IDENTIFICATION OF CYP1A2 AS THE MAIN ISOFORM FOR THE PHASE I HYDROXYLATED METABOLISM OF GENISTEIN AND A PRODRUG CONVERTING ENZYME OF METHYLATED ISOFLAVONES

MING HU, KRISTOPHER KRAUSZ, JUN CHEN, XIA GE, JIANQI LI, HARRY L. GELBOIN, AND FRANK. J. GONZALEZ

Laboratory of Molecular Carcinogenesis (M.H., K.K., H.L.G.), Laboratory of Metabolism (M.H., K.K., F.J.G.) National Cancer Institute, National Institute of Health, Bethesda, Maryland; Department of Pharmaceutical Sciences, College of Pharmacy, Washington State University, Pullman, Washington (M.H., J.C.); Department of Medicinal Chemistry, Shanghai Institute of Pharmaceutical Industries, Shanghai, China (X.G., J.L.)

(Received December 12, 2002; accepted March 25, 2003)

This article is available online at http://dmd.aspetjournals.org

ABSTRACT:
This study determined the cytochrome P450 (P450) isoforms responsible for metabolism of isoflavones using human liver microsomes (HLM) and expressed P450s. The primary metabolite of genistein is 3'-OH-genistein, as identified with an authentic chemically synthesized standard. CYP1A2 was predominantly responsible for 3'-OH-genistein formation since its formation was inhibited (>50%, p < 0.05) by a monoclonal antibody specific for CYP1A2, was correlated with CYP1A2 activities of HLM, and was catalyzed by expressed CYP1A2. In addition to CYP1A2, CYP2E1 also catalyzed, although to a lesser extent, its formation. The contribution of these P450s to the formation of 3'-OH-genistein was also confirmed with a panel of expressed enzymes. Methylated isoflavones biochanin A, prunetin, and formononetin (10–100 μM) were rapidly converted by HLM and expressed CYP1A2 to more active genistein and daidzein. The conversion of biochanin A to genistein appears to be mainly mediated by CYP1A2 because of the strong correlation between the conversion rates and CYP1A2 activities in HLM. Thus, CYP1A2 is an effective prodrug-converting enzyme for less active methylated isoflavones. CYP1A2-catalyzed conversion of biochanin A to genistein (Km, 7.80 μM; Vmax, 903 pmol/min/mg of protein; Vmax/Km, 116 μl/min/mg of protein) was much faster than 3'-hydroxylation of genistein (Km, 12.7 μM and Vmax, 109 pmol/min/mg of protein). The interaction studies showed that genistein inhibited formation of acetaminophen from phenacetin with an IC50 value of 16 μM. Additional studies showed that phenacetin and genistein were mutually inhibitory. In conclusion, CYP1A2 and CYP2E1 metabolized genistein and CYP1A2 acted as prodrug-converting enzymes for other less active methylated isoflavones.

The consumption of an isoflavonoid-rich diet has been associated with a decrease in the incidence of hormone-related cancers (e.g., breast and prostate cancer) (Kurzer and Xu, 1997; Birt et al., 2001; Yang et al., 2001 and references therein). Isoflavonoids, which include aglycones or isoflavones and their glucosides, are generally classified as phytoestrogens. The latter are plant-derived estrogen-like molecules. An important isoflavonoid is genistein, which has been extensively studied and widely publicized in the popular media for its health effects. The National Cancer Institute is currently engaged in a clinical trial of genistein (protocol CC#98-C-0098B) for prostate cancer prevention.

Despite the assertion that isoflavones are effective cancer prevention agents, the understanding of the absorption mechanism and metabolic pathways of genistein and other isoflavones is limited. For example, whether the active ingredient in vivo is the parent compound or a metabolite has not been established. This is a serious concern since the in vivo concentration (nanomolar range) achieved with a normal soy diet is much lower than that needed in vitro to achieve the reported anticancer and chemopreventive effects of genistein, which have IC50 values in the micromolar range (Kurzer and Xu, 1997; Birt et al., 2001; Yang et al., 2001; Busby et al., 2002). It is generally believed that the cause of this poor bioavailability is due to poor absorption of genistein-7-glucoside (natural form of genistein in soy products) and extensive metabolism of genistein following absorption (Xu et al., 1994, 1995; King et al., 1996, 1998; Watanabe et al., 1998).

The major metabolic pathways of genistein are sulfation and glucuronidation (King et al., 1996, 1998; Setchell et al., 2001). However, it is generally believed that conjugates are not active species, although one study has shown some activity for glucuronidated genistein (Zhang et al., 1999). Thus, identifying phase I metabolites of genistein, characterizing its metabolic pathways, and determining the biological activity of metabolites are of great importance for understanding the biological functions of isoflavones. Recently, several phase I metabolites of genistein, which are mainly hydroxylated metabolites, have been identified in vitro and in vivo (Kulling et al., 2000, 2001, 2002). Additionally, formononetin and biochanin A were shown to be converted to daidzein and genistein following oral administration to humans (Setchell et al., 2001). Currently, little is known about the specific
P450 isoforms responsible for phase I metabolism of isoflavones. A preliminary study published by Roberts-Kirchhoff et al. (1999) identified the formation of hydroxylated metabolites as a P450-catalyzed reaction. However, much important information about phase I metabolism of isoflavones is not available. For example, it is not known if P450 convert methylated isoflavones such as formononetin and biochanin A to more active daidzein and genistein. The goals of this study are to characterize the P450 isoform(s) responsible for the hydroxylation of genistein and the O-demethylation of its isoflavone analogs, and to determine the potential for drug-isoflavone interactions.

Materials and Methods

Materials. Human liver microsomes (HLM) were purchased from BD Gentest Corp. (Woburn, MA). Expressed P450s were obtained from BD Gentest Corp. Genistein, daidzein, biochanin A, formononetin, prunetin, and 4’,7-dimethoxyisoflavones (DMIF) (see Table 1 for structures) were purchased from Indofine Chemical Co. Inc. (Somerville, NJ). Hydroxylated metabolites of genistein, 2’-OH-genistein and 3’-OH-genistein were synthesized as described (see Appendix). Inhibitory monoclonal antibodies (mAbs) used in this study have been previously characterized (Gelboin et al., 1995, 1997; Krausz et al., 1997; Yang et al., 1998), and were used for single and combinatorial inhibitor studies. Monoclonal antibodies used were mAb1-599-16 (against CYP1A1), mAb26-7-5 (against CYP1A2), mAb151-45-4 (against CYP2B6), mAb49-10-20 (against CYP2B6), mAb281-1-1 (against CYP2C8), mAb1-7-4-4 (against CYP2C9), mAb1-58-11 (against CYP2C9), mAb512-1-8 (against CYP2D6), mAb2-106-12 (against CYP2E1), and mAb3-29-9 (against CYP3A4). The control mAb was Hy Hel raised against egg lysozyme.

Microsome Incubation Experiments. A typical assay contained 25 to 50 nmol expressed P450 or 150 to 300 nmol P450 in HLM in 0.5 ml 100 mM, pH 7.5, K2HPO4 buffer. For incubations with mAbs, 15–30 μl (10–30 μg of ascites protein) of ascites fluid was added to the enzyme and allowed to incubate for 5 min at 37°C. Monoclonal antibody Hy Hel, anti egg lysozyme (Gelboin et al., 1998), was used as a control. For combinatorial antibody inhibition studies, total protein content was kept at approximately the same level by using appropriate amounts of mAb Hy Hel. Substrate (60 μM final concentration, unless otherwise indicated) in 10 μl of methanol was added with additional buffer to a final volume of 0.5 ml. The oxidative metabolism reaction was initiated with the addition of NADPH (1 mM final concentration) or an NADPH-regenerating system (BD Gentest Corp.). The reaction proceeded for 30 min at 37°C in a shaking water bath (100 rpm), 7-OH-coumarin (10 nmol), used as an internal standard, was added and the metabolites were extracted with 8 ml of CH2Cl2 which ensured >85% recovery. For 3’-OH-genistein, an additional extraction with 4 ml of CH2Cl2 yielded >85% recovery.

Results

Metabolism of Genistein by HLM. The metabolic pattern of genistein was determined with HLM, which resulted in two major peaks that eluted earlier than genistein (designated as M1-M2) in the HPLC diagram (Fig. 1). This pattern is similar to those reported by earlier studies (Roberts-Kirchhoff et al., 1999; Kulling et al., 2000; Yang et al., 2001). Additional hydroxylated metabolites of genistein, which elutes earlier than genistein, have been previously reported (Kulling et al., 2000, 2001). Therefore, we did not further pursue the identities of these minor metabolites. Instead, we focused on the enzymatic and kinetic characterization of the P450 isoforms responsible for the formation of the primary phase I metabolite of genistein, M1.

Confirmation of Structures of M1. The M1 peak was purified from HPLC, and injected into a liquid chromatographer/mass spectrometer with atmospheric pressure ion-spray (not shown). The metabolite was found to be either 2’-OH or 3’-OH genistein (not shown). Authentic standards of 2’-OH-genistein and 3’-OH-genistein were synthesized as described (see Appendix). Comparison of the metabolites formed using HLM with the metabolites synthesized chemically indicated that M1 is 3’-OH genistein and not 2’-OH genistein. An earlier study of the hydroxylation of genistein did not show which metabolite (3’-OH genistein or 2’-OH genistein) was formed by CYP1A2 (Roberts-Kirchhoff et al., 1999).

Effect of mAbs on Genistein Metabolism by HLM. The contribution of each P450 isofrome was determined with a panel of well-characterized inhibitory monoclonal antibodies or mAbs (Gelboin et al., 1999) against various human P450 isoforms (Fig. 2). When they...
were added singly, it was found that mAb 26-7-5 against CYP1A2 was the most effective inhibitor of 3'-OH-genistein formation (p < 0.05). Monoclonal antibodies against CYP2E1 also significantly inhibited (p < 0.05) the formation of 3'-OH-genistein but to a lesser extent than mAb 26-7-5 against CYP1A2 (Fig. 2). Monoclonal antibodies against CYP2C8, CYP2B6, CYP2A6, and CYP3A4 caused minor inhibition of formation of 3'-OH-genistein, whereas mAbs against CYP1A1, CYP2C19, and CYP2D6 did not inhibit the metabolism of genistein into 3'-OH-genistein.

A combinatorial mAb approach was used to determine the contribution of liver P450 isoforms (Fig. 3). The results indicated the formation was approximately linear for each P450 isoform, whereas other expressed P450s including CYP3A4 and CYP3A5, the major P450 in the liver and intestine, did not form 3'-OH-genistein (Fig. 4). Expressed CYP2D6, CYP2C8, and CYP3A4 formed small amounts of 3'-OH-genistein (Fig. 4). Expressed CYP2B6, CYP2A6, and CYP3A4 formed substantial amounts of 3'-OH-genistein (Fig. 4).

Preparations of liver microsomes [HLM pool (lot 1 and lot 2), HG32, HG42, HG89, HG93, HK21, HK23, HK43, H56, H112 from BD Gentest Corp.] containing different CYP1A2 activities. We found a clear linear relationship (r² = 0.92) between CYP1A2 activity of the liver microsomes and the formation rate of 3'-OH-genistein (Fig. 5). We also plotted formation rates of 3'-OH-genistein against CYP2E1, CYP2A6, CYP3A4, CYP2C8, and CYP2C9 activities. None of these activities correlated with formation rates of 3'-OH-genistein except there was a weak correlation between formation rates of 3'-OH-genistein and CYP2E1 activities (r² = 0.23). Further studies were focused on CYP1A2-catalyzed metabolism of genistein and its isoflavone analogs.

Determination of the Kinetic Parameters of 3'-OH-Genistein Formation by CYP1A2. We determined initial rates of 3'-OH-genistein formation at genistein concentrations of 2, 2.5, 5, 10, 20, 40, 60, and 100 μM using expressed CYP1A2, and found that 3'-hydroxylayion of genistein was concentration-dependent with a Kₘ value of 12.7 μM and Vₘₐₓ value of 109 pmol/min/mg of protein (Fig. 6). The ratio of Vₘₐₓ/Kₘ value, which represents intrinsic clearance, is 8.6 μl/min/mg of protein. The Eadie-Hofstee plot shows that the involvement of one major enzyme (linear regression generated r² of 0.81).
CYP1A2-MEDIATED METABOLISM OF ISOFLAVONES

CYP1A2-Activation of Methylated Isoflavones. We determined whether CYP1A2 transform methylated isoflavones (Table 1) to genistein or daidzein through metabolic activation by CYP1A2. We used biochanin A, prunetin, formononetin, daidzein, 4′,5-dimethoxy-isoflavone to incubate with expressed CYP1A2. We found rapid conversion of biochanin A to genistein (see Fig. 6) and very slow formation (barely detectable but not quantifiable) of genistein from daidzein (100 μM). Prunetin (40 μM) was also rapidly converted and its rate of conversion (340 ± 47 pmol/min/mg of protein) was about 60% less than the conversion rates of biochanin A (40 μM) to genistein. At lower concentrations, the rate of conversion of prunetin to genistein (231 ± 5 pmol/min/mg of protein) was similar to that of biochanin A to genistein (202 ± 4 pmol/min/mg of protein). Formononetin was rapidly converted to daidzein and genistein, whereas 5,4′-dimethoxyisoflavone was rapidly converted to the daidzein but poorly converted to genistein. The rate of conversion from formononetin (100 μM) to genistein (295 ± 27 pmol/min/mg of protein) was similar to its conversion to daidzein (393 ± 68 pmol/min/mg of protein). The latter was approximately 40% slower than the conversion rate of biochanin A to genistein but was approximately 80 times greater than the conversion rate of 5,4′-dimethoxyisoflavone (100 μM) to daidzein. 5,4′-dimethoxyisoflavone (100 μM) was mainly converted to formononetin at a rate 4 times faster than the rate of conversion from biochanin A to genistein.

HLM-Activation of Methylated Isoflavones. Biochanin A, formononetin, and 5,4′-dimethoxyisoflavone at a high concentration (100 μM each) and at a low concentration (10 μM) were also incubated with HLM. At high isoflavone concentration, and the rate of metabolism was found in descending order as 5,4′-dimethoxyisoflavone (to formononetin) > biochanin A (to genistein) > 5,4′-dimethoxyisoflavone (to daidzein) > formononetin (to daidzein) ≈ formononetin (to genistein). The only change in the order, with comparison to using an expressed CYP1A2, was that 5,4′-dimethoxyisoflavone was metabolized to daidzein faster than formononetin to daidzein in HLM. At low isoflavone concentration, biochanin A, prunetin, and formononetin were converted to genistein, with the rates of conversion of 232 ± 5, 202 ± 4, and 52 ± 1 pmol/min/mg of protein for prunetin, biochanin A, and formononetin, respectively.

Formononetin was also converted to daidzein (40 ± 2 pmol/min/mg of protein) at a rate comparable with its conversion rate to genistein.

We then determined the rates of biochanin A conversion to genistein in a panel of nine human liver microsomes and plot them against the CYP1A2 activities supplied by the vendor (Fig. 7). The results indicated that the rates of conversion strongly correlated with the CYP1A2 activities (r² = 0.84, Fig. 7), but weakly correlated with CYP2E1 (r² = 0.45) and CYP2C9 (r² = 0.34) activities (not shown). These rates of conversion did not correlate with other P450 activities supplied by the vendor (BD Gentest Corp.).

Determination of Kinetic Parameters of CYP1A2-Catalyzed Metabolism of Biochanin A. We observed rapid conversion of biochanin A to genistein by CYP1A2. Similar to metabolism of genistein to 3′-OH-genistein, the metabolic rate of biochanin A (measured at 2, 5, 10, 20, 40, 70, and 100 μM) into genistein was also concentration dependent with a Kₘ value of 7.80 μM and a Vₘₐₓ value of 903 pmol/min/mg of protein (Fig. 6). The ratio of Vₘₐₓ/Kₘ, which represents intrinsic clearance, is 116 μl/min/mg of protein. The Eadie-Hofstee plot shows that the involvement of one major enzyme (linear regression generated r² of 0.89).

Interactions between CYP1A2-Catalyzed Metabolism of Genistein and Phenacetin. We determined the potential for drug interaction involving genistein and phenacetin, a typical substrate of CYP1A2. Formation rates of acetaminophen, the major metabolite produced by metabolism of phenacetin using expressed CYP1A2, was decreased in the presence of increasing concentration of genistein (Fig. 8). The IC₅₀ value was approximately 16 μM (Fig. 8). The interaction between phenacetin and genistein is mutually inhibitive in that increasing concentration of phenacetin also inhibited hydroxylation of genistein (10 μM). At a phenacetin concentration of 10 μM, the formation rate of 3′-OH-genistein was inhibited 56 ± 5%; at 20 μM, it was inhibited 93 ± 3%; and at 40 μM, it was inhibited 96 ± 4%.

Discussion

Phase I metabolism of isoflavones is an attractive target for metabolism studies since phase I biotransformation may generate metabolites that are more potent than the parent compounds (van Acker et al., 2000 and references therein). Recently, it has been shown that O-demethylation and hydroxylation were viable in vivo pathways for isoflavone activation, because less active biochanin A and formononetin were converted into genistein and daidzein (Setchell et al., 2001; Kulling et al., 2002).

The objectives of this study were to determine the primary P450 isoform responsible for the oxidative metabolism of isoflavones in vitro and to determine the potential for drug-isoflavone interactions. The results indicated that human liver microsomal P450 and expressed CYP1A2 were capable of metabolizing genistein into 3′-OH-genistein and converting methylated isoflavones into more biologically active genistein and daidzein. The results also indicated mutual inhibition of metabolism between genistein and phenacetin. Linear correlations between CYP1A2 activities and 3′-OH-genistein formation rates and between CYP1A2 activities and conversion rates of biochanin A to genistein using a panel of human liver microsomal samples supported this conclusion. Substantial inhibition of 3′-OH-genistein formation by specific mAbs validated the role played by P450 and CYP1A2 in the in vitro metabolism of genistein.

This study demonstrated that oxidative metabolism of genistein to 3′-OH-genistein is mainly by CYP1A2, and to a less extent by CYP2E1 in human liver microsomes. CYP2C8 and CYP3A4 may provide a minor contribution to its formation (Fig. 2). This con-
Conclusions are supported by the fact that the combined use of a panel of mAb to CYP1A2, CYP2E1, CYP2C8, and CYP3A4 inhibited the formation of 3'-OH-genistein by 87% (Fig. 3). This conclusion is also supported by the fact that the metabolism rate of genistein (into 3'-OH-genistein) correlated with the CYP1A2 activities in the human liver microsomes (Fig. 5). Expressed CYP1A2-catalyzed metabolism of genistein also confirmed the hypothesis that CYP1A2 is primarily responsible for the formation of 3'-OH-genistein, the major phase I metabolites of genistein in human liver microsomes (Fig. 4, Fig. 6). Secondary role played by CYP2E1 is also well supported in that expressed CYP2E1 formed 3'-OH-genistein, and inhibitory mAb against CYP2E1 inhibited formation of 3'-OH-genistein. There was also a significant albeit weak correlation between CYP2E1 activities and formation rates of 3'-OH-genistein (not shown). In addition to CYP1A2 and CYP2E1, CYP1A1 and CYP1B1, which are poorly expressed in human liver, may also contribute to the formation of 3'-OH-genistein in vivo and perhaps at the target sites other than liver, but further study is necessary to confirm their roles. Roles played by other P450 isoforms such as CYP2D6 and CYP2A6 may also need to be defined. For example, expressed CYP2A6 also did not form 3'-OH-genistein, but inhibitory mAb against CYP2A6 showed minor inhibition of 3'-OH-genistein formation when used alone. In another instance, expressed 2D6 formed small amounts of 3'-OH-genistein, but the inhibitory mAb against CYP2D6 did not inhibit the formation of 3'-OH-genistein. Previously, these two mAbs were shown to be specific against these two P450 isoforms with minimal cross inhibition (Gelboin et al., 1997, 1999). Although further studies are needed to definitively resolve these apparent inconsistencies, it is likely that one of the reasons is due to difference in how substrates are presented to the enzyme. In an expressed system, isoflavone has only one P450 pathway, whereas in the HLM, multiple P450 pathways could exist for the same isoflavone. Furthermore, in the HLM some P450 isoform may be polymorphic, and there could be large variations in individual P450 content between different HLM samples. The use of the mAbs as metabolism inhibitors is able to more accurately identify and quantify the individual P450 isoforms involved in the metab-

**Fig. 6.** Kinetic characterization of CYP1A2 catalyzed metabolism of genistein to 3'-OH-genistein (panel A) and biochanin A to genistein (panel B).

$K_m$ and $V_{max}$ values for hydroxylation of genistein were 12.7 μM and 109 pmol/min/mg of protein, respectively. The $K_m$ and $V_{max}$ values for O-demethylation of biochanin A were 7.80 μM and 903 pmol/min/mg of protein, respectively. The insert in each panel shows the Eadie-Hofstee plot for each metabolic reaction. Each point represents the mean value of two determinations.
We determined the potential for drug-isoflavone interactions since CYP1A2 is a major P450 isoflavone (≈13% total P450) in human liver and responsible for the metabolism of many drugs (Rendic and DiCarlo, 1997). The results indicated that genistein is a competitive inhibitor of CYP1A2-catalyzed metabolism of phenacetin to acetaminophen (Fig. 8), with an IC50 value (16 μM) close to its Km value (13 μM) (Fig. 6). Furthermore, phenacetin also inhibited the metabolism of genistein with its IC50 value (<10 μM) since 10 μM of phenacetin inhibited the metabolism of genistein (10 μM) by more than 50%. The potential for drug-isoflavone interaction is clearly present since the metabolism rate of phenacetin was decreased even at the lowest genistein concentrations (e.g., 5 μM). A genistein concentration in the low micromolar range is achievable in vivo (Setchell et al., 2001). Although the vast majority of the genistein is conjugated in vivo when measured at the systemic level (Kurzer and Xu, 1997; Setchell et al., 2001; Yang et al., 2001), it is entirely possible that the concentration of unconjugated genistein during its first-pass through the liver could reach that level. This is because genistein is rapidly absorbed and 40 to 60% absorbed genistein may reach liver as aglycone (Chen et al., 2003).

In conclusion, this study indicates that CYP1A2 is the main P450 isoform responsible for the formation of major phase I metabolites of genistein and for activation of methylated isoflavones to genistein and daidzein via the O-demethylation pathway. We have demonstrated the potential for drug-isoflavone interaction, but further studies are needed to show whether these interactions are pharmacokinetically relevant in vivo. The latter studies are important because many people are supplementing their diets with herbal supplements containing significant amounts of isoflavones.

APPENDIX

Synthesis of 2'-OH-genistein and 3'-OH-genistein. The synthetic pathways of these two compounds were similar, and only the synthesis of 3'-OH-genistein (see Fig. 9) is shown here. This synthetic scheme was developed based on earlier work of Robertson and Suckling (1949), Helena et al. (1999), and Nabaei-Bidhendi and Bannerjee (1991).

4,6-Di(methoxymethoxy)-2-hydroxyacetophenone (1). Anhydrous potassium carbonate (61.2 g, 0.45 mol) was added to phloroacetophenone (12.6 g, 0.0875 mol) in anhydrous acetone (400 ml), followed by rapidly dropping a solution of methoxymethyl chloride (23 ml, 0.3 mol) in acetone (70 ml). The mixture reacted at room temperature for 2 h. After the mixture was filtered to remove potassium carbonate, the filtrate was evaporated under vacuum. The residue was loaded into a column and eluted with EtOAc-petroleum ether to obtain white nee-
**FIG. 9.** A synthetic scheme for metabolic standards used in the present studies.

Example shown is the synthesis of 3'-OH-genistein, and a similar method was adapted to synthesize other isoflavones.

dles (12 g, 0.0517 mol, 59%). M.P.: 43–44°C; 1H NMR (DMSO-d$_6$) δ: 13.319 (s, 1H), 6.186–6.241 (dd, 2H), 5.226, 5.297 (2s, 4H), 3.389 (2s, 6H), 2.601 (s, 3H); MS m/z 256.

3,4-Di(methoxymethoxy)benzaldehyde (II). A mixture of 3,4-di-hydroxybenzaldehyde (6.9 g, 0.05 mol), methoxymethyl chloride (10 ml, 0.125 mol), and potassium carbonate (31 g, 0.225 mol) in acetone (250 ml) reacted at room temperature for 2 h. It was then worked up as above to yield white needles (7.8 g, 0.0345 mol, 69%). M.P.: 256.

2'-Hydroxy-4',6',3,4-tetramethoxymethoxychalkone (III). A solution of I and II (0.02 mol each) in anhydrous ethanol (10 ml) was treated by dropping of 50% potassium hydroxide (7.2 ml) in ice bath, it was then allowed to react at room temperature for 24 h. Afterwards, the reaction mixture was extracted with ethyl acetate and separated. The organic layer was washed sequentially with water and saturated saline and then treated with anhydrous magnesium sulfate. After the ethyl acetate was removed, the residue was crystallized from ethanol to yield III as yellow sheet crystals (4.2 g, 0.0011, 56%). M.P.: 267–269° C, Roberson and Suckling, 1949; 1H NMR (DMSO-d$_6$) δ: 12.972 (s, 1H), 12.259 (m, 3H), 7.305 (m, 2H), 6.253–6.298 (d, 2H, J = 13.6), 5.252(4s, 8H), 3.423 (4s, 12H); MS: m/z 464.

3',4',5,7-Tetrahydroxyisoflavone (IV). III (0.93 g, 0.0020 mol) in methanol (200 ml) was treated with thallium (III) trinitrate (1.95 g, 0.0044 mol) with stirring at room temperature for 4 h. Subsequently, 3 N hydrochloric (15 ml) was added to the mixture and refluxed for 5 h. The mixture was worked up as usual to yield pale yellow crystal (0.32 g, 0.0011, 56%). M.P.: 293°C, M.H was supported by Washington State University for a sabbatical leave at National Cancer Institute’s Laboratory of Metabolism and Laboratory of Molecular Carcinogenesis. The work at Washington State University was supported by a National Institutes of Health Grant CA87779. We thank Katherine Anderson for valuable editorial help.

**References**


