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RESISTANCE OF THE 2,2,2-TRIFLUOROETHOXY ARYL MOIETY TO THE CYTOCHROME P-450 METABOLISM IN RAT LIVER MICROSOMES

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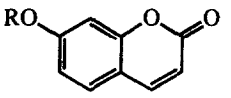
Abstract: 7-(2,2,2-trifluoroethoxy)coumarin (**1a**) or 4'-(2,2,2-trifluoroethoxy)acetanilide (**3a**), fluorinated analogs of the well known cytochrome P-450 deethylase (EC 1.14.14.1) substrates 7-ethoxycoumarin (**1b**) and phenacetin (**3b**), respectively, remained unaltered after incubation with rat liver microsomes in the presence of NADPH. In addition, compounds **1a** and **3a** showed a moderate activity as inhibitors of the above enzymes. Our results suggest that the CF_3CH_2O- group could play an important role in the design of bioactive compounds when a metabolic resistance at a specific position is desired.

Keywords: Cytochrome P-450 deethylase (EC 1.14.14.1). Dealkylation. 7-ethoxycoumarin. Phenacetin. 2,2,2-Trifluoroethoxy derivatives.

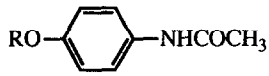
In the course of our research on inhibitors of lipid peroxidation based on simple 6,7-dialkoxy-2,2-dimethyl-1*H*-benzopyran derivatives,[1] we were interested in introducing alkoxy substituents that could exhibit different sensitivities towards the dealkylation mediated by cytochrome P-450 metabolism. Preliminary results showing that the 2,2,2-trifluoroethoxy group (CF_3-CH_2-O), a substituent previously used in our laboratory for the preparation of precocene analogues,[2] appeared to be resistant to the above metabolic cleavage, encouraged us to study this insusceptibility with more detail. To date, the only literature precedent on the metabolic cleavage of 2,2,2-trifluoroethoxy derivatives is that of fluroxene (2,2,2-trifluoroethyl vinyl ether, an anesthetic agent), and to flecainide (*N*-(2-piperidylmethyl)-2,5-bis(2,2,2-trifluoroethoxy)benzamide, an antiarrhythmic drug). In the former case, mediated cytochrome P-450 metabolism released 2,2,2-trifluoroethanol which meant that the cleavage occurred at the non fluorinated alkyl fragment of the ether.[3] Conversely, a major metabolite originated from a *O*-dealkylation at C-5 after oral administration of flecainide to humans was identified; nevertheless, cleavage of the second fluorinated substituent, i.e. that at C-2, was not observed.[4]

For our purpose, 7-(2,2,2-trifluoroethoxy)coumarin (**1a**) and 4'-(2,2,2-trifluoroethoxy)acetanilide (**3a**), fluorinated analogues of the well known substrates for cytochrome P-450 dealkylases 7-ethoxycoumarin (**1b**) [5] and phenacetin (**3b**),[5] respectively, were synthesized and their behavior towards metabolic deethylation was assayed by using rat liver microsomes in the presence of NADPH. These fluorinated substrates were chosen for two reasons. First, being 2,2,2-trifluoroethyl aryl ethers, they would not suffer oxidative cleavage at the aryl-oxygen bond of the ether linkage. Secondly, the potential metabolic attack at the 2,2,2-trifluoroethyl moiety would lead to the formation of strong chromophores (compounds **2** or **4**), thus facilitating their

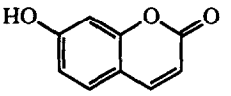
detection by HPLC. Among the known inducers of the cytochrome P-450 system, we used phenobarbital due to its selective stimulation of isoenzymes responsible for the *O*-dealkylation of the above substrates.[6]



1a: R = CF_3CH_2
1b: R = CH_3CH_2
1c: R = CH_3



3a: R = CF_3CH_2
3b: R = CH_3CH_2
3c: R = CH_3



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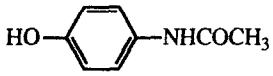

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The synthesis of fluorinated coumarin **1a** has been reported elsewhere.[7] 7-Ethoxycoumarin (**1b**) and 7-methoxycoumarin (**1c**) were prepared by reaction of 7-hydroxycoumarin (**2**) with ethyl or methyl iodide, respectively, in the presence of K_2CO_3 and *N,N*-dimethylformamide.[8] The same basic procedure was used for the preparation of phenacetin (**3b**) and 4'-methoxyacetanilide (**3c**) from 4'-hydroxyacetanilide (**4**).[8] The fluorinated acetanilide **3a** was synthesized in 65 % overall yield by reduction of 2,2,2-trifluoroethyl 4-nitrophenyl ether [7] with sulfurated borohydride [9] followed by acetylation of the intermediate aniline **5**.[10]

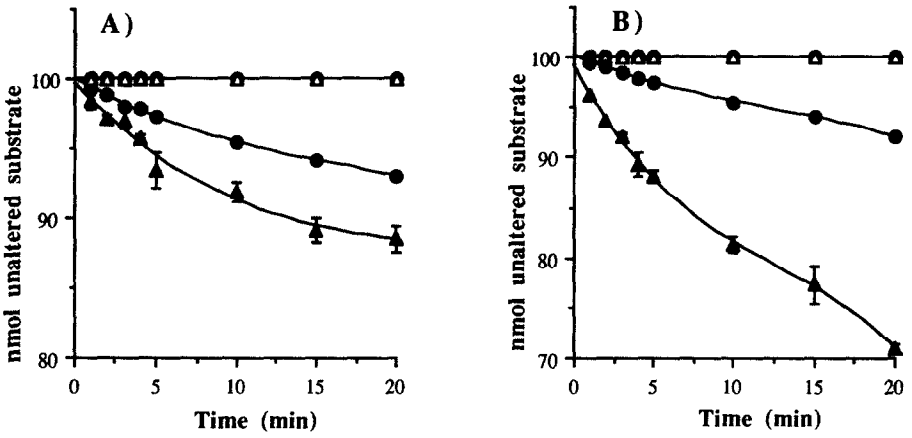

Figure 1. Time course metabolism of: A) 7-ethoxycoumarin (**1b**, full symbol) and 7-(2,2,2-trifluoroethoxy)coumarin (**1a**, empty symbol), and B) phenacetin (**3b**, full symbol) and 4'-(2,2,2-trifluoroethoxy)acetanilide (**3a**, empty symbol), in the presence of liver microsomes from non-treated rats (circles) or phenobarbital induced rats (triangles), and NADPH.

As shown in Fig. 1, substrates **1b** or **3b** suffered a cytochrome P-450 mediated deethoxylation when incubated with liver microsomes from treated or non-treated rats.[11] Calculated activities for **1b** were: 0.65 and 1.93 nmol of **2**/min·mg protein for control and treated rats, respectively. For the case of phenacetin (**3b**), activities found were 0.66 and 3.48 nmol of **4**/min·mg protein for control and treated rats, respectively. Conversely, the corresponding fluorinated derivatives **1a** or **3a** remained unaltered during the incubation period. On the other hand, the presence of metabolites derived from deacetylation of compounds **3a** and **3b** was not observed.

The metabolic resistance exhibited by the fluorinated compounds led us to test them as potential cytochrome P-450 inhibitors. Initially, incubation of **1a** or **3a** (0.5 mM) during 0, 5, 10 and 15 min with rat liver microsomes and NADPH before the addition of the respective substrate **1b** or **3b** showed that preincubation with the fluorinated compound did not alter or slightly decreased the inhibitory activity. Based on these results, subsequent assays were performed with the simultaneous addition of the inhibitor and the corresponding substrate. As shown in Figure 2, the inhibitory activity of fluorinated compounds **1a** or **3a** at different concentrations was not time dependent within the interval assayed,[12] which permitted the determinations of IC_{50} values for **1a** and **3a** (0.23 and 0.10 mM, respectively) by using a single incubation time (15 minutes).

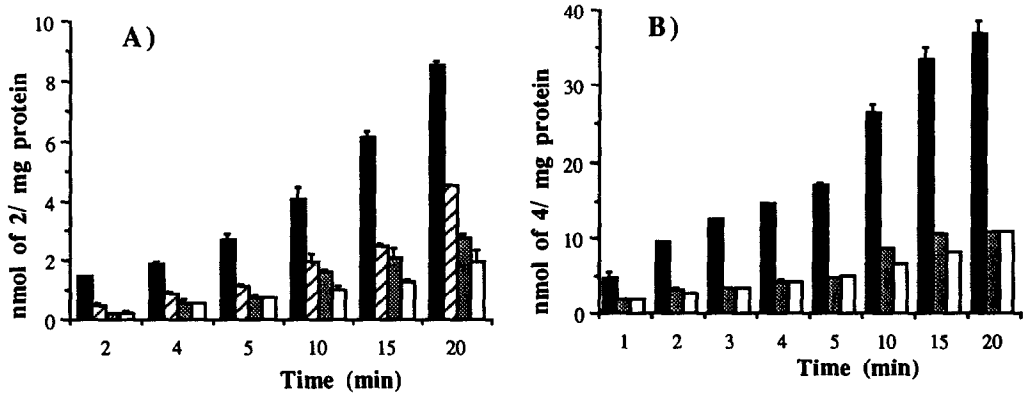

Figure 2. Inhibition of deethylase activity by: **A)** 7-(2,2,2-trifluoroethoxy)coumarin (**1a**) using 7-ethoxycoumarin (**1b**) as substrate and liver microsomes from non-treated rats and **B)** 4'-(2,2,2-trifluoroethoxy)acetanilide (**3a**) using phenacetin (**3b**) as substrate and liver microsomes from phenobarbital induced rats, at different times. Concentrations of inhibitor were: 1 mM (empty bars), 0.5 mM (dotted bars), 0.25 mM (hatched bars) and 0 (control, full bars).

In conclusion, the fact that the fluorinated derivatives **1a** and **3a** did not elicit high inhibitory activities suggests the possibility of using the 2,2,2-trifluoroethoxy group as a moiety which would exhibit a high resistance to the cytochrome P-450 metabolism at the site where the fluorinated group is located, without affecting seriously the activity of this enzymatic system. Use of this strategy for the design of potent lipid peroxidation inhibitors will be reported elsewhere.

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8. Compounds **1b**, **1c** and **3c** were identified by comparison of their spectral (^{1}H and ^{13}C NMR, MS) data with those reported in the literature.
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10. **3a**: IR (KBr): 3315, 1688, 1533, 1519, 1284, 1244, 1164 and 1076 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ, 2.00 (s, 3H), 4.25 (q, 2H, J = 8 Hz), 6.83 (d, 2H, J = 9 Hz), 7.1 (br, 1H), 7.37 (d, 2H, J = 9 Hz) ppm; ^{13}C NMR ($CDCl_3$): δ, 168.1, 154.2, 132.8, 121.7, 123.3 (q, J = 277 Hz), 115.6, 66.4 (q, J = 34 Hz), 24.38 ppm; MS (m/z): 233 (M^+), 191, 108.
    4-(2,2,2-Trifluoroethoxy)aniline (**5**): IR (film): 3425, 3188, 1514, 1286, 1278, 1174, 1157 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ, 6.74-7.84 (m, 2H), 6.6-6.7 (m, 2H), 4.25 (q, 2H, J = 8 Hz), 3.4 (br, 2H) ppm; ^{13}C NMR ($CDCl_3$): δ, 150.5, 141.7, 123.4 (q, J = 277 Hz), 116.5, 116.1, 67.0 (q, J = 34 Hz) ppm; MS (m/z): 191 (M^+), 108.
11. *Determination of deethylase activity.* Microsomes (from Sprague-Dawley males) were prepared as described in ref. 1. Induction by phenobarbital was done by daily intraperitoneal injections (75 mg/kg body wt) on four subsequent days.The incubation mixture for deethylase assays contained in 0.2 ml: Krebs-Ringer-Phosphate, pH 7.4, 2 mM NADPH, 0.5 mM substrate (either **1b** or **3b**, in acetonitrile solution, where the solvent did not exceed 1% of the overall test mixture) and 0.6 mg microsomal protein. The reaction was carried out at 37 °C for various lengths of time and terminated with 0.8 ml ^{t}butyl methyl ether. Then, compound **1c** or **3c** was added as internal standard, the organic phase was collected, evaporated to dryness and the residue was solved in acetonitrile (0.2 ml) and injected onto the HPLC system. Metabolic resistance experiments were performed by using either **1a** or **3a** as substrates under the above conditions. Inhibitory activity of fluorinated derivatives **1a** or **3a** was tested versus its respective ethoxylated analog **1b** or **3b**. In these cases, the inhibitor was preincubated at 37 °C in the presence of microsomes and NADPH for 0, 5 and 10 min before the addition of the corresponding substrate. IC_{50} values for fluorinated compounds **1a** and **3a** were determined by using 15 min incubations and simultaneous addition of the inhibitor and the corresponding substrate. Incubations were performed by duplicate and a minimum of two experiments per point were carried out. Protein was determined by the method of Bradford [13] adapted to a 96-well microplate and using bovine serum albumin as standard. Cytochrome P-450 contents were measured by the method of Omura and Sato [14] and values obtained for phenobarbital treated rats agreed with those reported in the literature.
    HPLC analyses were performed with a Lichrospher 100 RP-18 (125 x 4 mm, 5 μm, Merck) column under the following elution conditions: a) assays with 7-ethoxycoumarin (**1b**) (40% acetonitrile in water at 1 ml/min, λ = 320 nm); b) assays with phenacetin (**3b**) (20% methanol in water at 1 ml/min, λ = 250 nm). In order to detect the potential hydrolytic cleavage of the amide bond when compounds **3a** or **3b** were used as substrates, water was replaced by a 0.017 M H_3PO_4-NH_4OH buffer at pH 7.5.
12. Similar results were obtained when **1a** was tested versus **1b** with liver microsomes from phenobarbital induced rats or when **3a** was tested versus **3b** with liver microsomes from control rats.
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