Comparison of the Substrate Specificities of Human Liver Cytochrome P450s 2C9 and 2C18: Application to the Design of a Specific Substrate of CYP 2C18

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ABSTRACT: A series of 2-aroylthiophenes derived from tienilic acid by replacement of its OCH_2COOH substituent with groups bearing various functions have been synthesized and studied as possible substrates of recombinant human liver cytochrome P450s 2C9 and 2C18 expressed in yeast. Whereas only compounds bearing a negative charge acted as substrates of CYP 2C9 and were hydroxylated at position 5 of their thiophene ring at a significant rate, many neutral 2-aroylthiophenes were 5-hydroxylated by CYP 2C18 with k_{cat} values of >2 min^{-1}. Among the various compounds that were studied, those bearing an alcohol function were the best CYP 2C18 substrates. One of them, compound **3**, which bears a terminal $O(CH_2)_3$-OH function, appeared to be a particularly good substrate of CYP 2C18. It was regioselectively hydroxylated by CYP 2C18 at position 5 of its thiophene ring with a K_M value of 9 ± 1 μM and a k_{cat} value of 125 ± 25 min^{-1}, which are the highest described so far for a CYP 2C. A comparison of the oxidations of **3**, by yeast-expressed CYP 1A1, 1A2, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 3A4, and 3A5, showed that only CYP 2C8, 2C18, and 2C19 were able to catalyze the 5-hydroxylation of **3**. However, the catalytic efficiency of CYP 2C18 for that reaction was considerably higher (k_{cat}/K_M value being 3−4 orders of magnitude larger than those found for CYP 2C8 and 2C19). Several human P450s exhibited small activities for the oxidative O-dealkylation of **3**. The four recombinant CYP 2Cs were the best catalysts for that reaction (k_{cat} between 1 and 5 min^{-1}) when compared to all the P450s that were tested, even though it is a minor reaction in the case of CYP 2C18. All these results show that compound **3** is a new, selective, and highly efficient substrate for CYP 2C18 that should be useful for the study of this P450 in various organs and tissues. They also suggest some key differences between the active sites of CYP 2C9 and CYP 2C18 for substrate recognition.

Cytochrome P450s play a key role in the metabolism of exogenous compounds such as drugs. Their broad substrate specificity is now well-understood in terms of enzyme specificity (*1*). Cytochrome P450s of the 3A and 2C subfamilies are the major isozymes in human liver (*2*, *3*). To predict which P450 will be involved in the metabolism of a new biologically active molecule and to understand the structural basis that governs the specificity of a given P450 for a particular drug, it is important to determine the structure of the substrate binding sites of the main human liver P450s. In the absence of crystal structures for such membrane-bound mammalian P450s, the use of a series of indirect methods, combining biochemical results, spectroscopic studies, and theoretical models, is required. This has been successfully achieved for CYP 2D6 (see, for instance, ref *4* and references therein). In the case of CYP 2C9, a model for the interaction of this cytochrome with its substrates has been proposed on the basis of biochemical and UV−visible and ^{1}H NMR (*5*) results, as well as molecular modeling (*5*, *6*). In that model, CYP 2C9 substrates, which most often are anionic at pH 7.4, would interact through their anionic site with a cationic residue of the protein (*5*−*7*). This model takes into account the major interactions that seem to explain the highly specific inhibitory effects of sulfaphenazole toward CYP 2C9 (*7*). Other models derived from molecular modeling have proposed similar "pharmacophores" for CYP 2C9 substrates; however, these models involve a hydrogen bond between a polar residue of substrates with a CYP 2C9 amino acid instead of an ionic interaction (*8*−*10*).

Genetic analysis of the CYP 2C subfamily shows the presence of at least seven genes with several allelic variants. CYP 2C8, 2C9, and 2C19 are the isozymes of the 2C subfamily which are expressed at the highest level in human liver (*2*, *3*, *11*, *12*). The fourth member of the CYP 2C subfamily, CYP 2C18, appears to be expressed at a very low level in human liver (*13*, *14*). However, CYP 2C18 seems to be a major P450 2C in the skin and in the lung as judged from its mRNA levels (*15*, *16*).

Whereas specific substrates and inhibitors are known for CYP 2C8, 2C9, and 2C19, and the nature of substrate active sites of CYP 2C9 and 2C19 begins to be understood thanks to the aforementioned studies (*5*−*10*) and to recent results using CYP 2C9−2C19 chimeric proteins and mutants (*17*, *18*), almost nothing is known about the substrate active site of CYP 2C18. Moreover, to date, no reaction has been shown to be specifically catalyzed by this enzyme (*2*).

This paper reports the results of a comparative study of the substrate specificities of recombinant CYP 2C9 and CYP

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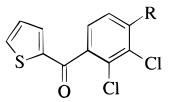


R =	
OCH_2COOH	Tienilic Acid
OH	**1**
OCH_3	**2**
$O(CH_2)_3OH$	**3**
$O(CH_2)_3CN$	**4**
$O(CH_2)_3CH_3$	**5**
$O(CH_2)_2OH$	**6**
$O(CH_2)_4OH$	**7**
$OCH_2CHOHCH_2OH$	**8**
$OCH_2CHOHCH_3$	**9**

FIGURE 1: Structure of 2-aroylthiophenes synthesized and used in this study.

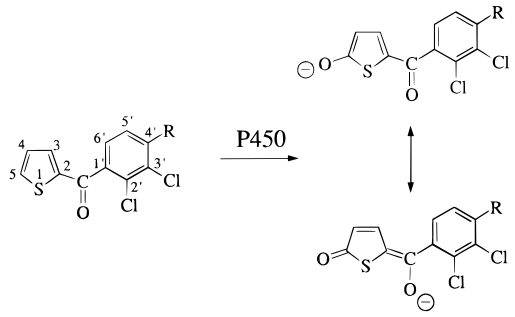


FIGURE 2: Major forms in which the 5-hydroxy metabolites of 2-aroylthiophenes exist at physiological pH. Labeling was used for ^{1}H NMR results.

2C18, which has been performed on a series of compounds derived from tienilic acid that have been synthesized for that purpose. These results provide a first idea of the different structural determinants that are involved in substrate recognition by CYP 2C9 and CYP 2C18. Moreover, they lead to the first substrate that is specifically hydroxylated by CYP 2C18 with a very high catalytic efficiency.

MATERIALS AND METHODS

Chemicals

All the chemicals that were used were of the highest quality commercially available. Tienilic acid and 2-(2,3-dichloro-4-methoxy)benzoylthiophene were provided by Anphar-Rolland (Chilly-Mazarin, France). 2-Parafluorobenzoylthiophene was purchased from Janssen.

Physical Measurements

UV−visible spectra were recorded on a Kontron Uvikon 820 spectrophotometer equipped with a diffusion sphere. ^{1}H NMR spectra were recorded at 27 °C on a Bruker ARX-250 instrument; chemical shifts are reported downfield from $(CH_3)_4Si$, and *J* values are in hertz. Labeling of the carbons of 2-aroylthiophenes corresponding to the following H assignments is given in Figure 2. Abbreviations used for peak description are s, d, t, m, bs, and dd for singlet, doublet, triplet, massif, broad singlet, and doublet of doublets, respectively. Mass spectroscopy (MS) was performed with electronic ionization (EI) on a Nermag R1010 apparatus. For all compounds related to tienilic acid that contain two chlorine atoms, all peaks corresponding to molecular ions or fragments involving two Cl atoms exhibited the isotope cluster shape expected for the presence of two Cl atoms (with a M:M + 2:M + 4 ratio of 10:6:1); *m/z* values described below are for ^{35}Cl. Elemental analyses were carried out at Centre Regional de Microanalyse (Paris).

Synthesis of Tienilic Acid Derivatives

2-Parachlorobenzoylthiophene, 2,3-dichloro-4-(2-thenoyl)-phenol (**1**), and 2-[2,3-dichloro-4-(2-thenoyl)phenoxy]ethanol (**6**) were prepared by previously described procedures (*6*, *19*). Their ^{1}H NMR spectra were in complete agreement with those reported previously (*6*).

*1-[2,3-Dichloro-4-(2-thenoyl)phenoxy]propan-2-ol (**9**).* Chloroacetone (45 μL, 0.53 mmol) was added to a solution of 2,3-dichloro-4-(2-thenoyl)phenol **1** (137 mg, 0.5 mmol) in anhydrous DMF (1 mL) containing 0.57 mmol of Na_2CO_3. After 3 h at 80 °C, DMF was evaporated and the residue dissolved in CH_2Cl_2. The organic phase was washed with water, neutralized with 1 N HCl, and dried over $MgSO_4$. [2,3-Dichloro-4-(2-thenoyl)phenoxy]acetone was purified by column chromatography (SiO_2, 98:2 CH_2Cl_2/acetone) and obtained as white crystals in a 70% yield: mp 85−86 °C; ^{1}H NMR ($CDCl_3$) δ 2.37 (s, 3H, CH_3CO), 4.64 (s, 2H, CH_2-CO), 6.76 (d, 1H, $H_{6'}$, $J = 8.4$), 7.11 (dd, 1H, H_4, $J_{43} = 3.6$, $J_{45} = 4.8$), 7.32 (d, 1H, $H_{5'}$, $J = 8.4$), 7.41 (d, 1H, H_3, $J_{34} = 3.6$), 7.75 (d, 1H, H_5, $J_{54} = 4.8$). Anal. Calcd for $C_{14}H_{10}Cl_2O_3S$: C, 51.08; H, 3.06. Found: C, 51.03; H, 3.11.

After being stirred for 3 min, a mixture of 60 mg of $NaBH_4$ in 15 mL of 1:1 MeOH/CH_2Cl_2 was cooled to −78 °C (dry ice/acetone), and a solution of [2,3-dichloro-4-(2-thenoyl)-phenoxy]acetone (60 mg, 0.18 mmol) in CH_2Cl_2 (1.5 mL) was added. After the mixture had been stirred for 10 min, the reaction was quenched (with 1.5 mL of acetone) and the mixture allowed to warm to room temperature. The solution was washed with 1 N NaOH, extracted with CH_2Cl_2, dried over $MgSO_4$, and concentrated. Compound **9** was purified by column chromatography (SiO_2, 98:2 CH_2Cl_2/acetone) and obtained in a 100% yield. Crystallization from diethyl ether gave white crystals: mp 105−106 °C; ^{1}H NMR ($CDCl_3$) δ 1.33 (d, 3H, CH_3, $J = 6.4$), 2.36 (d, 1H, OH, $J = 4$), 3.91 (m, 1H, CH_2, $J = 9$ and 7.4), 4.07 (m, 1H, CH_2, $J = 9$ and 2.9), 4.28 (m, 1H, C**H**OH), 6.90 (d, 1H, $H_{6'}$, $J = 8.4$), 7.11 (dd, 1H, H_4, $J_{43} = 3.8$, $J_{45} = 4.8$), 7.33 (d, 1H, $H_{5'}$, $J = 8.4$), 7.41 (dd, 1H, H_3, $J_{34} = 3.8$, $J_{35} = 1.2$), 7.74 (dd, 1H, H_5, $J_{54} = 4.8$, $J_{53} = 1.2$); MS (EI) *m/z* (relative intensity) 330 (M^+, 50%), 272 (65%), 189 (43%), 111 (100%). Anal. Calcd for $C_{14}H_{12}Cl_2O_3S$: C, 50.77; H, 3.65. Found: C, 50.82; H, 3.60.

*1-[2,3-Dichloro-4-(2-thenoyl)phenoxy]propane-2,3-diol (**8**).* A solution of phenol **1** (160 mg, 0.59 mmol) in water (0.8 mL) containing sodium hydroxide (24 mg, 0.6 mmol) was added to 250 μL of epibromohydrine and the mixture stirred vigorously for 43 h at room temperature. The product was extracted with CH_2Cl_2 and dried over $MgSO_4$. After removal of the solvent, 1-[2,3-dichloro-4-(2-thenoyl)phenoxy]-2,3-epoxypropane was purified by column chromatography (SiO_2, CH_2Cl_2 as the eluent) and obtained as white crystals in 60% yield: mp 115−116 °C; ^{1}H NMR ($CDCl_3$) δ 2.88 (m, 1H, OCH_2, $J = 4.8$ and 2.8), 2.98 (m, 1H, OCH_2, $J = 4.8$ and 4), 3.41 (m, 1H, C**H**O), 4.10 (1H, C**H**$_2$OAr, $J = 11.2$ and 5.6), 4.40 (1H, C**H**$_2$OAr, $J = 11.2$ and 2.8), 6.95 (d, 1H, $H_{6'}$, $J = 8.4$), 7.10 (dd, 1H, H_4, $J_{43} = 3.8$, $J_{45} = 4.8$), 7.32 (d, 1H, $H_{5'}$, $J = 8.4$), 7.40 (dd, 1H, H_3, $J_{34} = 3.8$, $J_{35} = 1.2$), 7.74 (dd, 1H, H_5, $J_{54} = 4.8$, $J_{53} = 1.2$). Anal. Calcd for $C_{14}H_{10}Cl_2O_3S$: C, 51.08; H, 3.06. Found: C, 50.97; H, 3.05.

1-[2,3-Dichloro-4-(2-thenoyl)phenoxy]-2,3-epoxypropane (46 mg, 0.14 mmol) was dissolved in dioxane (2 mL), and NaOH (2.3 mL, 0.3 N) was added to the solution and the mixture heated at 70−75 °C for 54 h. The solution was diluted with H_2O, extracted with CH_2Cl_2, washed with H_2O, and dried over $MgSO_4$. After recrystallization from CH_2Cl_2, compound **8** was obtained as white crystals in 53% yield: mp 121−122 °C; ^{1}H NMR ($CDCl_3$) δ 2.02 (t, 1H, OH), 2.67 (bs, 1H, OH), 3.88 (m, 2H, CH_2OH), 4.18 (m, 3H, OC**H**$_2$C**H**OH), 6.93 (d, 1H, $H_{6'}$, $J = 8.5$), 7.11 (dd, 1H, H_4, $J_{43} = 3.6$, $J_{45} = 4.8$), 7.34 (d, 1H, $H_{5'}$, $J = 8.5$), 7.40 (dd, 1H, H_3, $J_{34} = 3.6$, $J_{35} = 1.2$), 7.74 (dd, 1H, H_5, $J_{54} = 4.8$, $J_{53} = 1.2$); MS (EI) *m/z* (relative intensity) 346 (M^+, 38%), 272 (72%), 189 (35%), 111 (100%). Anal. Calcd for $C_{14}H_{12}Cl_2O_4S$: C, 48.43; H, 3.48. Found: C, 48.53; H, 3.50.

*3-[2,3-Dichloro-4-(2-thenoyl)phenoxy]propan-1-ol (**3**).* One milliliter of 3-bromopropan-1-ol (11 mmol) was added to a solution of phenol **1** (1.365 g, 5 mmol) in anhydrous DMF (10 mL) containing 540 mg of Na_2CO_3. After 3 h at 80 °C, evaporation of DMF, and extraction with CH_2Cl_2, the residue was washed with water, extracted, and dried over $MgSO_4$. Purification by column chromatography (SiO_2, 1−5% CH_2Cl_2/ether) does not allow one to obtain purified compound **3**. The acetylated derivative of **3** was then prepared.

The unpurified compound was dissolved in 5 mL of $CHCl_3$ and cooled to 0 °C. Pyridine (0.5 mL, 6.2 mmol) and then 0.5 mL of acetic anhydride (5.3 mmol) were added to the solution. The mixture was allowed to warm to room temperature, and then diluted with CH_2Cl_2, washed with 0.1 M HCl, and dried over $MgSO_4$. Acetylated **3** was purified by column chromatography (SiO_2, CH_2Cl_2): mp 60 °C; ^{1}H NMR ($CDCl_3$) δ 2.05 (s, 3H, CH_3), 2.20 (m, 2H, CH_2), 4.17 (t, 2H, OCH_2), 4.32 (t, 2H, C**H**$_2$OCO), 6.90 (d, 1H, $H_{6'}$, $J = 8.5$), 7.10 (dd, 1H, H_4, $J_{43} = 3.9$, $J_{45} = 4.9$), 7.32 (d, 1H, $H_{5'}$, $J = 8.5$), 7.41 (dd, 1H, H_3, $J_{34} = 3.9$, $J_{35} = 1.2$), 7.73 (dd, 1H, H_5, $J_{54} = 4.9$, $J_{53} = 1.2$). Anal. Calcd for $C_{16}H_{14}Cl_2O_4S$: C, 51.49; H, 3.78. Found: C, 51.32; H, 3.71.

The acetylated compound was then hydrolyzed in a mixture of MeOH and H_2O (5 and 5 mL, respectively) containing 240 mg of NaOH and refluxed for 1 h. The solution was diluted with water and extracted with CH_2Cl_2. The organic phase was dried over $MgSO_4$ and evaporated. Compound **3** (1.25 g) was obtained in a 75% global yield: mp 90−91 °C; ^{1}H NMR ($CDCl_3$) δ 1.81 (t, 1H, OH, $J = 5.35$), 2.13 (m, 2H, CH_2, $J = 5.8$), 3.92 (m, 2H, C**H**$_2$OH, $J = 5.8$, $J = 5.35$), 4.26 (t, 2H, CH_2OAr, $J = 5.8$), 6.92 (d, 1H, $H_{6'}$, $J = 8.5$), 7.10 (dd, 1H, H_4, $J_{43} = 3.9$, $J_{45} = 4.8$), 7.33 (d, 1H, $H_{5'}$, $J = 8.5$), 7.41 (dd, 1H, H_3, $J_{34} = 3.9$, $J_{35} = 1.1$), 7.73 (d, 1H, H_5, $J_{54} = 4.8$, $J_{53} = 1.1$); MS (EI) *m/z* (relative intensity) 330 (M^+, 58%), 272 (60%), 189 (27%), 111 (100%). Anal. Calcd for $C_{14}H_{12}Cl_2O_3S$: C, 50.77; H, 3.65. Found: C, 50.74; H, 3.58.

*4-[2,3-Dichloro-4-(2-thenoyl)phenoxy]butan-1-ol (**7**).* 4-Chlorobutan-1-ol (0.2 mL, 2 mmol) was added to a solution of phenol **1** (100 mg, 0.37 mmol) in anhydrous DMF (1 mL) containing 40 mg of Na_2CO_3. The mixture was heated at 80 °C for 6 h. After evaporation of DMF, the residue was dissolved in CH_2Cl_2, washed with water, and dried over $MgSO_4$. After purification by column chromatography (SiO_2, 95:5 CH_2Cl_2/AcOEt) and recrystallization from ether/cyclohexane, 30 mg of compound **7** was obtained in 24% yield: mp 80−81 °C; ^{1}H NMR ($CDCl_3$) δ 1.45 (t, 1H, OH), 1,81 (m, 2H, CH_2), 1,97 (m, 2H, CH_2), 3.75 (m, 2H, C**H**$_2$OH), 4.14 (t, 2H, CH_2OAr), 6.89 (d, 1H, $H_{6'}$, $J = 8.6$), 7.10 (dd, 1H, H_4, $J_{43} = 3.8$, $J_{45} = 4.8$), 7.32 (d, 1H, $H_{5'}$, $J = 8.6$), 7.41 (d, 1H, H_3, $J_{34} = 3.8$), 7.73 (d, 1H, H_5, $J_{54} = 4.9$); MS (EI) *m/z* (relative intensity) 344 (M^+, 6%), 272 (68%), 189 (24%), 111 (100%). Anal. Calcd for $C_{15}H_{14}Cl_2O_3S$: C, 52.19; H, 4.09. Found: C, 52.09; H, 4.12.

*4-[2,3-Dichloro-4-(2-thenoyl)phenoxy]butyronitrile (**4**).* 4-Bromobutyronitrile (110 μL, 1.1 mmol) was added to a solution of phenol **1** (100 mg, 0.366 mmol) in anhydrous DMF (1 mL) containing 40 mg of Na_2CO_3. After 3 h at 80 °C, DMF was evaporated and the residue dissolved in CH_2Cl_2. The organic phase was washed with water and dried over $MgSO_4$. Compound **4** was purified by column chromatography (SiO_2, CH_2Cl_2). Crystallization from diethyl ether gave white crystals in 77% yield: mp 131−132 °C; ^{1}H NMR ($CDCl_3$) δ 2.23 (m, 2H, CH_2), 2.68 (t, 2H, CH_2CN), 4.21 (t, 2H, CH_2OAr), 6.90 (d, 1H, $H_{6'}$, $J = 8.5$), 7.11 (dd, 1H, H_4, $J_{43} = 4$, $J_{45} = 4.8$), 7.34 (d, 1H, $H_{5'}$, $J = 8.5$), 7.41 (dd, 1H, H_3, $J_{34} = 4$, $J_{35} = 1.2$), 7.74 (dd, 1H, H_5, $J_{54} = 4.8$, $J_{53} = 1.2$); MS (EI) *m/z* (relative intensity) 339 (M^+, 38%), 272 (15%), 189 (20%), 111 (100%). Anal. Calcd for $C_{15}H_{11}Cl_2NO_2S$: C, 52.95; H, 3.26; N, 4.12. Found: C, 52.67; H, 3.12; N, 4.05.

*[2,3-Dichloro-4-(2-thenoyl)phenyl]butyl Ether (**5**).* 4-Bromobutane (80 μL, 0.74 mmol) was added to a solution of phenol **1** (107 mg, 0.39 mmol) in anhydrous DMF (1 mL) containing 45 mg of Na_2CO_3. After 3.5 h at 80 °C, DMF was evaporated and the residue dissolved in CH_2Cl_2. The organic phase was washed with water and dried over $MgSO_4$. Purification of the residue by column chromatography (SiO_2, 1:1 CH_2Cl_2/cyclohexane) afforded 128 mg (quantitative yield) of **5** as white crystals: mp 48−49 °C; ^{1}H NMR ($CDCl_3$) δ 0.99 (t, 3H, CH_3), 1.55 (m, 2H, CH_2), 1.86 (m, 2H, CH_2), 4.09 (t, 2H, CH_2OAr), 6.88 (d, 1H, $H_{6'}$, $J = 8.5$), 7.10 (dd, 1H, H_4, $J_{43} = 4$, $J_{45} = 4.8$), 7.32 (d, 1H, $H_{5'}$, $J = 8.5$), 7.42 (d, 1H, H_3, $J_{34} = 4$), 7.73 (d, 1H, H_5, $J_{54} = 4.8$); MS (EI) *m/z* (relative intensity) 328 (M^+, 48%), 272 (77%), 189 (26%), 111 (100%). Anal. Calcd for $C_{15}H_{14}Cl_2O_2S$: C, 54.72; H, 4.29. Found: C, 54.55; H, 4.25.

Yeast Transformation, Cell Culture, and Preparation of the Yeast Microsomal Fraction

The expression system used for human cytochrome P450s was based on a yeast strain W(R) fur 1 previously described (*20*), in which yeast cytochrome P450 reductase was overexpressed. Transformation by a pYeDP60 vector containing one of the human liver CYP 1A1, 1A2, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 3A4, and 3A5 cDNAs (*21*−*24*) was then performed according to a general method of construction of yeast strain W(R) fur 1 expressing various human liver P450s (*25*, *26*). Yeast culture and microsome preparation were performed by using previously described techniques (*27*). Microsomes were homogenized in 50 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA and 20% glycerol (v/v), aliquoted, frozen under liquid N_2, and stored at −80 °C until they were used. P450 contents of yeast microsomes were 200, 100, 40, 90, 40, 20, 40, 10, 200, and 40 pmol of P450 per mg of protein for CYP 1A1, 1A2, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 3A4, and 3A5, respectively. Activities of CYP 1A1, 1A2, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 3A4, and 3A5

in yeast microsomes were 10 (ethoxyresorufin O-deethylation), 2 (ethoxyresorufin O-deethylation), 0.2, 1.9, and 1.8 (5-hydroxylation of tienilic acid), 2 (4′-hydroxylation of *S*-mephenytoin), 5 (demethylation of dextrometorphan), 4 (6-hydroxylation of chlorzoxazone), and 1.8 and 1.2 (6β-hydroxylation of testosterone) nmol (nmol of P450)$^{-1}$ min^{-1}, respectively.

Microsomal P450 content was determined according to the method of Omura and Sato (*28*). The protein content in microsomal suspensions was determined by the Lowry procedure (*29*) using bovine serum albumin as the standard.

Enzyme Activity Assay

Specific Activities of the Different P450s. Specific assays for CYP 1A1 and 1A2 [ethoxyresorufin O-deethylation (*30*)], 2C8, 2C9, and 2C18 [tienilic acid 5-hydroxylation (*31*)], 2C19 [*S*-mephenytoin 4′-hydroxylation (*32*)], 2D6 [dextrometorphan demethylation (*33*)], 2E1 [chlorzoxazone 6-hydroxylation (*34*)], and 3A4 and 3A5 [testosterone 6β-hydroxylation (*35*)] were carried out by using previously reported procedures.

Spectroscopic Measurement of 2-Aroylthiophene 5-Hydroxylation. Quantitation of 5-hydroxy-2-aroylthiophenes was based on a spectrophotometric method (*19*) adapted to yeast microsomes expressing CYP 2C9 in the case of tienilic acid (*36*).

Incubations for metabolic activity with yeast microsomes were carried out at 28 °C, using glass tubes in a shaking bath. The incubation mixtures contained the yeast microsomal suspension, providing 0.2 μM P450, the substrate (as indicated in experiments), and a NADPH-generating system (1 mM $NADP^+$, 10 mM glucose 6-phosphate, and 2 units of glucose-6-phosphate dehydrogenase/mL) diluted in 0.1 M Tris buffer containing 1 mM EDTA and 8% glycerol (final concentrations). Activity assays were routinely initiated (t_o = 0 min) by incorporation of the NADPH-generating system into the incubation mixture after 3 min of separate preincubation at 28 °C for temperature equilibration. At t_0 (0 min) and regularly after, aliquots (140 μL) were taken and the reaction was quickly stopped by treatment with 70 μL of a cold CH_3CN/CH_3COOH (10:1) mixture. These conditions apply, unless noted otherwise, to all of the experiments described below.

For all 2-aroylthiophenes that were found to be 5-hydroxylated by yeast-expressed CYP 2C, controls were carried out to show that no hydroxylation occurred in the absence of NADPH or with microsomes from control yeast (not expressing a human P450) in the presence of NADPH. The hydroxylation of each substrate by yeast-expressed CYP 2C was studied as a function of time and was shown to be linear for at least 15 min for all the compounds that were studied. Therefore, the following activities were measured after 10 min incubation periods.

HPLC Analysis of the Oxidation of Tienilic Acid Derivatives by Human P450-Expressed Yeast Microsomes

In these experiments, incubations were carried out under the general conditions described above. At t_0 (0 min) and regularly after, aliquots (200 μL) were taken and the reaction was quickly stopped by treatment with 100 μL of a cold CH_3CN/CH_3COOH (10:1) mixture. Following addition of 2-(2,3-dichloro-4-methoxy)benzoylthiophene (10 μM final concentration) as an internal standard and centrifugation (10 000 rpm, 5 min in an Eppendorf centrifuge), the clear supernatants were analyzed by HPLC. HPLC analysis was performed by an optimized modification of the previously described method (*37*) on a Hypersil MOS column (5 μM, 4.6 × 250 mm, Hypersil) with a gradient from A [0.1 M acetate (pH 4.6)] to B (7:2:1 $CH_3CN/CH_3OH/H_2O$): A/B (83:17) from 0 to 1 min, and then at 1 min a linear gradient to 80% B in 21 min, and then at 22 min another linear gradient to 100% B in 2 min (a flow rate of 1 mL/min). For instance, in that system, retention times for compound **3** and its metabolites 5-OH-**3**, 5-OH-**1**, and **1** were 21, 10, 9, and 20 min, respectively.

Isolation of the Major Products of Oxidation of Tienilic Acid Derivatives by Microsomes of Yeast Expressing CYP 2Cs

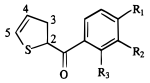
Incubations were carried out at 28 °C, using Erlenmeyer flasks in a shaking bath for a total volume of 50 mL. The incubation mixtures contained the yeast microsomal suspension, providing 0.1 μM P450, 200 μM substrate, and a NADPH-generating system (1 mM $NADP^+$, 10 mM glucose 6-phosphate, and 2 units of glucose-6-phosphate dehydrogenase/mL) diluted in 0.1 M Tris-HCl buffer containing 1 mM EDTA and 8% glycerol (final concentrations). After 20 min, the reaction was quickly stopped by addition of 1 mL of CH_3COOH. After centrifugation (15000*g* for 15 min), the clear supernatants were purified by solid phase extraction on Sep-Pak C18 columns. These columns were then washed with water and eluted with a 98:2 CH_3OH/NH_4OH mixture. After partial removal of the solvent, the collected fractions were purified by HPLC using the previously described gradient.

RESULTS

Synthesis of a Series of Compounds Related to Tienilic Acid as Potential Substrates for CYP 2C18. Tienilic acid (Figure 1, R = OCH_2COOH) is a good substrate of CYP 2C9 with K_M and k_{cat} values for the 5-hydroxylation of its thiophene ring of 6 μM and 2.4 min^{-1}, respectively (*6*). It has been shown that the presence of its carboxylate anion was essential for its recognition by CYP 2C9 (*6*). Recombinant CYP 2C18 is also able to catalyze the 5-hydroxylation of tienilic acid but with a much higher K_M value [150 μM (*31*)]. To determine the role of the para substituents of the aryl ring of 2-aroylthiophenes in substrate recognition by CYP 2C18 and 2C9, a series of compounds related to tienilic acid, in which the OCH_2COOH group was replaced by different substituents bearing various chemical functions, were synthesized. Their synthesis from the phenol precursor **1** (R = OH) is described in Materials and Methods. The structures of the new compounds were established from their ^{1}H NMR and mass spectra and elemental analysis (see Materials and Methods); they are shown in Figure 1.

Assay Used for Studying the P450 2C-Dependent 5-Hydroxylation of 2-Aroylthiophenes. It has been reported that many 2-aroylthiophenes are hydroxylated at position 5 of their thiophene ring by cytochrome P450s from liver microsomes. The corresponding 5-hydroxy-2-aroylthiophene metabolites are easily detected by UV−visible spectroscopy

Table 1: Comparison of the Abilities of Yeast-Expressed P450 2C9 and 2C18 To 5-Hydroxylate Various 2-Aroylthiophene Derivatives



compound	R_1	R_2	R_3	5-hydroxylation activity[a] CYP 2C9	CYP 2C18
tienilic acid	OCH_2-COOH	Cl	Cl	1.9 ± 0.1	1.4 ± 0.1
1	OH	Cl	Cl	0.6 ± 0.1	1.4 ± 0.2
2	OCH_3	Cl	Cl	<0.02[b]	1.7 ± 0.2
	Cl	H	H	<0.02[b]	1.7 ± 0.3
	F	H	H	<0.02[b]	0.8 ± 0.2

[a] Initial rates in nanomoles of 5-hydroxylated product per nanomole of P450 per minute were measured after a 10 min incubation of microsomes from W(R) fur 1 yeast expressing either CYP 2C9 or CYP 2C18 (0.2 μM P450) in 0.1 M Tris buffer containing 1 mM EDTA and 8% glycerol in the presence of 100 μM substrate and a NADPH-generating system, assuming an ϵ at 385 nm identical for all 5-hydroxy-2-aroylthiophene metabolites (see the text). Values are the mean $\pm$ standard deviation from three to five independent experiments. [b] Under the detection limit of the assay; it was determined that no activity could be detected even in incubations using 500 μM substrate.

at wavelengths around 385 nm (*19*). This spectral feature is due to the existence of 5-hydroxy-2-aroylthiophenes in a highly conjugated anionic form at pH 7.4, because of their low pK_a ($\sim$3) (*19*) (Figure 2). This was the basis of the assay used for following the 5-hydroxylation of TA derivatives by rat liver microsomes or by CYP 2C9 expressed in yeast (*6*, *19*, *31*, *36*). The λ_{max} and $\epsilon(\lambda_{max})$ values exhibited by 5-hydroxy-2-aroylthiophenes are very similar whatever the nature of the substituents of the aryl group: λ_{max} between 382 and 386 nm and $\epsilon(\lambda_{max})$ between 27 and 30 mM^{-1} cm^{-1} (*19*). Therefore, the 5-hydroxylation of compounds **1**−**9** can be easily followed by UV−visible spectroscopy at 385 nm; it should lead to 5-hydroxy-2-aroylthiophene metabolites exhibiting very similar UV−visible spectra.

Comparison of the Ability of CYP 2C9 and CYP 2C18 To Hydroxylate Various Tienilic Acid Derivatives at Position 5 of Their Thiophene Rings. As a first approach, the ability of various tienilic acid derivatives to act as substrates of recombinant CYP 2C9 and 2C18 was studied by following the appearance of 5-hydroxy metabolites by the procedure described above. Recombinant CYP 2C9 and CYP 2C18 were obtained from expression of the corresponding cDNA in yeast strain W(R) fur 1 in which yeast cytochrome P450 reductase was overexpressed, as described previously (*22*, *24*, *26*). Yeast microsomes were directly used to measure the CYP 2C9 and CYP 2C18 activities. Table 1 shows that CYP 2C9 was only able to 5-hydroxylate compounds bearing an anionic charge (at pH 7.4), such as tienilic acid or phenol **1**, in agreement with previously reported results (*6*). It failed to 5-hydroxylate compound **2** and the two derivatives bearing a para Cl or F substituent mentioned in Table 1. Moreover, CYP 2C9 was found to be a poor catalyst for the 5-hydroxylation of compounds **3**−**9** (data not shown). By contrast, yeast microsomes expressing CYP 2C18 catalyzed the 5-hydroxylation of the three neutral compounds listed in Table 1 as well as that of anionic substrates tienilic acid and **1**. These first results suggested that the active site of CYP 2C18 was less selective toward anionic substrates than that of CYP 2C9.

Table 2: Kinetic Values Determined for the 5-Hydroxylation of Various 2-Aroylthiophenes by Yeast-Expressed P450 2C18[a]

substrate	R	k_{cat} (min^{-1})	K_M (μM)	k_{cat}/K_M (min^{-1} μM^{-1})
tienilic acid	O-CH_2-COOH	2.0 ± 0.3	150 ± 10	0.01
1	OH	4.8 ± 0.4	11 ± 1	0.43
2	OCH_3	1.7 ± 0.2	10 ± 1	0.17
3	O-CH_2-CH_2-CH_2-OH[b]	149 ± 10	15 ± 1	9.6
4	O-CH_2-CH_2-CH_2-CN	3.6 ± 0.1	26 ± 4	0.2
5	O-CH_2-CH_2-CH_2-CH_3	3.3 ± 0.2	29 ± 2	0.1

[a] R refers to the para substituent of the aryl ring of various 2-aroylthiophenes (see Figure 1). Kinetic values were derived from the rates of appearance of the 385 nm peak which is characteristic of the 5-hydroxy-2-aroylthiophene metabolite, in incubations of microsomes of yeast expressing CYP 2C18 (0.2 μM P450) in 0.1 M Tris buffer containing 1 mM EDTA and 8% glycerol in the presence of 2−100 μM substrate and a NADPH-generating system. It was assumed that ϵ values at 385 nm were identical for all 5-hydroxy-2-aroylthiophene metabolites (see the text). Values are the mean $\pm$ standard deviation from three to five independent experiments. [b] It is noteworthy that the 5-hydroxylation of **3** is subject to an inhibition by **3** in excess. It follows classical saturation kinetics for concentrations of **3** of <80 μM, and inhibition by excess substrate at higher concentrations. The indicated K_M and k_{cat} values were deduced from Lineweaver−Burk plots for concentrations of **3** between 1 and 50 μM.

Other compounds bearing various functions on para position of the dichlorophenyl group were then synthesized so we could better understand the structural features recognized by CYP 2C18. All the compounds that were studied (**1**−**9**) were found to be 5-hydroxylated by yeast microsomes expressing CYP 2C18 in the presence of NADPH, as they gave rise to the appearance of a peak around 387 nm as a function of time. The UV−visible spectra of the corresponding metabolites were almost identical. This 5-hydroxylation of compounds **1**−**9** did not occur in identical incubations but in the absence of NADPH, or in incubations using microsomes from yeast transformed by the pYeDP60 vector not containing CYP 2C18 cDNA (data not shown). 5-Hydroxylation of **1**−**9** was shown to be linear as a function of time for at least 15 min, and as a function of CYP 2C18 concentration between 0.05 and 0.5 μM (data not shown). Table 2 compares the K_M and k_{cat} values obtained for the 5-hydroxylation of tienilic acid and compounds **1**−**5** by CYP 2C18. It shows that compounds bearing OCH_3, $O(CH_2)_3$-CN, and $O(CH_2)_3CH_3$ para substituents are more efficiently hydroxylated than tienilic acid, with K_M values 1 order of magnitude lower and k_{cat}/K_M values 1 order of magnitude higher than the values for tienilic acid. However, the most spectacular result was obtained with compound **3** that bears a $O(CH_2)_3OH$ substituent. The k_{cat} value determined for its 5-hydroxylation (149 ± 10 min^{-1}) is, to our knowledge, the highest one reported so far for a P450 2C. The catalytic efficiency of CYP 2C18 toward **3** is also illustrated by its k_{cat}/K_M value which is 20−1000-fold larger than those measured for all the other CYP 2C18 substrates.

Comparison of Various Derivatives of Tienilic Acid Bearing an Alcohol Function as Substrates of CYP 2C18. Several analogues of **3**, all bearing an alcohol function on the chain in the para position of the dichlorophenyl ring, were then synthesized to determine whether the presence of such an alcohol function is important for recognition by CYP 2C18. As shown in Table 3, the two primary alcohols, **6** and **7**, that have two and four CH_2 groups, respectively,

Table 3: Kinetic Values Determined for the 5-Hydroxylation of Various 2-Aroylthiophenes Bearing an Alcohol Function by Yeast-Expressed P450 2C18[a]

Substrate	R	k_{cat} (min^{-1})	K_M (μM)	k_{cat}/K_M ($min^{-1}.\mu M^{-1}$)
6	$O-CH_2-CH_2-OH$	16 ± 1	40 ± 3	0.4
3	$O-CH_2-CH_2-CH_2-OH$	149 ± 10	15 ± 1	9.6
7	$O-CH_2-CH_2-CH_2-CH_2-OH$	13 ± 1	42 ± 2	0.3
8	$O-CH_2-CH(OH)-CH_2(OH)$	25 ± 3	52 ± 4	0.5
9	$O-CH_2-CH_2-CH_3$ (OH)	43 ± 3	26 ± 1	1.7

[a] R refers to the para substituent of the aryl ring of various 2-aroylthiophenes (see Figure 1). Conditions were identical to those described in Table 2.

between their ether and alcohol functions, the diol **8**, and the secondary alcohol **9** all were efficiently 5-hydroxylated by CYP 2C18. The corresponding k_{cat} values were all larger than 10 min^{-1} and varied between 13 and 43 min^{-1}. The K_M values varied between 26 and 52 μM, and the k_{cat}/K_M values were either similar or larger than the one described for the 5-hydroxylation of tienilic acid by CYP 2C9 (*6*).

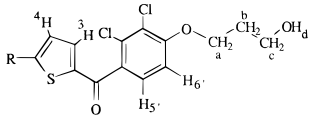
These results confirmed that the presence of an alcohol function on the para substituent of the dichlorophenyl ring is an important factor for recognition by CYP 2C18. However, Table 3 also shows that alcohol **3** is by far the best substrate for 5-hydroxylation by CYP 2C18, with the best k_{cat} and K_M values and a k_{cat}/K_M value that is 5−30-fold larger than those of the other alcohol substrates. When the three primary alcohols **6**, **3**, and **7** are compared (Table 3), it is clear that the presence of three CH_2 groups between the ether and alcohol functions is optimal for efficient hydroxylation by CYP 2C18.

*Complete Study of the Hydroxylation of Alcohol **3** by Recombinant CYP 2C18, Including Identification of the Products.* As these preliminary studies, based only on the UV−visible detection of 5-hydroxy metabolites, indicated that **3** was the best substrate of CYP 2C18, a more complete study of the oxidation of **3** by CYP 2C18 was then performed. HPLC analysis of incubation mixtures of **3** with microsomes from yeast expressing CYP 2C18 in the presence of NADPH revealed the formation of a very major metabolite, further characterized as 5-hydroxy-**3**, and two minor metabolites. One of these minor metabolites exhibited an UV−visible spectrum and a retention time identical to those of phenol **1** (Figure 1) which was previously prepared by a nonambiguous method and completely characterized.

The major metabolite (95% of total metabolites), which exhibited an UV−visible spectrum characteristic of 5-hydroxy-2-aroylthiophenes, was produced in great amounts, allowing its easy purification. For instance, incubations performed on 2 mg of **3** led, after purification by preparative HPLC, to about 1.5 mg of this major metabolite. Its mass spectrum, with a molecular peak at 347 (M^+), and ^{1}H NMR spectrum (compared to that of **3** in Table 4) were in complete agreement with a 5-hydroxy-**3** structure (Figure 3).

The third metabolite (around 2% of total metabolites) exhibited a retention time and an UV−visible spectrum identical to the only metabolite obtained upon oxidation of **1** by microsomes of yeast expressing CYP 2C18 in the presence of NADPH. This compound exhibited an UV−visible spectrum typical of 5-hydroxy-2-aroylthiophenes, and should be derived from the 5-hydroxylation of **1**.

Table 4: ^{1}H NMR Characteristics of Compound **3** and Its Major CYP 2C18-Dependent Metabolite[a]



	H for compound **3**	OH for 5-hydroxylated compound **3**
H_3	7.41 (dd, 1H, J_{34} = 3.9, J_{35} = 1.1)	7.35 (d, 1H, J = 4.8)
H_4	7.10 (dd, 1H, J_{43} = 3.9, J_{45} = 4.8)	5.95 (d, 1H, J = 4.8)
H_5	7.73 (dd, 1H, J_{53} = 1.1, J_{54} = 4.8)	
$H_{5'}$	7.33 (d, 1H, J = 8.5)	7.4 (d, 1H, J = 7.8)
$H_{6'}$	6.92 (d, 1H, J = 8.5)	7.25 (d, 1H, J = 7.8)
H_a	4.26 (t, 2H, J = 5.8)	4.36 (t, 2H, J = 6.5)
H_b	2.13 (m, 2H)	2.15 (m, 2H)
H_c	3.92 (m, 2H)	3.89 (t, 2H, J = 6.8)
H_d	3.89 (t, 1H, J = 5.35)	

[a] ^{1}H NMR spectra of compound **3** and its major metabolite were recorded in $CDCl_3$ and D_2O, respectively.

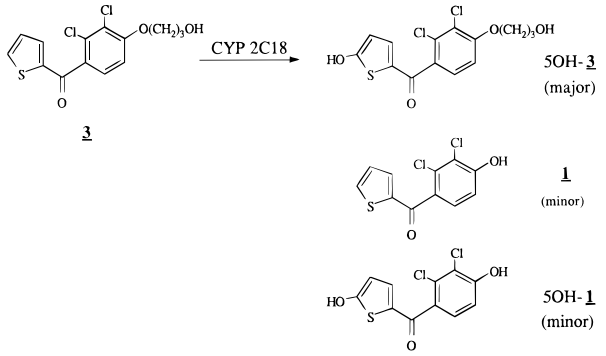


FIGURE 3: Various oxidations of **3** catalyzed by yeast-expressed CYP 2C18.

The CYP 2C18-dependent oxidation of **3** thus leads to a very major metabolite (>95% of the total metabolites) deriving from the 5-hydroxylation of its thiophene ring, and to two minor products (around 2% each of the total metabolites) deriving either from the oxidative O-dealkylation of its $O(CH_2)_3OH$ chain (**1**) or from two successive oxidations (5-hydroxy-**1**) (Figure 3).

Interestingly enough, similar HPLC and isolation studies performed with CYP 2C18-dependent oxidations of alcohols **6**, **8**, and **9** led to very similar results. In each case, the major metabolite that was found (>90% of the total metabolites) was derived from the 5-hydroxylation of the thiophene ring. The ^{1}H NMR characteristics of the isolated major metabolites are shown in Table 5. The minor metabolites observed for **6**, **8**, and **9** were also the O-dealkylation product **1** and its 5-hydroxy metabolite (data not shown).

*Oxidation of **3** by Recombinant Human Liver CYP 1A1, 1A2, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5.* Oxidations of alcohol **3** by microsomes of W(R) fur 1 yeasts expressing the other principal human liver cytochrome P450s were then studied, to determine whether this substrate was specific for CYP 2C18. The HPLC chromatograms of the different incubates showed the formation of five metabolites: the three metabolites previously observed in CYP 2C18-dependent oxidation of **3** (5-hydroxy-**3**, **1**, and 5-hydroxy-**1**), as well

Table 5: ^{1}H NMR Characteristics of the Major Metabolite Derived from Hydroxylation of Substrates **6**, **8**, and **9** by Yeast-Expressed P450 2C18[a]

	5-Hydroxylated **6**	5-Hydroxylated **8**	5-Hydroxylated **9**
R[b]	−O−CH$_2$−CH$_2$−OH (a, b)	OH OH −O−CH$_2$−CH−CH$_2$ (a, b, c)	OH −O−CH$_2$−CH−CH$_3$ (a, b, c)
H3	7.25 (d, 1H, J=4.8)	6.92 (d, 1H, J=4.8)	6.87 (d, 1H, J=4.8)
H4	5.35 (d, 1H, J=4.8)	5.30 (d, 1H, J=4.8)	5.25 (d, 1H, J=4.8)
H5'	7.28 (d, 1H, J=8.4)	7.23 (d, 1H, J=8.5)	7.15 (d, 1H, J=8.5)
H6'	7.02 (d, 1H, J=8.4)	7.04 (d, 1H, J=8.5)	6.95 (d, 1H, J=8.5)
Ha	4.15 (t, 2H, J=6.2)	4.12 (m, 2H)	4.09 (d, 2H, J=6.0)
Hb	3.95 (t, 2H, J=6.2)	3.98 (m, 1H)	3.64 (m, 1H)
Hc	-	3.66 (d, 2H, J=5.0)	masked by trace impurities from HPLC

[a] ^{1}H NMR spectra in D_2O. [b] R refers to the para substituents of the aryl rings of **6**, **8**, and **9** (see Figure 1). Labeling of the hydrogens as described in the legend of Figure 2.

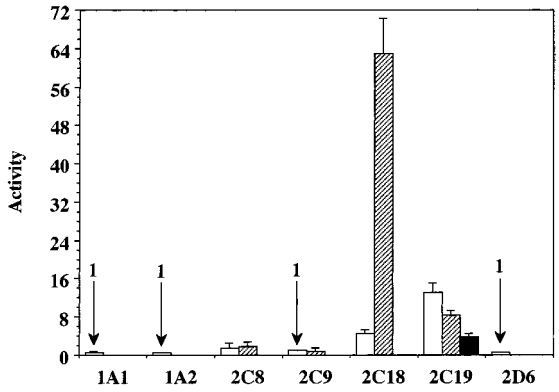


FIGURE 4: Oxidation of **3** by microsomes of yeast expressing various human liver P450s. The striped bars represent data for 5-hydroxy-**3**, the black bars **8**, and the white bars **1**. Activities are in nanomoles per nanomole of P450 per 5 min. Conditions of Table 2 with 200 μM **3**.

as **8**, which is derived from a C-hydroxylation of **3**, and its 5-hydroxy derivative. The three major metabolites were derived from three possible monooxygenations of **3**: the 5-hydroxylation of its thiophene ring (5-hydroxy-**3**) and the hydroxylations of the (O-)C**H**$_2$ and (O-CH$_2$-)C**H**$_2$ groups of its O-alkyl chain (**1** and **8**, respectively). Figure 4 compares the abilities of the various human liver cytochrome P450s to catalyze those three hydroxylations. CYP 2E1, 3A4, and 3A5 were found to be unable to catalyze these hydroxylations as no metabolite could be detected even after incubation for 30 min. CYP 1A1, 1A2, and 2D6 appeared to be poor catalysts, only leading to the product of O-dealkylation **1** with very small initial rates [$<$0.2 nmol (nmol of P450)$^{-1}$ min^{-1}]. The four members of the CYP 2C subfamily appeared to be much better catalysts for the oxidation of **3**. These four isozymes catalyzed the O-dealkylation of **3** into **1** with rates between 0.2 and 3 nmol (nmol of P450)$^{-1}$ min^{-1}. Interestingly enough, CYP 2C19 was the only member of the CYP 2C subfamily to efficiently C-hydroxylate **3** with formation of **8** [1 nmol (nmol of P450)$^{-1}$ min^{-1}], the other CYP 2Cs producing initial rates of $<$0.1 nmol (nmol of P450)$^{-1}$ min^{-1}. The four P450 2Cs catalyzed the 5-hydroxylation of the thiophene ring of **3** with very different rates (Figure 4). To better appreciate the different efficiencies of CYP 2Cs in catalyzing the O-dealkylation, C-hydroxylation, and 5-hydroxylation of **3**, further experiments were performed to measure the K_M and k_{cat} values for these reactions for each CYP 2C, by following the formation of the corresponding metabolites by HPLC. Table 6 confirms that the k_{cat} values determined for the O-dealkylation of **3** by the four CYP 2Cs are similar (between 1.2 $\pm$ 0.3 min^{-1} for CYP 2C9 and 5 $\pm$ 1 min^{-1} for CYP 2C19). The k_{cat}/K_M values varied between 0.002 min^{-1} μM^{-1} for CYP 2C9 and 0.1 min^{-1} μM^{-1} for CYP 2C18, mainly because of relatively large variations of the K_M values. As mentioned above, CYP 2C19 is the only CYP 2C exhibiting a significant C-hydroxylation activity with formation of **8**. However, the k_{cat}/K_M value for that CYP 2C19-dependent reaction is rather low (0.005 min^{-1} μM^{-1}).

Table 6: Kinetic Values Determined for the Various Oxidations of **3** by Yeast-Expressed CYP 2Cs[a]

	CYP	k_{cat} (min^{-1})	K_M (μM)	k_{cat}/K_M (min^{-1} μM^{-1})
O-dealkylation	2C8	2.2 $\pm$ 0.5	530 $\pm$ 100	0.004
	2C9	1.2 $\pm$ 0.3	650 $\pm$ 90	0.002
	2C18	4 $\pm$ 2	45 $\pm$ 10	0.1
	2C19	5 $\pm$ 1	160 $\pm$ 50	0.03
5-hydroxylation	2C8	0.4 $\pm$ 0.1	180 $\pm$ 70	0.002
	2C18	125 $\pm$ 25	9 $\pm$ 1	13
	2C19	1.7 $\pm$ 0.3	75 $\pm$ 25	0.02
aliphatic hydroxylation	2C19	1.0 $\pm$ 0.5	190 $\pm$ 70	0.005

[a] Kinetic values were derived from rates of appearance of the various metabolites of **3** detected by HPLC from incubations under the conditions described in Table 2. Values are the mean $\pm$ standard deviation from four independent experiments.

The most striking feature of Table 6 is the very high efficiency of CYP 2C18 for the 5-hydroxylation of **3**, when compared to those of the other CYP 2Cs. This is illustrated by the k_{cat}/K_M value determined for this reaction which is 3−4 orders of magnitude higher for CYP 2C18 than for CYP 2C8 or CYP 2C19. Kinetic values could not be determined in the case of CYP 2C9 because too little 5-hydroxy-**3** formed. This high efficiency of CYP 2C18 is the result of the k_{cat} values being 2 orders of magnitude higher when compared to those of CYP 2C8 and CYP 2C19 (125 min^{-1} instead of 0.4 and 1.7 min^{-1}), and of K_M values that are about 10 times lower with CYP 2C18 than with CYP 2C8 and CYP 2C19 (9 $\pm$ 1 μM instead of 75 $\pm$ 25 and 180 $\pm$ 70 μM).

DISCUSSION

The above comparison of the aptitudes of several newly synthesized compounds derived from tienilic acid to be 5-hydroxylated by recombinant CYP 2C9 and CYP 2C18 clearly showed that CYP 2C18 is much more permissive than CYP 2C9 for nonanionic substrates. In fact, suppression of the acid function of tienilic acid results in a dramatic decrease in the rates of hydroxylation by CYP 2C9, whereas the reverse is true for CYP 2C18 (Table 1). Several neutral molecules derived from tienilic acid, **2**−**9**, are 5-hydroxylated by CYP 2C18 with k_{cat} values ranging from 2 to 150 min^{-1}. Among the products that were tested, those bearing an alcohol function in the *O*-alkyl chain of the phenyl ring (**3** and **6**−**9**) gave the best results (Tables 2 and 3). Alcohol **3**, which bears a O-(CH$_2$)$_3$-OH substituent, is by far the best substrate of CYP 2C18. Its 5-hydroxylation is characterized by a k_{cat} value of 149 $\pm$ 10 min^{-1} which is, to our knowledge,

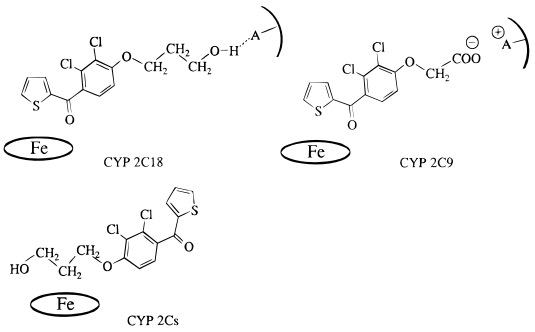


FIGURE 5: Two main possible positionings of **3** in the active sites of CYP 2Cs. The main positioning of **3** in the CYP 2C18 active site is compared to that proposed for tienilic acid in the CYP 2C9 active site (from ref *5*).

the highest rate described so far for a P450 of the 2C subfamily. Moreover, the k_{cat}/K_M value determined for this reaction is remarkably high (approximatively 10 min^{-1} μM^{-1}) (Table 2). These results suggest that whereas CYP 2C9 is particularly prone to recognizing and oxidizing substrates bearing an anionic site, CYP 2C18 would prefer molecules bearing an alcohol function. This could be the result of the presence of a different key amino acid residue in the substrate active site of these two cytochrome P450s. It has been proposed that the CYP 2C9 active site (*5*−*7*) would involve a cationic amino acid residue; our results would suggest that the CYP 2C18 active site contains an amino acid residue that is able to act as a hydrogen bond acceptor.

A more detailed study of the oxidation of compound **3** by various recombinant human liver cytochrome P450s showed that only the cytochrome P450s of the 2C subfamily are really efficient for catalyzing the oxidation of **3**. Most of the human P450s that were used either are inactive (CYP 2E1, 3A4, and 3A5) or exhibit a low oxidative O-dealkylation activity leading to compound **1** (CYP 1A1, 1A2, and 2D6). It is noteworthy that CYP 2C8, 2C9, 2C18, and 2C19 also catalyze this oxidative O-dealkylation of **3** with similar rates (k_{cat} between 1 and 5 min^{-1}, Table 6). By contrast, there is a spectacular difference in the abilities of the different CYP 2Cs to catalyze the 5-hydroxylation of **3**. CYP 2C18 is by far the best catalyst for that reaction with a reasonably low K_M value (9 ± 1 μM) and a very high k_{cat} (125 ± 25 min^{-1}) for a P450 2C. From all the recombinant P450s that were tested, only CYP 2C19 and CYP 2C8 catalyze this 5-hydroxylation, however, with a much lower catalytic efficiency (k_{cat}/K_M being 3−4 orders of magnitude lower than in the case of CYP 2C18). Thus, compound **3** appears to be a very good substrate for CYP 2C18; the 5-hydroxylation of its thiophene ring is regioselectively performed by CYP 2C18 with a rare efficiency. Compound **3** should be a substrate of choice for the research of active CYP 2C18 in various organs and tissues. Research in that direction is currently being carried out in this laboratory.

Again with regard to the different kinds of oxidation of **3** observed with recombinant human liver P450s, the aforementioned results may be interpreted by considering two positionings of **3** in the P450 active sites (Figure 5). In one of them, the O-$(CH_2)_3$-OH chain would be held above the heme iron in the active site, enabling the hydroxylation of the CH_2 groups α or β to the ArO moiety. This should mainly result in the oxidative O-dealkylation observed with several recombinant human P450s. This should be the chemically preferred reaction as it is well-known that cytochrome P450s easily hydroxylate C−H bonds α to an ether function, the carbon radical involved as an intermediate being stabilized by the presence of the α oxygen atom. Accordingly, this reaction occurs with several P450s, CYP 1A1, 1A2, 2D6, 2C8, 2C9, 2C18, and 2C19, the levels of activity ranging from 0.1 min^{-1} for CYP 1A2 to 5 min^{-1} for CYP 2C19.

The second possible mode of positioning of **3**, with the thiophene ring above the heme iron in the active site (Figure 5), which results in the 5-hydroxylation of **3**, seems only to occur with the three P450s, CYP 2C8, 2C19, and 2C18. However, it is less favored than the first positioning in CYP 2C8 and 2C19. CYP 2C18 is unique in that it is the only P450 that would greatly favor this second positioning over the first one. This unique behavior of CYP 2C18 could be due to the presence in its active site of a well-located protein amino acid residue that is able to specifically recognize the O-$(CH_2)_3$-OH moiety. We propose that it could be a hydrogen acceptor located in a position similar to that of the putative cationic amino acid residue of CYP 2C9 which would recognize the anionic part of CYP 2C9 substrates. This would explain the high regioselectivity of CYP 2C18 and 2C9 for their respective substrates, **3** and tienilic acid. Many experiments to confirm this suggestion remain to be carried out.

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