Z., Yamashita, Y., Ochiai, K., Ando, K., Iwasaki, T., Takiguchi, T. and Nakano, H. (1995) Terpentecin and UCT4B, New Family of topoisomerase II Target antitumor Antibiotics Produced by Streptomyces: Producing Organism, Fermentation and Large Scale Purification. J. Antibio. 48, 211-216.

Kelly, G. E., Nelson, C., Waring, M. A., Joannou, G. E., and Reeder, A. Y. (1993) Metabolites of Dietary (Soya) Isoflavones in Human Urine. *Clinica Chimica Acta* **223**, 9-22.

King, H., and Locke, F. B. (1980) Cancer Mortality Among Chinese in the United States. *J. Natl. Cancer Insti.* **65**, 1141-1148.

King, H., Li, J., Locke, F. B., Pollack, E. S., Tu, J. T. (1985) Patterns of Site-Specific Displacement of Cancer Mortality Among Migrants: The Chinese in the United States. *Am. J. Publ. Health* **75**, 237-242.

Kosslak, R. M., Bookland, R., Barkei, J., Paaren, H. E., and Appelbaum, E. R. (1987) Induction of *Brandyrhizobium japanicum* Common Nod Genes by Isoflavones Isolated from *Glycine max*. *Proc. Natl. Acad. Sci. USA* **84**, 7428-7432.

Kramer, R. P., Hindorf, H., Jha, H. C., Kallage, J., and Zilliken, F. (1984) Antifungal Activity of Soybean and Chickpea Isoflavones and Their Reduced Derivatives. *Phytochemistry* **23**, 2203-2205.

Krishnamurty, H. G., and Sathyanarayana, S. (1986) Catalytic Transfer Hydrogenation, a Chemo-selective Reduction of Isoflavones to Isoflavanones. *Syn. Comm.* **16**, 1657-1663.

Kudou, S., Fleury, Y., Welti, D., Magnolato, D., Uchida, T., Kitamura, K., and Okubo, K. (1991) Malonyl Isoflavone Glycosides in Soybean Seeds (Glycine max Merrill). *Agric. Biol. Chem.* **55**, 2227-2233.

Lamberton, J. A., Suares, H., and Watson, K. G. (1978) Catalytic Hydrogenation of Isoflavones. The Preparation of ( )-Equol and Related Isoflavans. *Aust. J. Chem.* **31**, 455-457.

Lee, H. P., Goruley, L., Duffy, S. W., Esteve, J., Lee, J., and Day, N. E. (1991) Dietary Effects on Breast-Cancer Risk in Singapore. *The Lancet* **337**, 1197-1200.

Liu, L. F. in "DNA Topology and Its Biological Effects" Ed. by N. R. Cozzarelli and J. C. Wang, Cold Spring Harbor Laboratory Press, New York, 1990, Chapter 14, pp. 371-389.

Locke, F. B., and King, H. (1980) Cancer Mortality Risk Among Japanese in the United States. *J. Natl. Cancer Insti.* **65**, 1149- 1151.

Lombardi, P., Goldin, B., Boutin, E., and Gorbach, S. L. (1978) Metabolism of Androgens and Estrogens by Human Fecal Microorganisms. *J. Steroid Biochem* **9**, 795-801.

Lubin, F., Wax, Y., and Modan, B. (1986) Role of Fat, Animal Protein, and Dietary Fiber in Breast Cancer Etiology: A Case-Control Study. *J. Natl. Cancer Insti.* **77**, 605-612.

Markovits, J., Linassier, C., Fosse, P., Couprie, J., Jacquemin- Sablon, A., Saucier, J. M., Le Pecq, J. B., and Larsen, A. K. (1989) Inhibitory Effects of the Tyrosine Kinase Inhibitor Genistein on Mammalian DNA Topoisomerase II. *Cancer Reserach* **49**, 5111-5117.

Meselhy, M. R., Kadota, S., Hattori, M., and Namba, T. (1993) Metabolism of Safflor Yellow B by Human Intestinal Bacteria. *J. Nat. Prod.* **56**, 39-45.

Messina, M. J., Persky, V., Setchell, K. D. R., and Barnes, S. (1994) Soy Intake and Cancer Risk: A Review of the In Vitro and In Vivo Data. *Nutr. Cancer* **21**, 113-31.

Messina, M. and Barnes, S. (1991) The Role of Soy Products in Reducting Risk of Cancer. *J. Natl. Cancer Insti.* **83**, 541-546.

Messina, M. and Messina, V. (1991) Increasing Use of Soyfoods and Their Potential Role in Cancer Prevention. *J. Amer. Diet. Assoc.* **91**, 836-840.

Murakami, H., Asakawa, T., Terao, J., and Matsushita, S. (1984) Antioxidative Stability of Tempeh and Liberation of Isoflavones by Fermentation. *Agric. Biol. Chem.* **48**, 2971-2975.

Murphy, P. A. (1982) Phytoestrogen Content of Processed Soybean Products. *Food Technology*, 60-64.

Naim, M., Gestetner, B., Zikah, S., Birk, Y., and Bondi, A. (1974) Soybean Isoflavones. Characterization, Determination, and Antifungal Activity. *J. Agric. Food Chem.* **22**, 806-810.

Nair, M G., Safir, G. R., and Siqueira, J. O. (1991) Isolation and Identification of Vesicular-Arbuscular Mycorrhiza-Stimulatory Compounds from Clover (*Trifolium repens*) Roots. *Applied and Environmental Microbiology* **57**, 434-439.

Nair, M. G., Chandra, A., and Thorogood, D. L. (1993) Griseulin, a New Nitro-containing Bioactive Metabolites Produced by *Streptomyces spp. J. Antibio.* **46**, 1762-1763.

Nitiss, J. L., Liu, Y.-X., and Hsiung, Y. (1993) A Temperature Sensitive Topoisomerase II Allele Confers Temperature Dependent Drug Resistance on Amsacrine and Etoposide: A Genetic System for Determining the Targets of Topoisomerase II Inhibitors. *Cancer Res.* **53**, 89-93.

Pelter, A. and Foot, S. (1976) A New Convenient Synthesis of Isoflavones. *Synthesis* 326.

Peterson, H. and Barnes, S. (1991) Genistein Inhibition of the Growth of Human Breast Cancer Cells: Independence from Estrogen Receptors and the Multi-Drug Resistance Gene. *Biochem. Biophy. Res. Comm.* **179**, 661-667.

Safir, G. R.; Nair, M. G.; Siqueira, J. O. Method and Compositions for Stimulating Vesicular-Arbuscular Mycorrhizal Fungi. *US Patent No.* 5,002,603. *1991*; 5,085,682. *1992*; 5,125,955. *1992*; *Taiwan Patent No.* 60604. *1993.*

Sekizake, K., Yokosawa, R., Chinen, C., Adachi, H., and Yamane, Y. (1993) Studies on Zoospore Attracting Activity. II. Synthesis of Isoflavones and Their Attracting Activity to Aphamomyces Euteiches Zoospore. *Biol. Pharm. Bull.* **16**, 698-701.

Setchell, K. D. R., Borriello, S. P., Hulme, P., Kirk, D. N., and Axelson, M. (1984) Nonsteroid Estrogens of Dietary Origin: Possible Roles in Hormone-Dependent Disease. *Amer. J. Clin. Nutri.* **40**, 569-578.

Shriner, R. L. and Hull, C. J. (1945) Isoflavones. III. the Structure of Prunetin and a New Synthesis of Genistein. *J. Org. Chem.* **10**, 288-291.

Shu, Y. Z., Kingston, D. G. I., Tassell, R. L. V., and Wikins, T. D. (1991) Metabolism of 1,4-Dinitro-2-Methylpyrrole, a Mutagen Formed by a Sorbic Acid-Nitrite Reaction, by Intestinal Bacteria. *Environ. Mol. Mutagenesis* **17**, 181-187.

Shutt, D. A., and Braden, A. W. H. (1968) The Significance of Equol in Relation to the Oestrogenic Responses in Sheep Ingesting Clover with a High Formononetin Content. *Aust. J. Agric. Res.* **19**, 545-553.

Shutt, D. A., Weston, R. H., and Hogan, J. P. (1970) Quantitative Aspects of Phyto-Oestrogen Metabolism in Sheep Fed on Subterranean Clover (Trifolium Subterraneum Cultivar Clare) or Red Clover (Trifolium Pratense). *Aust. J. Agric. Res.* **21**, 713- 722.

Siqueira, J. O.; Safir, G. R.; Nair, M. G. (1991[a]) Stimulation of Vesicular-Arbuscular Mycorrhiza Formation and Growth of White Clover by Flavonoid Compounds. *New Phytol.* **118**, 87-93.

Siqueira, J. O.; Safir, G. R.; Nair, M. G. (1991[b]) VA-Mycorrhiza and Mycorrhiza Stimulating Isoflavonoid Compounds Reduce Plant Herbicide Injury. *Plant and Soil* **134**, 233-242.

Sit, K. H., Wong, K. P., and Bay, B. H. (1991) Effects of Genistein on ATP Induced DNA Synthesis and Intracellular Alkalinization in Chang Liver Cells. *Japan J. Pharmacol.* **57**, 109- 111.

Staszewski, J., McCall, M. G., and Stenhouse, N. S. (1971) Cancer Mortality in 1962-66 Among Polish Migrants to Australia. *Br. J. Cancer* **25**, 509-610.

Szabo, V., and Antal, E. (1973) The Selective Reduction of Isoflavone. *Tetrahedron Lett.* 1659-1662.

Talamini, R., La Vecchia, C., Decarli, A., Franceschi, S., Grattoni, E., Grigoletto, E., Liberati, A. And Togoroni, G. (1984) Social Factors, Diet and Breast Cancer in a Northern Italian Population. *Br. J. Cancer* **49**, 723-729.

Tominaga, S. (1985) Cancer Incidence in Japanese in Japan, Hawaii and Western United States. *Natl. Cancer Inst. Monogr.* **69**, 83-92.

Troll, W., Wiesner, R., Shellabarger, C. J., Holtzman, S., and Stone, J. P. (1980) Soybean Diet Lowers Breast Tumor Incidence in Irradiated Rats. *Carcinogenesis* **1**, 469-472.

Uckun, F. M., Evans, W. E., Forsyth, C. J., Waddick, K. G., Ahlgren, L. T., Chelstrom, L. M., Burkhardt, A., Bolen, J., and Myers, D. E. (1995) Biotherapy of B-Cell Precursor Leukemia by Targeting Genistein to CD 19-Associated Tyrosine Kinases. *Science* **267**, 886-891.

Wang, H. J., and Murphy, P. A. (1994[a]) Isoflavone Composition of American and Japanese Soybeans in Iowa: Effects of Variety, Crop Year, and Location. *J. Agric. Food Chem.* **42**, 1674-1677.

Wang, H. J., and Murphy, P. A. (1994[b]) Isoflaovne Content in Commercial Soybean Foods. *J. Agric. Food Chem.* **42**, 1666-1673.

Wei, H., Wei, L., Frenkel, K., Bowen, R., and Barnes, S. (1993) Inhibition of Tumor Promoter-Induced Hydrogen Peroxide Formation In Vitro and In Vivo by Genistein. *Nutrition and Cancer* **20**, 1-12.

Willett, W. C., Stampfer, M. J., Colditz, G. A., Rosner, B. A., Hennekens, C. H., and Speizer, F. E. (1987) Dietary Fat and the Risk of Breast Cancer. *N. Engl. J. Med.* **316**, 22-28.

Winter, J., Popoff, M. R., Grimont, P., and Bokkenheuser, V. D. (1991) *Cloustridium orbiscindens* sp. nov., a Human Intestinal Bacterium Capable of Cleaving the Flavonoid C-Ring. *Intl. J. Sys. Bacteriol.* **41**, 355-357.

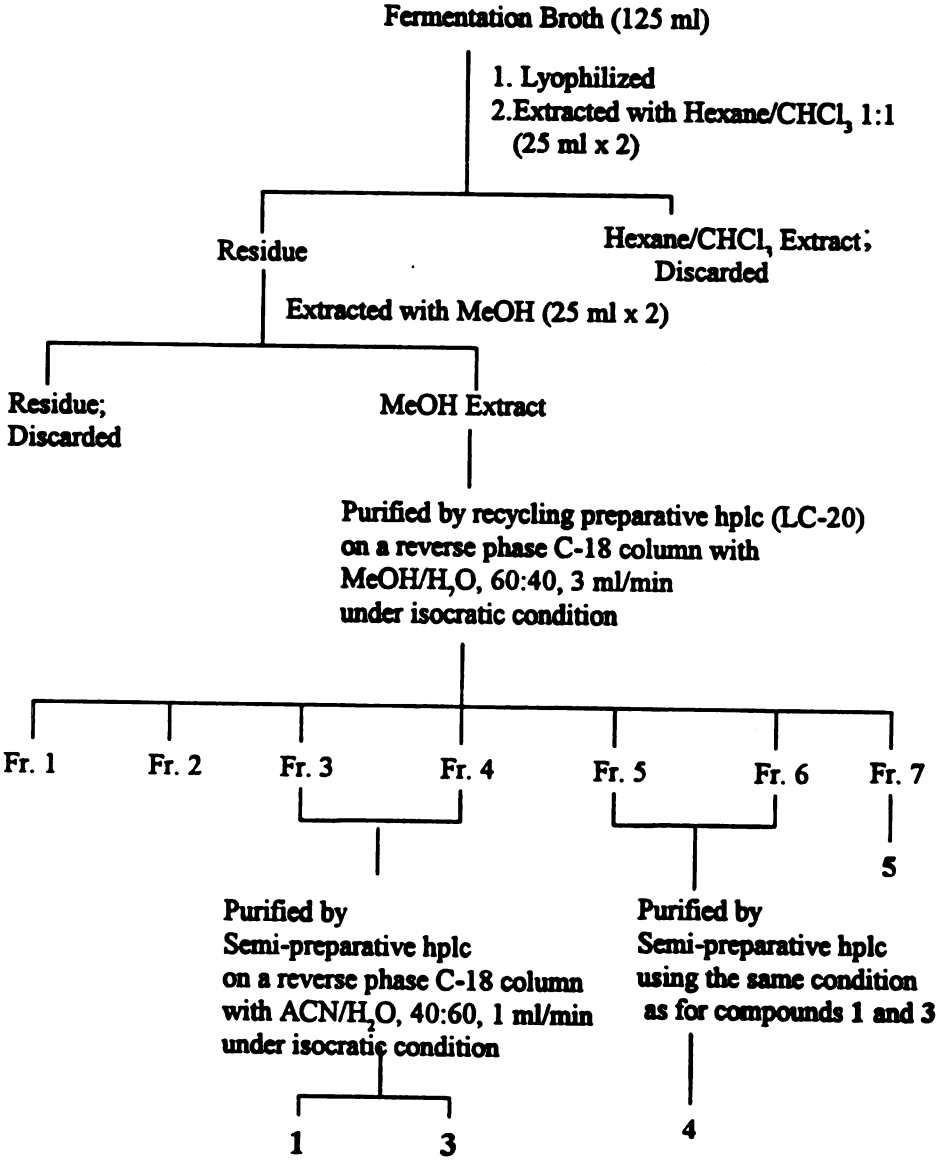
Yanagihara, K., Ito, A., Toge, T., and Numoto, M. (1993) Antiproliferative Effects of Isoflavones on Human Cancer Cell Lines Established from the Gastrointestinal Tracts. *Cancer Reserach* **53**, 5815-5821.

Yasuda, T., Kano, Y., Saito, K. I., and Ohsawa, K. (1994) Urinary and Biliary Metabolites of Daidzin and Daidzein in Rats. *Biol. Pharm. Bull.* **17**, 1369-1374.

Yoder, L., Cheng, E., and Burroughs, W. (1954) Synthesis of Estrogenic Isoflavone Derivatives. *Iowa Academy of Science* **61**, 271-277.

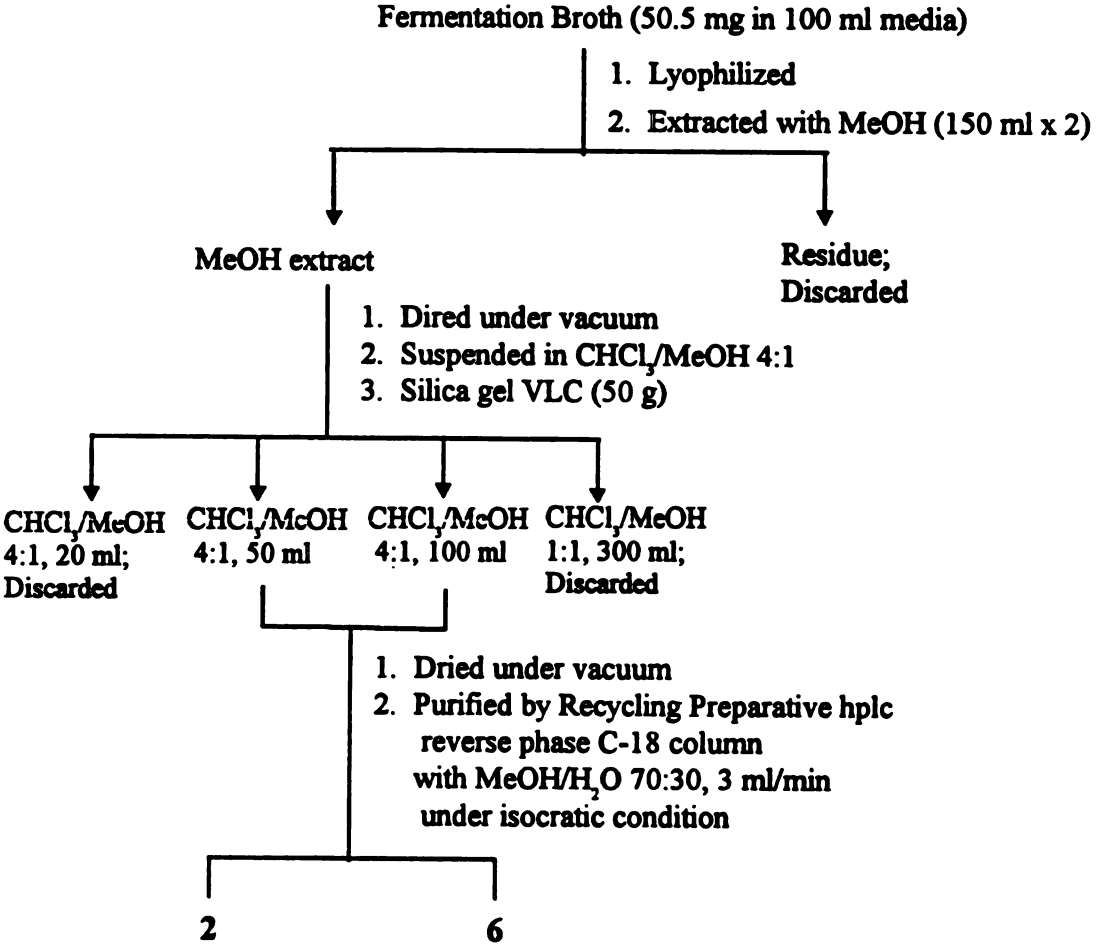
APPENDIX I

Isolation and purification of daidzein and its metabolites from the fermentation broth



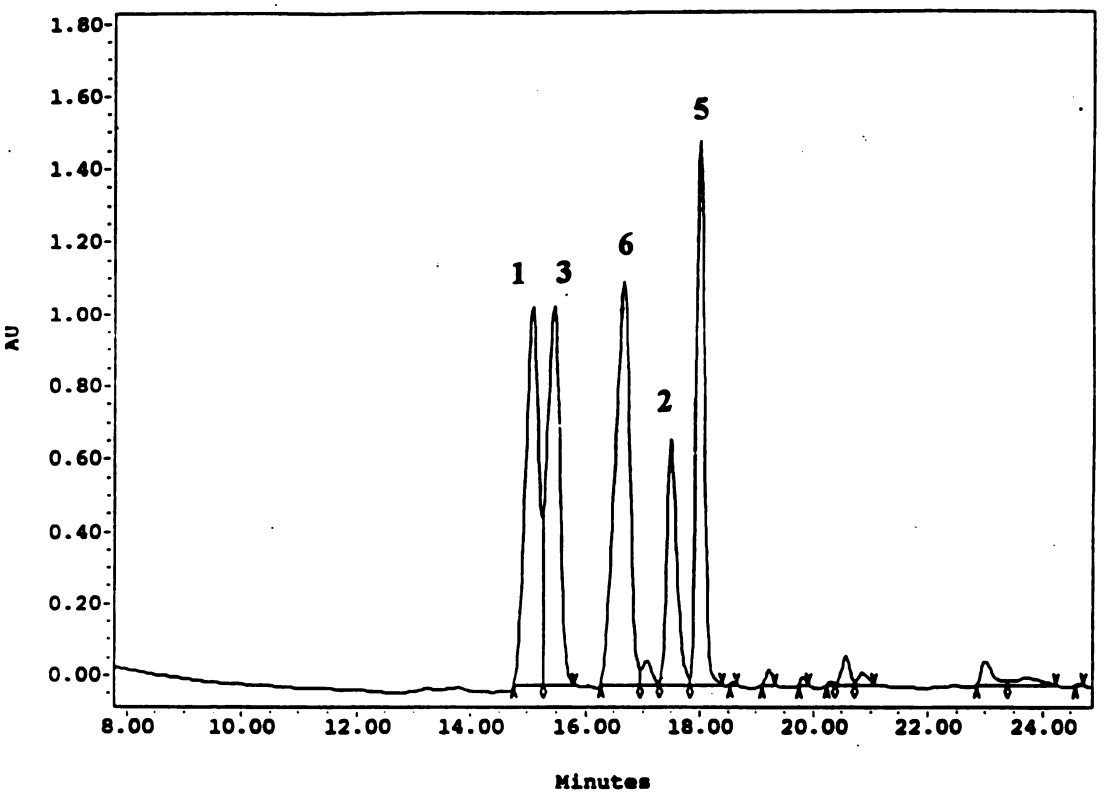
APPENDIX II

Isolation and purification of genistein and its metabolites from the fermentation broth



APPENDIX III

HPLC of daidzein and genistein and their metabolites



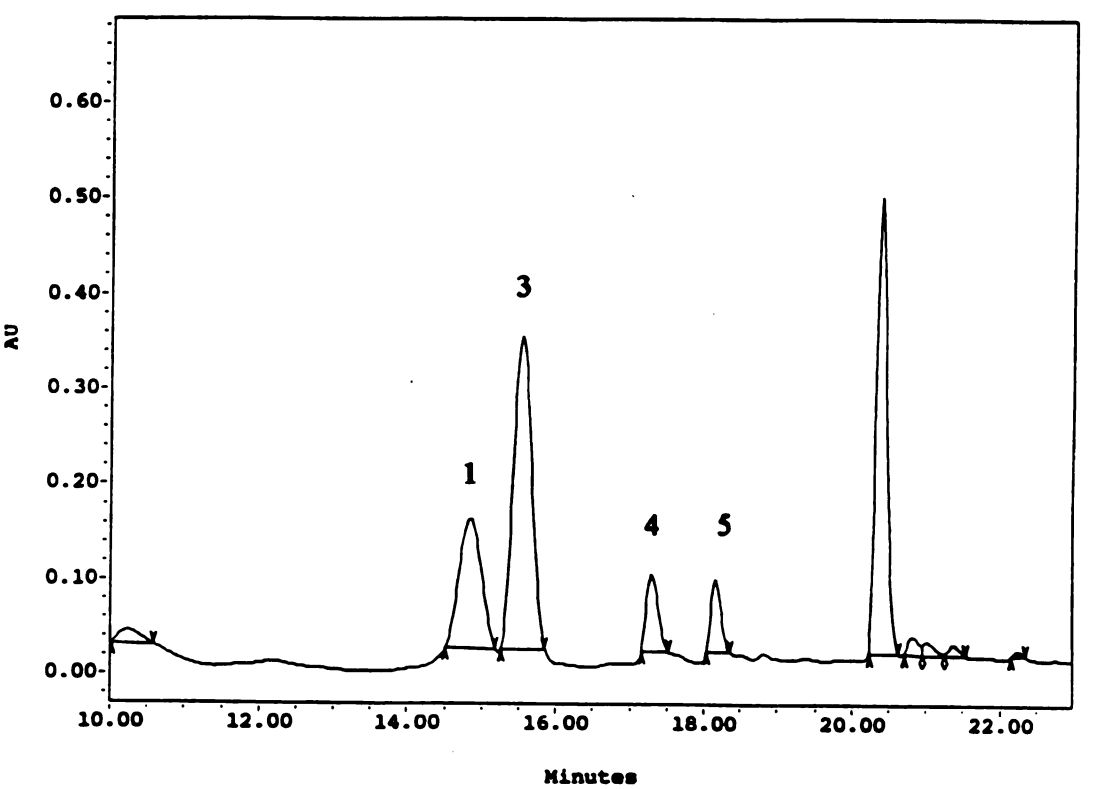
HPLC condition:

Column:	C-18 reverse phase column (4.6 x 250 mm)
Mobile phase:	ACN and H_2O under linear gradient of ACN/H_2O 30:70 to ACN 100 % (final) in 15 min. The column was eluted with ACN 100 % for an addition 10 min.
Flow rate:	0.5 ml/min
Detection:	Monitored by a PDA detector and the data was collected at 200 - 360 nm and processed to obtain the results at 210 nm.

1: Daidzein; 2: Genistein; 3: Dihydrodaidzein; 5: Equol; 6: Dihydrogenistein

APPENDIX IV

HPLC of the fermentation broth of daidzein



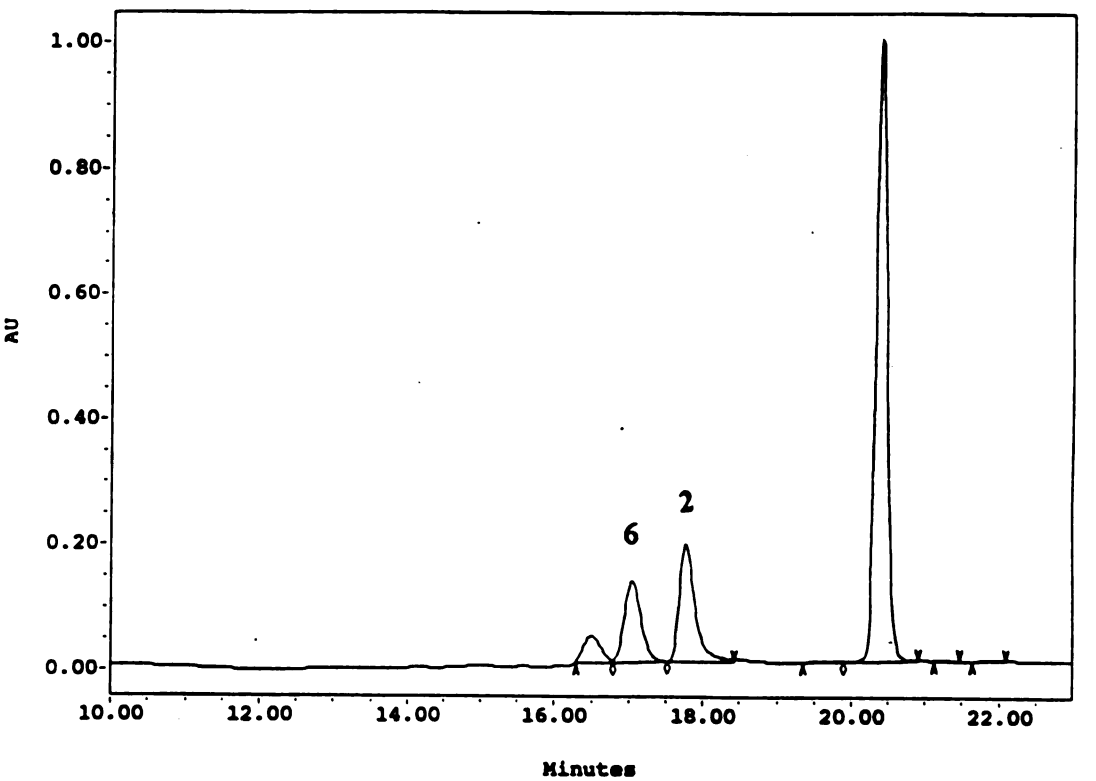
HPLC condition:

Column:	C-18 reverse phase column (4.6 x 250 mm)
Mobile phase:	ACN and H_2O under linear gradient of ACN/H_2O 30:70 to ACN 100 % (final) in 15 min. The column was eluted with ACN 100 % for an addition 10 min.
Flow rate:	0.5 ml/min
Detection:	Monitored by a PDA detector and the data was collected at 200 - 360 nm and processed to obtain the results at 210 nm.

1: Daidzein; 3: Dihydrodaidzein; 4: Benzopyran-4,7-diol, 3-(4-hydroxyphenyl); 5: Equol

APPENDIX V

HPLC of the fermentation broth of genistein



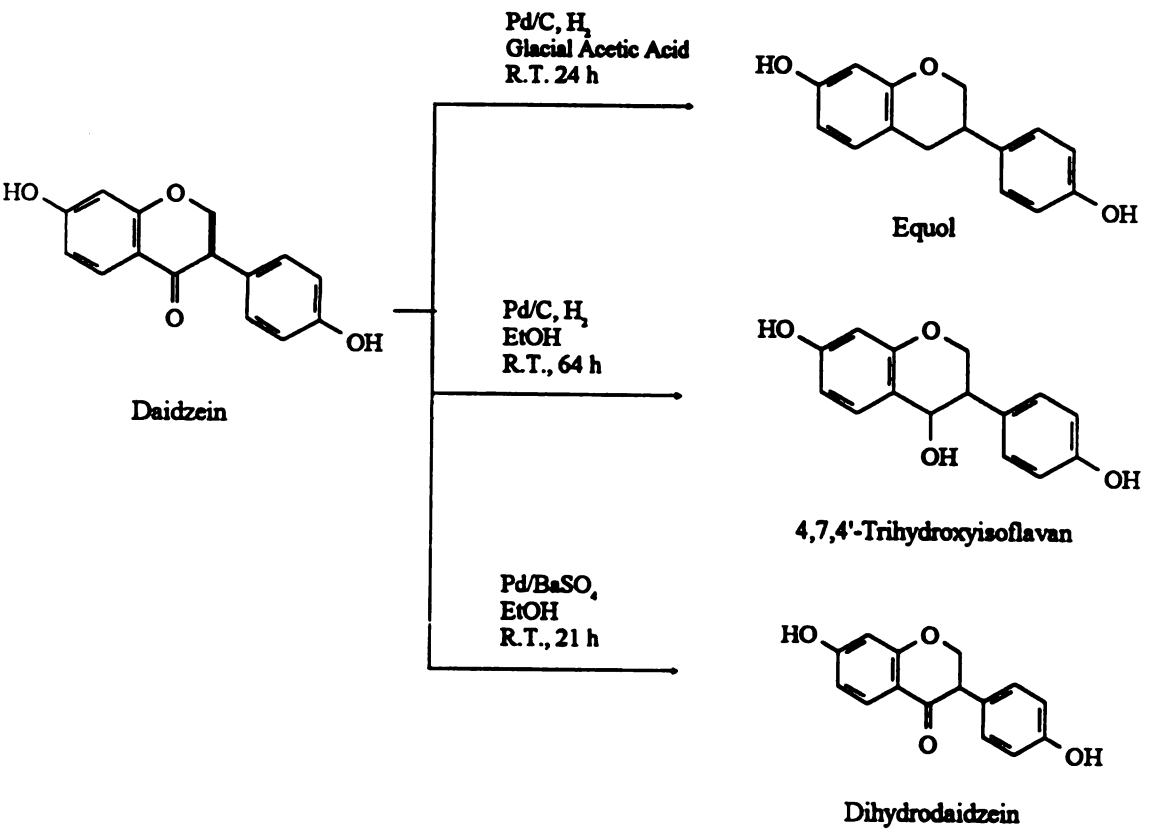
HPLC condition:

Column:	C-18 reverse phase column (4.6 x 250 mm)
Mobile phase:	ACN and H_2O under linear gradient of ACN/H_2O 30:70 to ACN 100 % (final) in 15 min. The column was eluted with ACN 100 % for an addition 10 min.
Flow rate:	0.5 ml/min
Detection:	Monitored by a PDA detector and the data was collected at 200 - 360 nm and processed to obtain the results at 210 nm.

2: Genistein; 6: Dihydrogenistein

APPENDIX VI

Hydrogenation scheme for daidzein



APPENDIX VII

Hydrogenation scheme for genistein