reviews

molecular pharmaceutics

Methylation of Dietary Flavones Greatly Improves Their Hepatic Metabolic Stability and Intestinal Absorption

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Received May 22, 2007; Revised Manuscript Received September 14, 2007; Accepted September 14, 2007

Abstract: Dietary flavonoids and other polyphenols have many biological properties that could make them useful as chemopreventive agents. However, very poor oral bioavailability makes them largely ineffective *in vivo*. The low bioavailability is mainly due to highly efficient glucuronic acid and sulfate conjugation of these mono- or polyhydroxylated agents in the intestinal/hepatic barrier. This review describes how the methyl capping of all free hydroxyl groups of flavones results in dramatically increased metabolic stability, as the metabolism is shifted to less efficient CYP-mediated oxidation. This was demonstrated best by using the human liver S9 fraction with an appropriate selection of cofactors. In addition, the intestinal transport of flavones was much improved through methylation, as shown in Caco-2 cell Transwell experiments. *In vivo* in the rat, oral administration of one methylated flavone resulted in high bioavailability and tissue distribution with no detectable levels of its unmethylated analogue. In addition to increased metabolic stability, methylation resulted in markedly increased inhibition of cancer cell proliferation. Thus, methylation appears to be a simple and effective way of increasing both metabolic resistance and transport of the flavonoids and, most important, some of their major biological activities.

Keywords: Flavonoids; flavones; methylation; bioavailability; metabolic resistance; cell proliferation

Introduction

Dietary flavonoids and other polyphenols, while showing promise as potential disease-preventing agents,[1] suffer from very poor oral bioavailability, which makes their utility as such agents very tenuous.[2–4]

Most naturally occurring flavonoids exist as glycosides with various sugars. Numerous studies have concluded that these glycosides are poorly absorbed and require prior hydrolysis to the aglycones in the intestinal lumen[2,5,6] and oral cavity,[7] which appears to be an efficient process. However, the aglycons also have great bioavailability problems, which will be the focus of this review article

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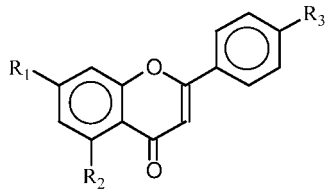
10.1021/mp700071d CCC: $37.00
Published on Web 10/25/2007



	R_1	R_2	R_3
Chrysin	OH	OH	H
Apigenin	OH	OH	OH
5,7-DMF	OCH_3	OCH_3	H
5,7,4'-TMF	OCH_3	OCH_3	OCH_3

Figure 1. Chemical structures of chrysin (5,7-dihydroxyflavone) and apigenin (5,7,4′-trihydroxyflavone) and their methylated analogues 5,7-dimethoxyflavone (5,7-DMF) and 5,7,4′-trimethoxyflavone (5,7,4′-TMF).

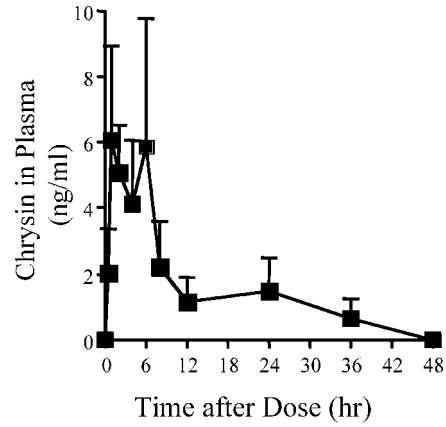


Figure 2. Plasma concentration–time curve for chrysin in seven normal volunteers (mean values ± SEM) after a single oral 400 mg dose.[15]

together with one possible solution through methylation of these agents. For simplicity, this paper has been limited to a few model compounds, such as chrysin and apigenin and their methylated analogues (Figure 1). Parts of this topic have been addressed in separate publications.[8,9]

Chrysin and apigenin are two of the most simple flavones in diets commonly consumed by humans.[10,11] A number of *in vitro* studies have suggested potential chemopreventive properties of both compounds, including aromatase inhibition for chrysin to protect against hormone-sensitive cancers[12] and inhibition of cancer cell proliferation for apigenin through interaction with other signaling events important to the cancer cells.[13,14] These and other claims have made them appear in health food stores for consumption as food supplements, sometimes in quite high doses. As an aromatase inhibitor chrysin is touted as an androgen-boosting flavone to help build muscle strength in young athletes. Its *in vivo* activity is, however, very much in doubt.

Low Oral Bioavailability of Unmethylated Flavones. A 400-mg dose of chrysin, the dose recommended in health food stores, was given to seven healthy volunteers, and the pharmacokinetics and metabolism were determined by HPLC analysis.[15] The major finding was that the plasma chrysin concentrations were very low, with maximum levels reaching only 6 ng/mL (Figure 2). This corresponds to 24 nM, which is 100-fold less than the 2.6 μM K_i value for its aromatase inhibition.[12] In addition, the plasma protein binding of chrysin was estimated to be as high as 99%, leaving a miniscule unbound concentration. It would thus be difficult to invoke any aromatase inhibiting effects of chrysin *in vivo*. This conclusion was supported by the finding that chrysin had no effect on urinary testosterone levels in men.[16] Also, in androstenedione-treated immature rats, very high doses of chrysin failed to inhibit the uterine growth expected with aromatase-induced estrogen formation.[17]

Although both chrysin sulfate and chrysin glucuronide were recognized as metabolites of chrysin in humans, the most striking observation was that a large fraction of the dose was excreted unchanged in the stool. This may be a reflection of poor oral absorption due to limited solubility (see below). However, it is more likely due to a combination of biliary and enteric secretion of the conjugates followed by bacterial hydrolysis back to chrysin in the intestinal lumen.

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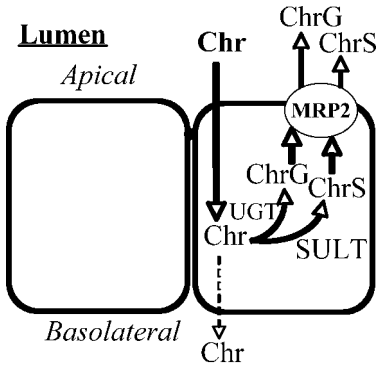

Figure 3. Fluxes and fate of chrysin (Chr) in the human enterocytes Caco-2 cell monolayer model: G, glucuronic acid; S, sulfate; UGT, UDP-glucuronosyltransferase; SULT, sulfotransferase; MRP2, multiresistance-associated protein 2. Small amounts of ChrG and ChrS also appear on the basolateral side.

The biliary secretion was strongly supported by the finding of large amounts of both glucuronide and sulfate conjugates in the bile after either oral or intravenous chrysin doses in the rat.[15] The enteric secretion was strongly supported in a study using human intestinal epithelial Caco-2 cell grown in Transwells.[18] Chrysin was well absorbed across the apical cell membrane and conjugated to both glucuronic acid and sulfate. Both conjugates were exported from the cells back to the apical buffer chamber by MRP2 and possibly other transporters[4] (Figure 3). The efficiency of both conjugation reactions as well as the outwardly directed transport of the metabolites was very high.

There is no similar *in vivo* human study of apigenin. However, when human volunteers consumed a single dose of blanched apigenin glycoside-rich parsley, corresponding to about 20 mg of apigenin, the maximum plasma apigenin levels (after enzymatic hydrolysis) were about 100 nM,[19] which is somewhat higher than for chrysin, but lower than the plasma levels of chrysin sulfate (800 nM) in the above study. Like chrysin, apigenin has been shown to be metabolized by glucuronidation and sulfation in rats[20] as well as in mouse and human liver preparations[21] and human hepatic Hep G2 cells[22] and intestinal Caco-2 cells.[23]

These studies and clinical studies of other polyphenols, such as quercetin[3,24] and resveratrol,[25,26] strongly indicate that polyhydroxylated dietary compounds are very efficiently metabolized *in vivo* with minimal concentrations of unchanged polyphenols reaching the general circulation.

Mechanism of Low Oral Bioavailability. It is clear that extensive conjugation of the free hydroxyl groups is the main reason for the low oral bioavailability of the dietary flavonoids and other polyphenols,[2,3,27] although transporters may play a role for certain polyphenols, e.g., tea catechins and anthocyanins.[28–30] However, whether oxidation also might be important is less clear.[31–33] Human liver microsomal studies of CYP-mediated oxidation include the flavonoids galangin, kaempferide, biochanin A, prunetin, formononetin, genistein, and tangeretin.[34–37] However, this

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commonly used *in vitro* model does not reflect the complete metabolic profile, in particular for those polyphenols mainly metabolized by conjugation. Thus, using the human liver homogenate 9000*g* supernatant (S9 fraction) with appropriate cofactors and the flavonoid galangin (3,5,7-trihydroxyflavone) as the model substrate, the role of the CYPs decreased dramatically in comparison with glucuronidation and sulfation with oxidation accounting for only about 2% of total metabolism.[35] This was well reflected in the metabolism of galangin by freshly plated human hepatocytes, the model system closest to the *in vivo* situation.[35] The intrinsic clearance through glucuronidation, mediated mainly by UGT1A9, was as much as five times higher than of bilirubin, one of the best UGT substrates known, and much higher than for most drugs.[38] The rate of sulfation was lower than that of glucuronidation, although still much higher than the rate of oxidation.

A more recent study using the human liver S9 fraction[39] showed that for some polyphenols, e.g., resveratrol and 7-hydroxyflavone, sulfation was more extensive than glucuronidation. However, when examining the combined effects of glucuronidation and sulfation in the S9 fraction, the difference in the extent of elimination between flavonoids was surprisingly small.[39] Thus, if the free hydroxyl groups were removed, the clearance would be expected to decrease dramatically because of the absence of both glucuronidation and sulfation.

Metabolic Stability of the Methoxyflavones. Methylation was examined as a generic approach to cap all free hydroxyl groups in the polyphenols. This article is focused exclusively on flavones, a flavonoid subclass (Figure 1) with the basic idea that blocking the free hydroxyl groups should eliminate conjugation as the primary metabolic pathway. If the oxidative demethylation rate was slow enough, great improvements in metabolic stability may result. Parts A and B of Figure 4 show the metabolic stability of unmethylated compared with methylated flavones in pooled human liver S9 fraction incubations supplemented with the cofactors for glucuronidation, sulfation, and oxidation to create a metabolically competent *in vivo*-like system. The disappearance of chrysin and apigenin was very rapid, complete within 20 min, due to extensive metabolism mainly through glucuronidation.[39] This is consistent with the very low oral bioavailability of both compounds in humans (see above). In sharp contrast, the corresponding methylated flavones, i.e., 5,7-DMF and 5,7,4′-TMF, both showed remarkable stability with no disappearance of 5,7-DMF over the 60-min incubation period (Figure 4A) and only about 20% disappearance of 5,7,4′-TMF (Figure 4B). Similar large differences in metabolic stability were also seen for other pairs of unmethylated and methylated flavones.[39]

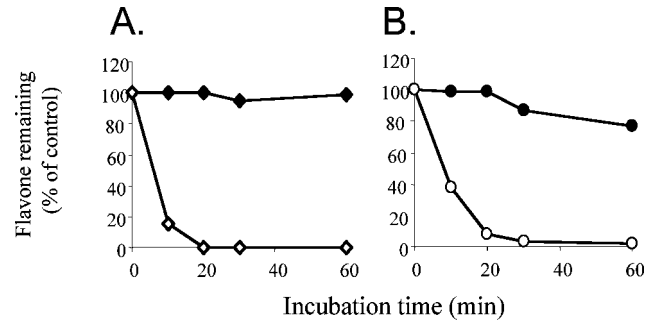

Figure 4. Time-dependent metabolic depletion of unmethylated and methylated polyphenols in pooled human liver S9 fraction:[39] (A) 5,7-DMF (filled symbols) and chrysin (open symbols); (B) 5,7,4′-TMF (filled symbols) and apigenin (open symbols). Human liver S9 fraction was incubated with UDPGA, PAPS, and NADPH and 5 μM polyphenols and analyzed by HPLC.

The rate of intestinal absorption may also play an important role in determining the bioavailability of xenobiotics. In Caco-2 cell monolayers grown on permeable support, considered the best model of human intestinal absorption,[40,41] considerably higher permeability was observed for the two methylated compounds compared with their unmethylated analogues (Figure 5A,B). This is most likely related to the greater metabolic stability of the methylated compounds, as in Figure 4A,B. The unmethylated flavones were mainly metabolized by sulfation, rather than glucuronidation, consistent with the fact that sulfation in the intestine is relatively more important than glucuronidation.

On the basis of these observations with the hepatic S9 fraction and the intestinal Caco-2 cells, the oral bioavailability of 5,7-DMF could be predicted to be much greater than that for chrysin. This was tested *in vivo* in the rat. 5,7-DMF and chrysin were coadministered by oral gavage at 5 mg/kg, a common dose for chrysin as a dietary supplement in humans.[42] Only 5,7-DMF was detectable in the plasma, with a C_{max} of 2.3 μM at 1 h (Figure 6A). 5,7-DMF accumulated in liver, lung, and kidney tissue at quite high

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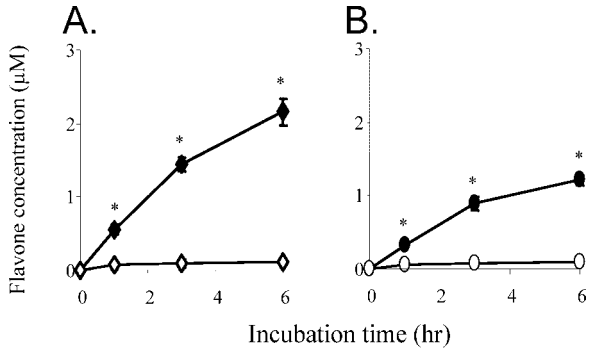

Figure 5. Caco-2 cell transport of methylated versus unmethylated flavones (C and D):[39] (A) 5,7-DMF (filled symbols) and chrysin (open symbols); (B) 5,7,4′-TMF (filled symbols) and apigenin (open symbols). A 5 μM concentration of the flavones (10 μM for 5,7-DMF and chrysin) in transport buffer was added to the apical chambers of Transwells. Samples were taken from the basolateral side at 0.5, 1, 3, and 6 h.

concentrations compared to plasma (Figure 6B). Chrysin was not detectable in any tissue but started to appear in the fecal pellets after 2 h (Figure 6C). This is likely the result of intestinal absorption of chrysin, enzymatic conjugation with glucuronic acid and sulfate, MRP2-mediated export of the conjugates, followed by enzymatic hydrolysis by β-glucuronidase and sulfatase back to chrysin in the intestinal lumen and fecal excretion, as previously described in humans *in vivo* and *in vitro*[15,18] (see also Figure 3).

The oral bioavailability, based on tissue measurements, was also determined for 5,7-DMF and chrysin in a small fish model, the Atlantic killifish.[43] The fishes were exposed to the flavones in the water at a concentration of 5 μM, a low human dietary flavonoid concentration, for 8 h prior to sacrifice. The 5,7-DMF concentrations greatly exceeded those of chrysin in all tissues, most notably in the brain, where they were 150-fold higher. The findings are similar to those in the rat study. It should be noted that the killifish, being a saltwater species, ingests foreign chemicals by swallowing water, i.e., similar to oral administration in mammals.

Microsomal Oxidation of the Methoxyflavones. As can be noted from the rat study, even though the methylated flavones are metabolically much more stable than the unmethylated analogues, they will be metabolized *in vivo*, in particular in the rat, which is well-known to have high oxidative capacity. Using human liver microsomes, the intrinsic oxidative clearance of 5,7-DMF was 13 mL min^{-1} kg^{-1},[44] which is similar to the oxidative clearance of many common drugs.[45]

In continuing studies with human liver microsomes, the oxidative metabolism of a number of methoxyflavones was

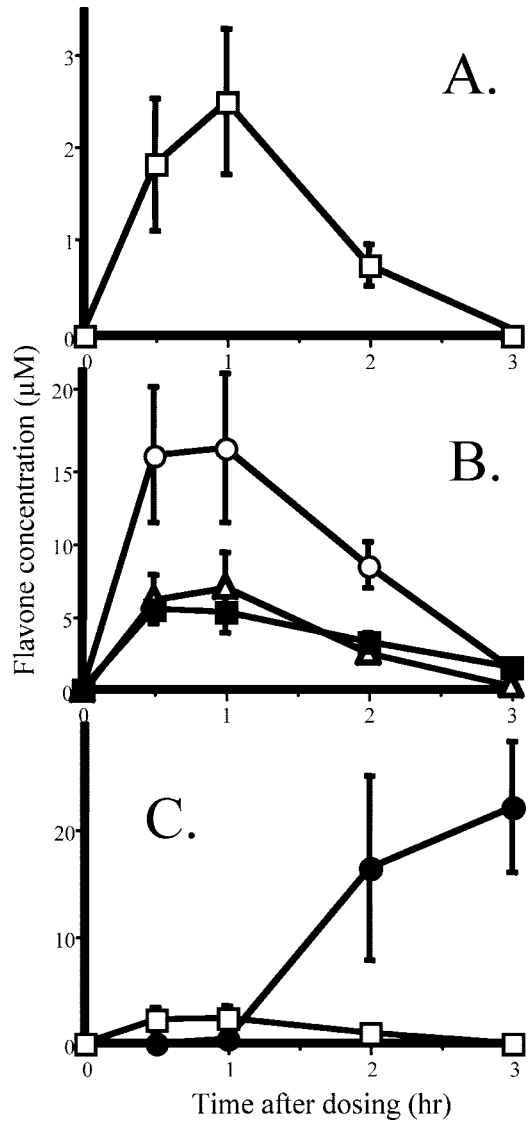

Figure 6. Plasma and tissue levels of 5,7-DMF and chrysin after oral administration of 5 mg/kg in rats: (A) plasma 5,7-DMF (no chrysin could be detected); (B) tissue 5,7-DMF in liver (○), lung (■) and kidney (Δ) (no chrysin could be detected); (C) 5,7-DMF (□) and chrysin (●) in colon with associated fecal pellet (mean ± SEM of five animals at each time-point).[42]

compared with that of 5,7-DMF.[46] The metabolically most stable compound was 5,7-DMF followed closely by 5-methoxyflavone, whereas 7-methoxyflavone was much more susceptible to oxidation. It also shows the rather rapid oxidation of the well-known polymethoxylated citrus flavonoid tangeretin (5,6,7,8,4′-pentamethoxyflavone). In gen-

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eral, there appears to be a wide range of stabilities among the methoxyflavones investigated so far.

Biological Effects of Methoxyflavones. Methylation of the flavonoids results in methyl ethers, which cannot be considered prodrugs, as their *O*-demethylation, as observed, is a slow process, in contrast to conjugation reactions. A critically important question is therefore if methylation of the flavonoids results in loss of beneficial biological properties. It has long been assumed that the hydroxyl groups are important for biological actions, as they are for the antioxidant properties.[47] However, more recent studies have clearly demonstrated that the antioxidant properties of flavonoids are much less important than their effects as direct modulators of protein and lipid kinase signaling.[48]

I. Effects on Carcinogen Bioactivating Enzymes. Both chrysin and apigenin have clearly been shown to be activators of CYP1A1 as measured by the ethoxyresorufin deethylation (EROD) assay.[49] In the same study, the methylated form of chrysin, i.e., 5,7-DMF, was a very potent inhibitor not only of benzo[*a*]pyrene-induced CYP1A1 but also of basal CYP1A1 activity. This resulted in potent inhibition of benzo[*a*]pyrene-DNA covalent binding. Even such a low concentration as 2 μM 5,7-DMF was able to inhibit the covalent binding significantly. This should be compared to the 18 μM reached in the liver after an oral dose of 5,7-DMF (cf. Figure 6). Several studies report on the down-regulation of CYP1A1 protein expression by 5,7-DMF not only in the liver,[49] but also in oral[50] and lung[51] cells. 5,7-DMF is also a potent direct inhibitor of CYP1A1[49] as well as CYP1B1[50] protein.

In combination with high bioavailability, 5,7-DMF and other methoxyflavones are therefore predicted to be able to effectively inhibit carcinogen-bioactivating enzymes in the liver, lungs as well as other sites. This should now be tested directly *in vivo*.

II. Effects on Cancer Cell Proliferation and Aromatase Activity. Inhibition of cell proliferation by the flavones was tested in the human oral squamous carcinoma cell line SCC-9,[52] using the BrdU incorporation assay. Both

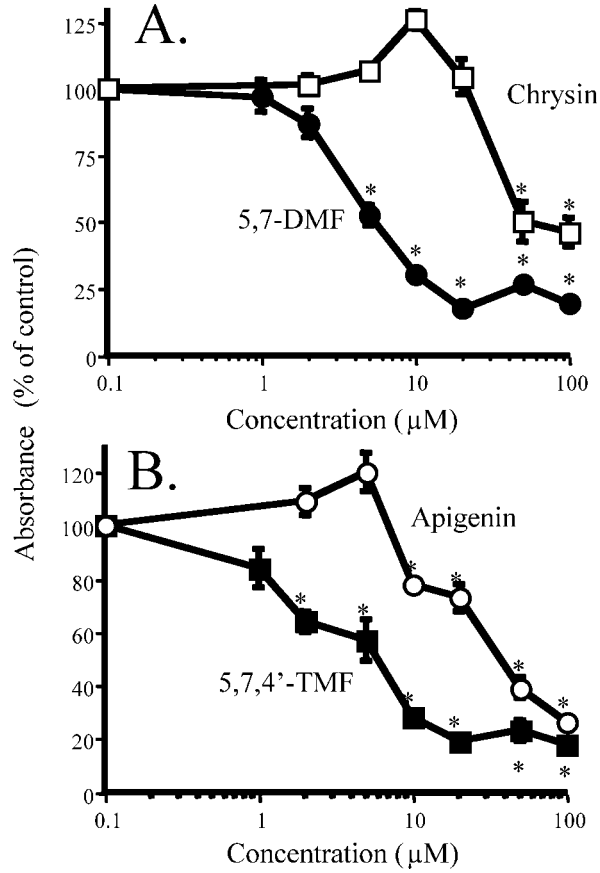

Figure 7. Effect of the methylated flavones (closed symbols) 5,7-DMF (A) and 5,7,4′-TMF (B) compared to their unmethylated analogues (open symbols) chrysin (A) and apigenin (B) on SCC-9 cell proliferation (24 h exposure), as measured by BrdU incorporation into cellular DNA.[42]

5,7-DMF and 5,7,4′-TMF were 8–10 times more potent than the unmethylated analogues chrysin and apigenin, with IC_{50} values as low as 5–8 μM (Figure 7).[42] Not only was this highly unexpected in a comparative sense, but the high tissue distribution in our rat study (cf. Figure 6) suggests that these methylated flavones may have activity also *in vivo*. The higher potency of the methylated flavones could be due to higher cell accumulation of these compounds compared to the unmethylated flavones. However, when SCC-9 cells were exposed to 25 μM chrysin or 5,7-DMF for up to 24 h, the cell content was virtually identical.[42] In addition, exactly the same potencies were observed for effects of these compounds on the cell cycle.[42] Thus, methylation does not only greatly improve oral bioavailability and tissue uptake of flavones, but also their intrinsic cancer antiproliferative activity. The latter point should be studied much more thoroughly.

As discussed in the Introduction, previous studies had shown chrysin to inhibit aromatase activity with a K_i value of 2.6 μM,[12] however, without the ability to reach such concentrations *in vivo* due to the very low oral bioavailability.[15] Methylation of chrysin to 5,7-DMF essentially abolished this effect, apparently due to steric hindrance. However,

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two other metabolically stable flavones, 7-methoxyflavone and 7,4′-dimethoxyflavone, were potent inhibitors *in vitro*[53] and may have the ability to inhibit aromatase activity *in vivo*. This is another example of flavones where methylation maintains intrinsic biological activity. As with the antiproliferative activity, the aromatase inhibiting effect should now be examined *in vivo* together with the pharmacokinetic properties.

Where Can the Methoxyflavones Be Found?

Although the methylated flavones investigated were synthetic compounds, some of them have been identified in plants. 5,7,4′-TMF is a citrus flavonoid,[54] which also is present in other plants used in folk medicine.[55,56] 7,4′-DMF has been identified in nutmeg species[57,58] as well as in propolis[59] and 5,7-DMF is highly abundant in pepper vine leaves.[60,61] The presence of some of these methylated flavones in citrus fruits is of interest in regard to a very recent epidemiological study demonstrating citrus fruits and juices to be protective against oral premalignant lesions.[62]

Acknowledgment. These studies were supported by the National Institutes of Health Grant No. GM55561. The assistance from Kristina Walle in carrying out ongoing experiments and editing the manuscript is greatly appreciated.

MP700071D

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