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SYNTHESIS AND BIOLOGICAL EVALUATION OF 6′,7′-DIHYDROXYBERGAMOTTIN (6,7-DHB), A NATURALLY OCCURRING INHIBITOR OF CYTOCHROME P450 3A4

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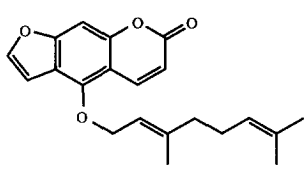
Abstract: The recently isolated inhibitor of cytochrome P450 CYP3A4, 6′,7′-dihydroxybergamottin (6,7-DHB), was synthesized by an efficient route that is readily adaptable to the production of analogues. The compound was evaluated as an inhibitor of 3A4 in a purified enzyme preparation, as well as against human liver microsomes and human 3A4 expressed in *Escherichia coli* membrane. In each case, 6,7-DHB proved to be a potent NADPH- and time dependent inactivator of 3A4. © 1997 Elsevier Science Ltd.

We[1,2] and others[3,4] have recently shown that co-administration of grapefruit juice can reduce the observed pre-systemic metabolism of a number of drugs by inhibition of CYP3A4, the major cytochrome P450 drug metabolizing enzyme in the gut.[5] A number of psoralens, coumarins, and flavonoids found in grapefruit have been hypothesized to produce this inhibition. We recently reported the isolation and identification of 6′,7′-dihydroxybergamottin (6,7-DHB, **2**), a derivative of the previously described furanocoumarin bergamottin (**1**), from a methylene chloride extract of grapefruit juice.[6] Although 6,7-DHB had previously been identified as a



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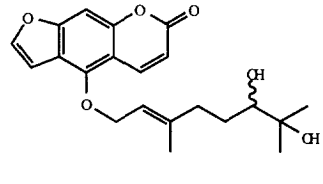

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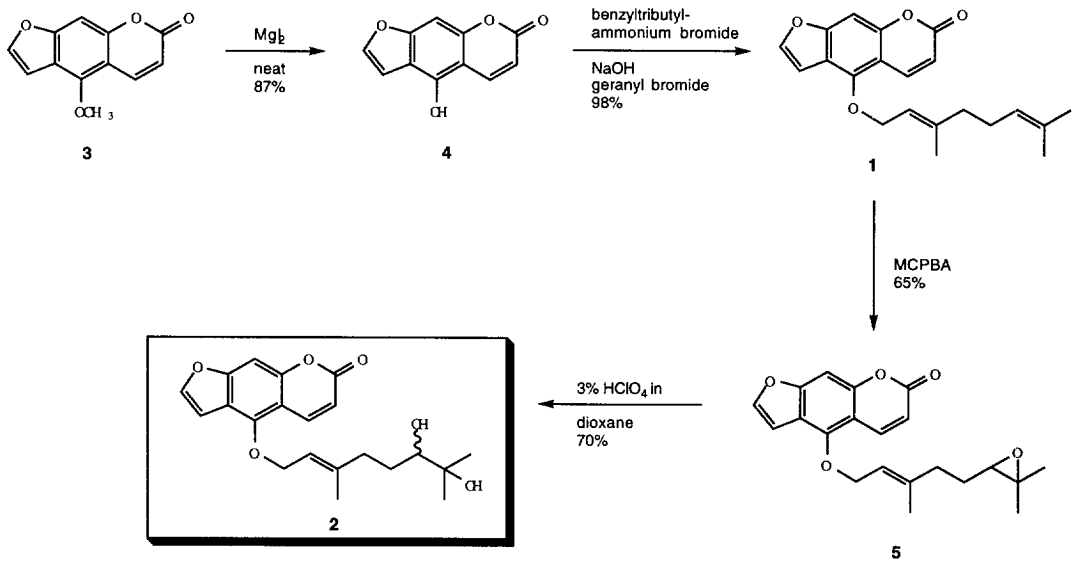
component of grapefruit (but not orange) juice,[7,8] our data was the first to suggest that it acts as a potent inhibitor of CYP3A4.[6] 6,7-DHB and its analogues could theoretically find therapeutic use as agents to increase the oral bioavailability of certain drugs, and/or to prevent the activation of carcinogens, by inhibiting CYP3A4-mediated gut metabolism in vivo. For this reason, we sought to develop an efficient synthesis of 6,7-DHB that could be readily adapted to the production of rationally designed analogues. A synthesis leading to **1** has been reported,[9] (10% overall yield for two steps), and a preparation of **2** from **1** has also appeared in the literature.[8] However, the extremely low yield resulting from these syntheses is not satisfactory for the efficient production of either **1** or **2**.

The synthetic route used by our group to afford 6,7-DHB is outlined in Scheme 1. The commercially available furanocoumarin bergapten (**3**) was demethylated using a previously published procedure,[10] producing the corresponding phenolic derivative bergaptol, **4**. Geranylation of **4** under phase transfer conditions[11] (geranyl bromide, benzyl tributylammonium bromide, NaOH) then afforded bergamottin **1** in 98% yield. Subsequent treatment of **1** with *m*-chloroperbenzoic acid then resulted in selective epoxidation of the terminal double bond

Scheme 1



of the geranyl sidechain, affording bergamottin-6,7-epoxide **5** in 65% yield.[8] Opening of the epoxide (3% perchloric acid in dioxane)[8] then produced the desired 6,7-DHB **2** in 70% yield.

Preincubation of 6,7-DHB with NADPH and human lymphoblast-expressed CYP3A4. 6,7-DHB (5 μM) was pre-incubated with 4.0 mg/mL of human lymphoblast-expressed CYP3A4 (Gentest Corporation, Woburn, MA), 30 mM $MgCl_2$, and either phosphate buffer (50 mM) or an NADPH regenerating system (G6PD 1 u/mL, G6P 5 mM and $NADP^+$ 1 mM). After a 10 min pre-incubation period, 50 μL of this solution was added to fresh tubes containing testosterone (100 μM), phosphate buffer, $MgCl_2$, and the same NADPH regenerating system in a total volume of 0.5 mL. Samples were incubated for 15 min, and the formation of 6-β-testosterone was measured by HPLC as previously described.[6] During the 15 min incubation, further inactivation of P450 3A4 was neglible, since 6,7-DHB was significantly diluted, and the concentration of testosterone was relatively high.

Coexpression of CYP3A4 and reductase in *E. coli*. The plasmid pB216 containing human CYP3A4 cDNA and human P450 reductase cDNA was transformed into JM 109 cells.[12] The growth of the transformed *E. coli* was carried out in the modified Terrific Broth and the expression of CYP3A4 and reductase was induced by

addition of 1 mM IPTG. δ-Aminolevulinic acid (0.5 mM) was added for heme synthesis. The membrane fraction was prepared from the bacterial cells by sonication after treatment with lysozyme. It was subsequently isolated by differential centrifugation from the bacterial cell homogenate.[13]

Inactivation of CYP3A4 in human liver microsomes and in *E. coli* membranes. The human liver microsomes or the *E. coli* membrane fractions containing 1 nmol of CYP3A4 were incubated with various concentrations of 6′,7′-dihydroxybergamottin (DHB) in 1 mL of 50 mM Hepes buffer (pH 7.5) containing 2 mM GSH, 1000 U catalase, 0.5 mM EDTA , 30 mM $MgCl_2$ and 20% glycerol at 37 °C for various time periods. At end of the incubation, 100 μL of the incubation mixture was taken for determination of testosterone 6β-hydroxylation activity. The other aliquot was used for P450 determination by the method of Omura and Sato.[14] The human liver microsomes were prepared by differential centrifugation.

Determination of testosterone 6β-hydroxylation. The human liver microsomes or the CYP3A4 and reductase containing *E. coli* membrane fractions (100 μmol of P450) were incubated with 200 μM testosterone in 1 mL of 50 mM Hepes buffer (pH 7.5). The reactions were initiated by the addition of 1 mM NADPH and terminated by 1 ml of ethyl acetate in ice. The 6β-hydoxytestosterone product was determined by HPLC on a C18 column (Microsorb-MV, 5 μm, 4.6 × 15 cm) eluted with a mobile phase of 65% of methanol at flow rate of 1 mL/min and the eluate was monitored by UV detection at 254 nm.

Results and Discussion. 6,7-DHB has been implicated as the primary compound in grapefruit juice that is responsible for inhibition of testosterone 6-β-hydroxylase activity in liver microsomes from dexamethasone-induced rats.[6] It has been suggested that P450 inhibition by 6,7-DHB in grapefruit juice contributes to the enhancement of plasma concentrations of some drugs metabolized by CYP3A4 (e.g., felodipine, nifedipine, midazolam, terfenadine and cyclosporine). However, it has not been shown previously that 6,7-DHB inhibits

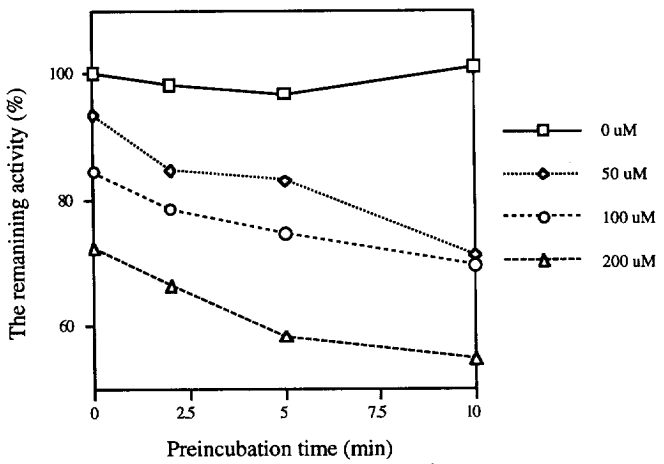


Figure 1. Time and concentration dependent inhibition of testosterone 6β-hydroxylase activity in human liver microsomes by 6, 7-DHB. Incubation and enzyme activity assays were performed as described in Materials and Methods. Each data point is the average of two determinations which in no case differed by more than 10%.

human CYP3A4, and if it does, by what mechanism. In the present study, 6,7-DHB reduced the activity of human lymphoblast-expressed CYP3A4 to 54.4% of control values when pre-incubated with the enzyme in the absence of NADPH. Activity was further inhibited to 34.1% of control when the preincubation was conducted in the presence of NADPH. CYP3A4 activity was significantly inhibited by 6,7-DHB in either human liver microsomes (66.7%) or *E. coli* membranes containing the expressed human CYP3A4 and reductase (94.5%) following preincubation with NADPH. The inhibition of 6β-hydroxytestosterone formation activity in human liver microsomes was time and concentration dependent, and required catalytic turnover of 6,7-DHB (Figure 1), suggesting that the observed inhibition resulted from mechanism based-inactivation of CYP3A4.[15] This inactivation appeared to be due to a reaction at the active site, since it was not inhibited by 2 mM GSH in the incubation system. Additional kinetic studies involving 6,7-DHB revealed K_i and k_{inact} values of 59 μM and 0.61 min^{-1}, respectively, when measured in an assay system containing purified recombinant human P450 3A4.[16] Reversible inhibition of catalytic activity was also observed for samples run without preincubation with NADPH. Testosterone 6β-hydroxylase activity was inhibited 31.5% when 6,7-DHB was incubated with human liver microsomes at a final concentration of 20 μM (Figure 1), and 45.3% in the expressed CYP3A4/reductase system under similar conditions (Table 1).

Table 1. The 6′,7′-dihydroxybergamottin (DHB) mediated inactivation of CYP3A4.*

	Testosterone 6-β hydroxylation (nmol/nmol P450/min)	P450 content (nmol/mL)
Human liver microsomes		
+NADPH/-DHB	0.54	0.94
-NADPH/+DHB	0.37	0.93
+NAHPH/+DHB	0.18	0.87
Coexpressed CYP3A4 with reductase in *E. coli* membrane		
+NADPH/-DHB	8.13	1.20
-NADPH/+DHB	4.45	1.16
+NADPH/+DHB	0.45	1.20

*Human liver microsomes or *E. coli* membrane containing CYP3A4 coexpressed with reductase (1 nmol/mL) were incubated with 400 μM DHB for 15 min at 37 °C in 50 mM Hepes buffer (pH 7.5) containing 30 mM $MgCl_2$, 2 mM GSH, 0.5 mM EDTA, 1000 u/mL of catalase and 20% glycerol. Each data point is the average of two determinations which in no case differed by more than 10%.

Despite the loss of 70% of CYP3A4 catalytic activity by preincubation with 6,7-DHB and NADPH, human liver microsomes still retained about 90% of the P450 as measured by the reduced-CO spectrum (Table 1). Similar results were obtained in the *E. coli* expressed CYP3A4 system, where catalytic activity was reduced by 94.5% after 15 minute preincubation with DHB and NADPH with no loss of spectrally detectable P450 (Table 1). Previous studies[17,18] involving inactivation by another furanocumarin, 8-methoxypsoralen, have also shown that there was no heme fragment, metabolic intermediate complex or heme adduct formation in in vivo studies (8-

methoxypsoralen treated rats) or in in vitro studies (8-methoxypsoralen incubated with rat liver microsomes). However, 8-methoxypsoralen was extensively bound to microsomal apoP450, and the binding was only partially diminished by cysteine. Another related study involved inactivation of P450 1A1 and 1A2 by coriandrin, a linear furoisocoumarin. In this case, covalent binding of coriandrin to apoP450 was demonstrated without significant heme destruction.[19] Therefore, 6,7-DHB-mediated inactivation of CYP3A4 appears to be primarily a result of modification of the apoprotein, as has been observed with 2-or 9-ethynylphenanthrene for the mechanism based inactivation of P450 2B1 and 2B4.[20,21] Determination of the precise mechanism of inactivation of CYP3A4 by 6,7-DHB, as well as the synthesis and evaluation of analogues of 6,7-DHB, are ongoing concerns in our laboratories.

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12. ^{1}H NMR spectral data for compound **1**: δ 8.16 (d, 1H, H4), 7.59 (d,1H, H7), 7.16 (s, 1H, H8), 6.96 (d, 1H, H6), 6.27 (d, 1H, H3), 5.54 (t, 1H, vinyl H2′), 5.06 (t, 1H, vinyl H6′), 4.96 (d, 2H, H1′), t, 4H, H4 and H5), 1.69 (s, 3H, methyl at C3′), 1.60 (s, 3H, terminal CH_3), 1.57 (s, 3H, terminal CH_3). ^{1}H NMR spectral data for compound **2**: δ 8.15 (d, 1H, H4), 7.59 (d,1H, H7), 7.15 (s, 1H, H8), 6.95 (d, 1H, H6), 6.27 (d, 1H, H3), 5.60 (t, 1H, vinyl H2′), 4.95 (d, 2H, H1′), 3.32 (dd, 1H, H6′), 2.34 and 2.14 (m, 1H each, H4′), 1.71 (s, 3H, methyl at C3′), 1.57 and 1.46 (m, 1H each, H5′), 1.20 (s, 3H, terminal CH_3), 1.17 (s, 3H, terminal CH_3).

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