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A DIRECT, HIGHLY SENSITIVE ASSAY FOR CYTOCHROME P-450 CATALYZED *O*-DEETHYLATION USING A NOVEL COUMARIN ANALOG

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Abstract—The microsomal *O*-deethylation of a novel coumarin analog, 7-ethoxy-4-trifluoromethylcoumarin (EFC), to a fluorescent product was characterized. Results indicate that this analog provides a rapid, convenient and highly sensitive means to assay cytochrome P-450-mediated metabolism. Like microsomal 7-ethoxycoumarin (7-EC) *O*-deethylation, EFC *O*-deethylation responded to both phenobarbital and methylcholanthrene induction. The increase in EFC *O*-deethylase activity seen with phenobarbital was greater than that seen with 7-EC (5- to 6-fold over control after 50 mg/kg/day for 4 days in Sprague–Dawley rats compared to ~2-fold for 7-EC). Since the reaction was monitored by direct fluorometry of the product, any departures from linearity under a particular set of reaction conditions (e.g. with highly induced samples) were immediately apparent. In the absence of an NADPH-regenerating system, background drift was very low (<0.01 fluorescent units), so the sensitivity of the assay was limited primarily by that of the fluorometer employed. This makes the assay particularly useful in situations where test material is limited, e.g. when measuring activity in cultured hepatocytes. Its simplicity, reproducibility, and response to a variety of inducing agents also make it suitable for a rapid screening assay for cytochrome P-450 induction.

The measurement of cytochrome P-450-mediated oxidative metabolism and its induction as a result of xenobiotic administration is an integral part of many research and safety assessment programs. While there are a variety of assays available for this purpose such as benzphetamine *N*-demethylation or ethoxycoumarin *O*-deethylation, most of them are cumbersome and time-consuming, involving the use of difficult-to-obtain radiolabeled substrates and/or tedious solvent extraction separations [e.g. Refs. 1 and 2)]. The need to separate product from substrate prior to analysis also means that, unless multiple time points are taken for each sample, a linear rate of reaction over the course of the incubation must be assumed in order to calculate a reaction rate. Finally, many of the currently available assays suffer from a lack of sensitivity, often requiring hundreds of micrograms of microsomal protein for a single determination. While this lack of sensitivity is not a problem with *in vivo* systems (1 g of liver yields 20–40 mg of microsomes), it can be a serious handicap with *in vitro* systems such as cultured hepatocytes, where material is scarce (20–200 μg of microsomes/10^6 cells) and has lower cytochrome P-450 activity than does *in vivo* material.

We report here the development and characterization of a direct fluorometric assay for cytochrome P-450 catalyzed *O*-deethylation. It is based on the use of 7-ethoxy-4-trifluoromethylcoumarin (EFC§), a novel analog of the widely employed substrate 7-ethoxycoumarin. While such an assay utilizing 7-ethoxycoumarin itself has been described [3], it suffers from the fact that the excitation and emission maxima of 7-ethoxycoumarin (340 and 450 nm respectively) correspond to those of NADPH. Since NADPH is a required co-factor, its presence limits the sensitivity of the direct 7-EC assay [1].

MATERIALS AND METHODS

Materials. 7-Ethoxy-4-trifluoromethylcoumarin (EFC) and its deethylated derivative, 7-hydroxy-4 trifluoromethylcoumarin (HFC), were obtained from Enzyme Systems Products, Livermore, CA. Glucose-6-phosphate (G6P), glucose-6-phosphate dehydrogenase (G6PDH), NADPH, phenobarbital (PB), pyrazole (PYR), dexamethasone (DEX), and 3-methylcholanthrene (MC) were obtained from Sigma, St. Louis, MO. NADP was from Boehringer Mannheim, Indianapolis, IN. 7-Ethoxycoumarin (7-EC) and 7-hydroxycoumarin (7-HC) were obtained from Aldrich, Milwaukee, WI. Pregnenolone carbonitrile (PCN) was a gift from the Upjohn Co., Kalamazoo, MI.

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§Abbreviations: EFC, 7-ethoxy-4-trifluoromethylcoumarin; 7-EC, 7-ethoxycoumarin; G6P, glucose-6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase; KPB, 0.1 M potassium phosphate buffer; HFC, 7-hydroxy-4-trifluoromethylcoumarin; PB, phenobarbital; MC, 3-methylcholanthrene; DMSO, dimethyl sulfoxide; F.U., fluorescent units; DEX, dexamethasone; PCN, pregnenolone carbonitrile; and ETOH, ethanol.

Cytochrome P-450 induction and microsome preparation. The microsomes used for the kinetic studies were prepared from the pooled livers of three 180–220 g male Sprague–Dawley rats as follows. Cytochromes P-450 were induced either by i.p. injection of PB (80 mg/kg/day) in phosphate-buffered saline for 3 days, with the livers excised on day 4, or by a single injection of MC (20 mg/kg; 10 mg/ml in corn oil) with 1 day allowed for induction. Livers were weighed and homogenized in 3 vol. of 0.1 M KPO_4, pH 7.4 (KPB), using 15 strokes of a Potter–Elvehjem homogenizer, rpm = 2000. The homogenate was centrifuged at 9000 *g* for 30 min, and the resulting supernatant fraction was centrifuged at 105,000 *g* for 1 hr to precipitate microsomes. Pellets were resuspended in KPB at 10–20 mg/ml by brief sonication, and 0.2-ml aliquots were stored at −70°.

A variation of the above protocol was used to prepare microsomes for comparison of the effects of different classes of cytochrome P-450 inducing agents. Preparations were from the individual livers of four male and four female rats per group (80–150 g). Cytochromes P-450 were induced by four daily doses (by gavage in 0.5% methylcellulose) of PB (50 mg/kg), PCN (300 mg/kg), DEX (300 mg/kg) or ETOH (5, 6, 7, and 8 g/kg) with microsomes preparation on day 5, approximately 20 hr after the last dose. Homogenization was accomplished by a 5- to 10-sec burst at maximum power of a polytron tissue homogenizer (Kinematica GmbH), and the homogenate was centrifuged for 1 min at 15,000 *g*. All other procedures were identical.

The variations in preparation methodology had no measurable effect on activity. Protein was determined by the method of Lowry *et al.* [4] using bovine serum albumin (BSA) as a standard.

EFC O-deethylase assay. Since the solubility limit of EFC in KPB is ~50 μM (determined spectrophotometrically), stock solutions were prepared in DMSO. DMSO at 0.2% increased the solubility to ~100 μM and at this low concentration only slightly inhibited the reaction (~5% decrease in activity). Stocks stored at 4° appeared to be relatively stable for a month or more; however, prolonged storage at 4° (~5 months) appeared to result in slight decreases in reaction rates. When stocks were stored at −20°, EFC gave reproducible results for >1 year, and this is the protocol we currently use. Aliquots (100 μl) of 0.5 M G6P and 0.1 M NADP were kept frozen at −70° and mixed with 200 μl of 70 units/ml G6PDH (which was filter sterilized and stored at 4°) and diluted to 1 ml immediately prior to use. When 20 μl of this was added to 1 ml of reaction mix, the final concentrations were 0.5 mM, 0.1 mM and 0.14 units/ml for G6P, NADP and G6PDH respectively. Microsomes and complete NADPH-regenerating systems were kept on ice during the course of a study.

An assay was conducted as follows: 0.96 ml of KPB, prewarmed to 41°, was added to a cuvet, mixed with 20 μl of microsomal suspension and 20 μl of complete NADPH-regenerating system (final volume = 1.00 ml), and placed in a jacketed cuvet holder warmed to 37° by a circulating water bath (bath temperature = 41°). EFC was added in 2 μl DMSO and mixed with a disposable plastic pipette, and the increase in fluorescence with time was recorded on a Perkin–Elmer 650-40 fluorescence spectrophotometer with an excitation of 410 nm, an emission of 510 nm, and slit widths of 5–20 nm, depending on the desired degree of sensitivity. In the kinetic studies, the final microsome concentration was 10 μg/ml, but it was 150 μg/ml in all other studies; also, the final volume for all studies other than the kinetic studies was 2 ml and additions were increased proportionately.

Slit widths can be varied to achieve the desired degree of sensitivity since the overlap of HFC excitation and emission spectra with those of other assay components is negligible (see Results). Background fluorescence increased with increasing slit width and decreasing microsome concentration but was extremely stable with time (<0.01 F.U./min, see Results, Fig. 4B) and thus did not interfere with the assay of very low activity samples.

In our experience, the rate of change usually stabilizes within 15 sec and remains linear for up to 15 min or more, depending on the substrate concentration and level of activity. We generally run to reaction for 1–3 min and determine the slope from a recorded tracing. We have recently automated data collection for this system using a series of programs which capture the fluorescence reading at user-selected time intervals and convert this raw data directly to rates in picomoles per minute per milligram. This system has greatly reduced data analysis time (e.g. for a study of 120 assays, manual slope determination, number crunching and data table compilation can take as much as 2 days but takes about 15 min using the automated system).

Each time a set of assays was run, deethylated standards in complete reaction mix were used to determine the yield of fluorescence units per picomole of product. At a given machine setting, the fluorescence yield was constant over a wide range of substrate concentrations but decreased slightly as microsome concentration was increased, presumably due to light scatter. We find that, at slit widths of 3 nm (excitation) and 20 nm (emission), 1 F.U. is approximately equal to 33 pmoles of HFC/ml.

Thin-layer chromatography. TLC was performed on either whole reaction mix or an acidified (1 N HCl) extract (partitioned into petroleum ether–hexane–ethyl acetate, 1:1:1). Samples were spotted onto silica gel TLC plates (Sigma) and developed in hexane–ethyl acetate, 5:2. Developed plates were examined under 365 nm illumination and after exposure to I_2 vapor or H_2SO_4 treatment.

RESULTS

General assay characteristics. Figure 1 shows the excitation and emission spectra of HFC compared to that of EFC and NADPH. It can be seen that, at the wavelengths of 410 and 510 nm, the combined interfering fluorescence of both components was more than 1000-fold lower than that of HFC on a molar basis (Fig. 1, panel 4; note difference in scales).

Initially, we found a dependence of specific activity (rate/mg microsomal protein) on microsome concentration at less than ~50 μg/ml (Fig. 2). The dependence was most pronounced with PB-induced

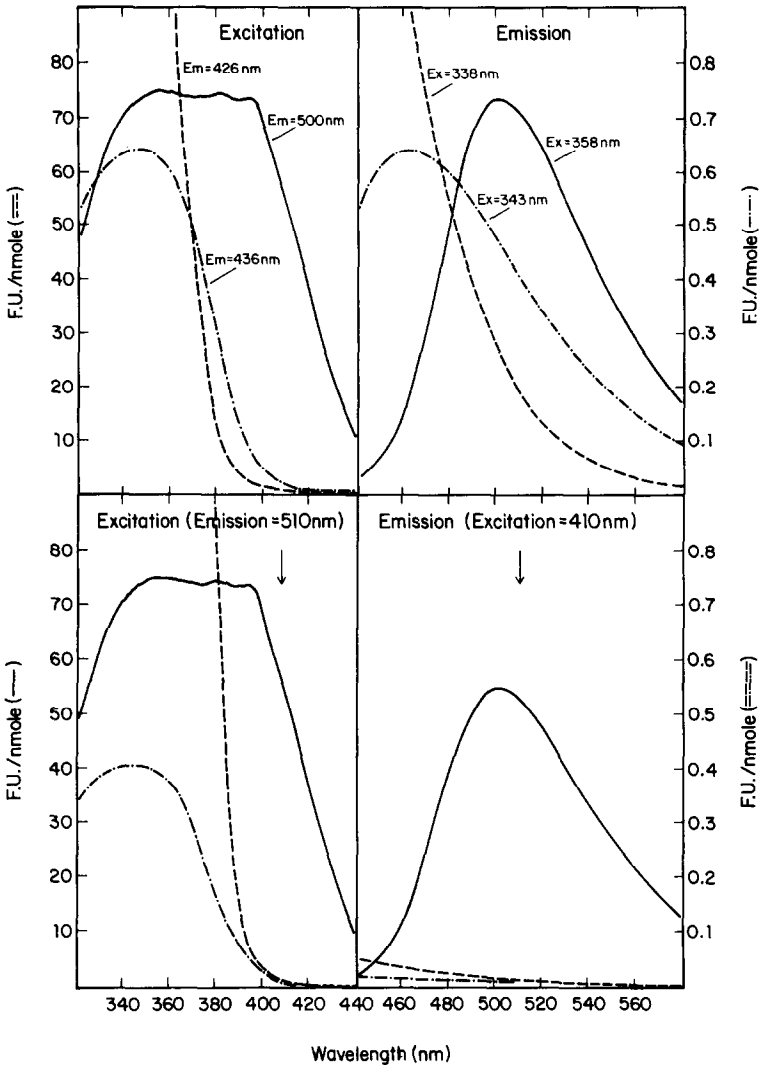


Fig. 1. Fluorescence spectra of EFC, HFC and NADPH. Top panels show excitation and emission with corresponding emission or excitation wavelength set to λ_{max} (indicated in figure). Bottom panels show spectra with excitation at 410 and emission at 510. Note different scale for NADPH (all panels) and EFC (lower panels). Key: (—) HFC; (- - -) EFC; and (- · · ·) NADPH. Spectra were taken in complete reaction mix with 10 μg/ml heat-inactivated microsomes, slits = 5 × 5, normal PMT gain. F.U. = fluorescence units. Arrows in lower panels indicate the corresponding excitation (left panel) and emission (right panel) wavelengths used in the assay.

microsomes, less so with MC-induced microsomes, and very small with uninduced microsomes. Further investigation revealed that this appears to result from some non-specific protein concentration phenomenon since addition of BSA to the reaction mix abolished the decrease in specific activity (data not shown). We are not sure why the specific activity for MC-induced microsomes appeared to decreased with increasing microsome concentration (slopes of F.U. vs time remained linear) but, for all three preparations, the range of 50–200 μg/ml appeared to represent an "optimal" one when sample was not limited. Therefore, we routinely used 150 μg/ml in our current studies, or where sample was limited, we added BSA to achieve the same final protein concentration.

Mediation of cytochrome P-450 system. The reaction requirements shown in Table 1 were examined as indications of the involvement of the cytochrome P-450 system in the generation of HFC from EFC. The reaction was O_2 dependent, and there was a strict requirement for exogenous NADPH; under our standard conditions, NADPH produced by the generating system was in excess since reducing the concentration of all compounds by a factor of two had no effect on the reaction rate (data not shown). The incomplete CO inhibition was probably the result of not directly gassing the microsomes (see legend). Inhibition of the reaction by *n*-octylamine makes involvement of the microsomal flavin monooxygenase (amine oxidase) in EFC *O*-deethylation unlikely [5, 6]. Another hallmark of cytochrome P-

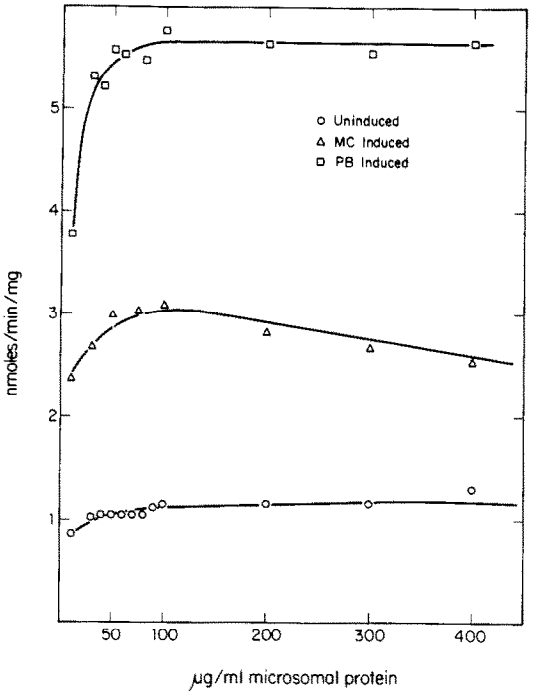


Fig. 2. Relationship of microsome concentration to apparent specific activity. Each point represents the average of two to six determinations. EFC concentration was 30 μM. Note that the ordinate represents specific activity, i.e. nanomoles per minute per milligram of microsomal protein, not activity.

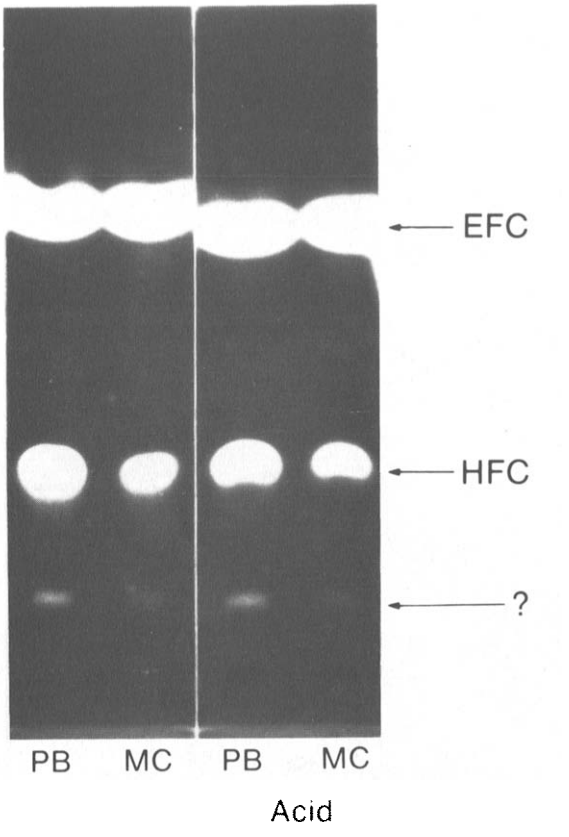


Fig. 3. TLC of reaction products. Reactions were either spotted directly or acidified and extracted before development on silica gel plates (see Materials and Methods). Plates were then photographed under 365 nm illumination. The unidentified band had the same color fluorescence as EFC (purple) rather than HFC (green).

450 catalyzed reactions is xenobiotic inducibility. This was examined for various agents and is discussed below.

Reaction products. TLC of reaction products from incubations with uninduced, PB- and MC-induced microsomes was performed to determine if HFC is the major reaction product. Examination and photography of the TLC plate under 365 nm light revealed only two major bands, EFC and HFC. Overexposure of the film revealed a third faint band (Fig. 3) which appeared to be proportional in intensity to the product band but to have the same fluorescence wavelength as substrate, indicating that a small percentage of the substrate is converted to some product other than HFC. This band was not seen in uninduced incubations (data not shown).

Table 1. Cytochrome P-450 mediation of EFC *O*-deethylation

Reaction mix	% Activity
Complete	100
$-O_2$*	8
−NADP	0.16
+CO†	34
+3 mM Octylamine	5

* Complete reaction mix (−substrate) was gassed for 5 min with N_2.

† Buffer (0.96 ml) was gassed with CO for 5 min prior to addition of other reaction components. This procedure using N_2 was shown to result in no inhibition, thereby ruling out the possibility of CO gassing resulting in O_2 depletion.

Staining of the plate with H_2SO_4 or I_2 failed to reveal any non-fluorescent bands which were not apparent in "no EFC" controls (data not shown), indicating that HFC is by far the most abundant of all reaction products.

Linearity and sensitivity. One advantage of a direct assay is assurance of a linear reaction rate over the time course examined, even when assaying very high activity samples. Examples of this are shown in Fig. 4A for a standard microsome concentration of 150 μg/ml (both uninduced and PB-induced microsomes) and in Fig. 4B for an assay using either 150 or 50 ng/ml of uninduced microsomes (supplemented with BSA, see above). It can be seen that, while the absolute level of background fluorescence varied from assay to assay, the background rate of change with time was extremely small, allowing measurements of very low activities (see "Comparison of EFC with 7-EC").

Xenobiotic inducibility. Initially, we examined induction with the prototypic-inducing agents PB and MC. While both agents clearly induced EFC *O*-deethylase activity, the isozymes induced by MC appeared to have high affinity for EFC, whereas those induced by PB had low affinity relative to control. This is illustrated in Fig. 5 where linear regression analysis of the data presented gives the

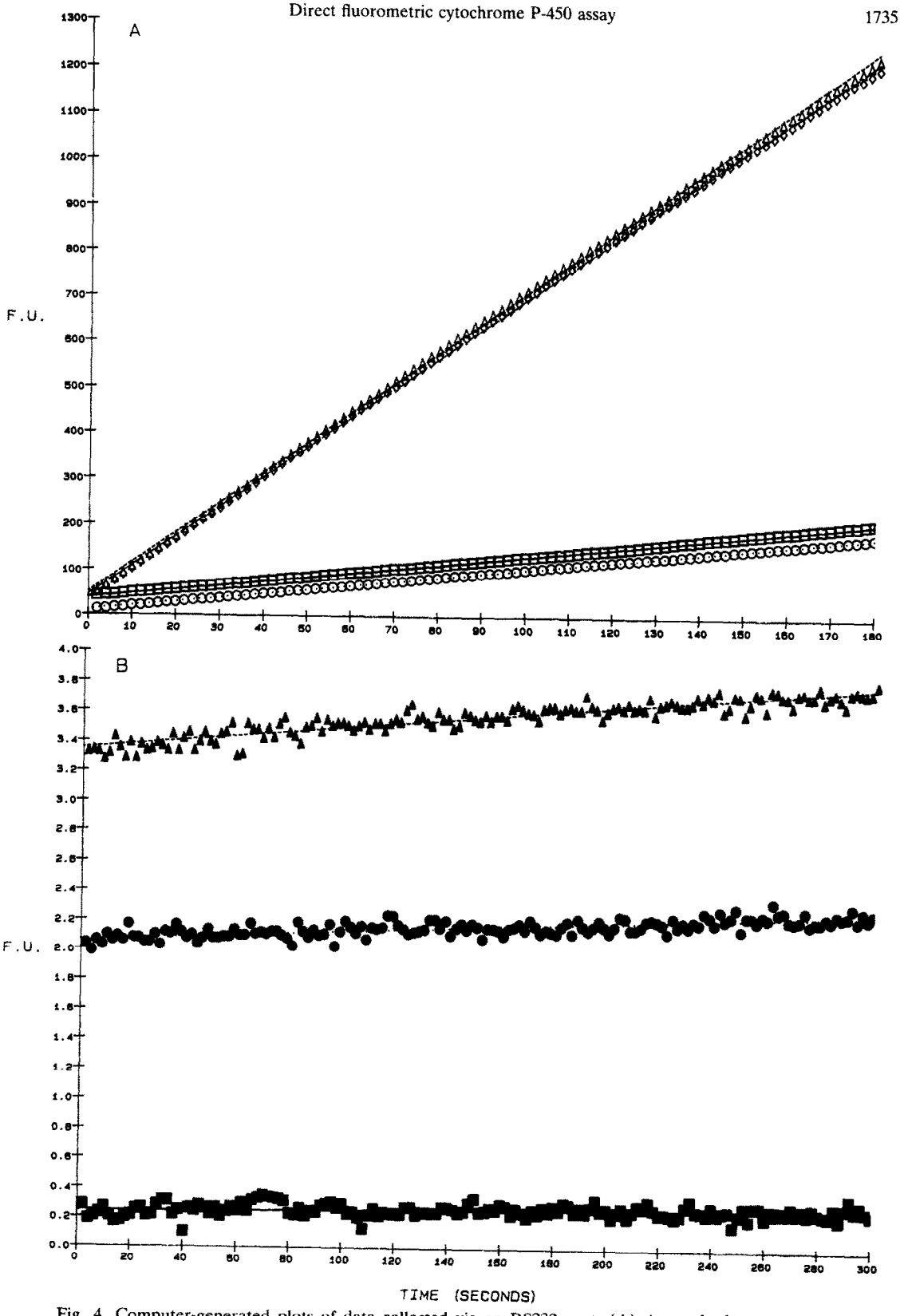


Fig. 4. Computer-generated plots of data collected via an RS232 port. (A) A standard microsome concentration of 150 μg/ml was used. Two independent runs with each microsome sample are shown. Uninduced microsomes: (□) Y = 0.94X + 38.2, and (○) Y = 0.93X + 11.1. PB-induced microsomes: (△) Y = 6.85X + 50.2, and (◇) Y = 6.50X + 44.4. (B) Uninduced microsomes at very low concentrations (supplemented to 150 μg/ml with BSA, see text). Key: (■) background (-NADP), Y = 9.35×10^{-5}X + 0.24, (●) 0.05 μg/ml, Y = 6.68×10^{-4}X + 2.07, and (▲) 0.15 μg/ml, Y = 1.45×10^{-3}X + 3.35. Linear regression slopes are given in F.U./sec.

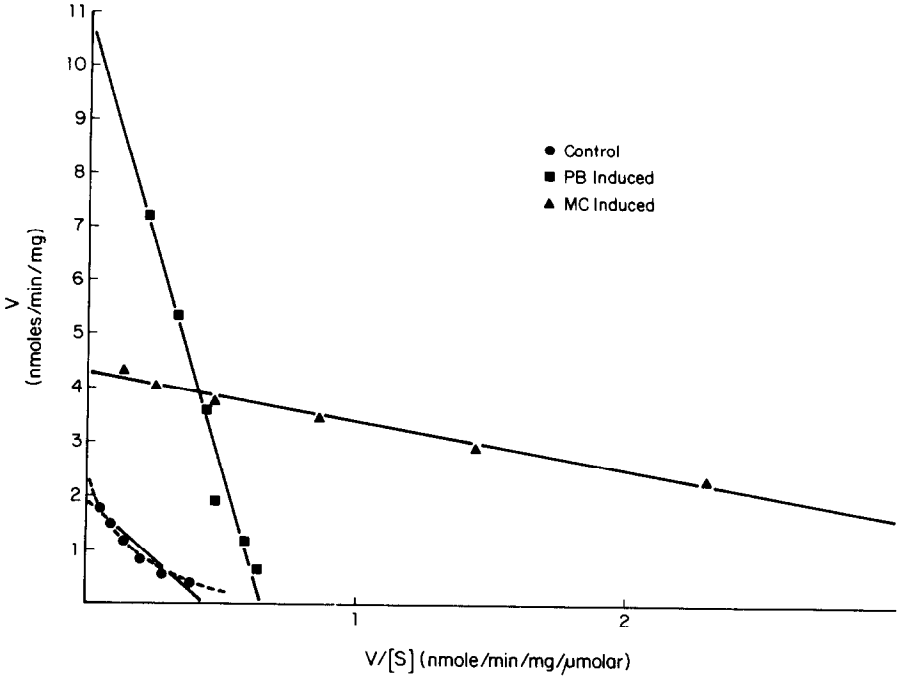


Fig. 5. Eadie–Hofstee plots for control, PB-induced and MC-induced microsomes. Points represent the average of six to nine independent determinations. The average standard error of the mean for control, PB and MC microsomes was 5.4, 5.0 and 2.8% respectively. Least squares linear regression is shown (solid lines). Dotted line is drawn by eye, see text.

Table 2. Comparison of EFC with 7-EC

(A*) Specific activity (nmol/min/mg)			
Microsomes (μg/ml)	7-EC, Direct†	7-EC, Indirect‡	EFC
150	1.41 (0.023)	1.44 (0.038)	1.08 (0.062)
15	1.28 (0.061)	1.41 (0.040)	1.03 (0.035)
1.5	1.14 (0.45)	1.04 (0.084)	1.05 (0.043)
0.5	NR	0.81	1.04 (0.061)
0.15	NR	NR	1.27 (0.26)§
0.05	ND‖	ND	1.91 (0.66)§

(B¶) Fold increase over control				
Inducing agent	7-EC, Direct†		EFC	
	Male	Female	Male	Female
PB	1.4**	ND	6.7††	5.4††
MC	4.2††	ND	2.0††	ND
DEX	2.5††	2.0††	1.3††	0.9††
ETOH	1.8††	1.8††	1.1**	1.2††

* A: NR = no response over background or replicates gave highly variable responses. All incubations were run in the presence of 150 μg/ml protein (BSA replaced microsomal protein). Numbers in parentheses represent standard deviations of two to four replicate determinations (not all values were replicated).

† Method of Ullrich and Weber [3].

‡ Method of Greenlee and Poland [1].

§ Assayed for 5 min.

‖ ND = not done.

¶ B: Four animals of each sex per group were used; each was assayed independently (microsomes were not pooled). Student's *t*-test with equal variance was used for statistical analysis. Control values: EFC, 0.97 nmol/min/mg microsomal protein; and 7-EC, 0.98 nmol/min/mg microsomal protein.

** Significantly different from control, $P < 0.05$.

†† Significantly different from control, $P < 0.01$.

following parameters (V_{max} is given in nanomoles per minute per milligram and K_m in micromolar): uninduced microsomes—V_{max} = 1.84, K_m = 4.15; MC-induced—V_{max} = 4.27, K_m = 0.90; and PB-induced—V_{max} = 10.68, K_m = 16.43. These changes in apparent K_m are similar to those noted by Ullrich and Weber [3] in mice and by Greenlee and Poland [1] in rats for 7-EC *O*-deethylation; however, unlike these investigators we were unable to detect two distinct populations of differing K_m in induced microsome preparations. This may be due to the limited solubility of EFC which restricted our ability to examine concentrations greater than 100 μM. Alternatively, EFC may have a different set of isozyme affinities than 7-EC. This is suggested by the different response of EFC to various inducing agents (see below) as well as by the suggestion of heterogeneity seen in uninduced microsomes (dotted line, Fig. 5), a property not seen with 7-EC.

Comparison of EFC with 7-EC. Many laboratories have examined the characteristics of 7-EC assays for a variety of liver and hepatocyte preparations using both direct [3, 7] and indirect [1, 8] methods.

7-EC seems to be a good "general purpose" substrate, in that it does not appear to have high specificity for particular cytochrome P-450 isozymes, unlike, for example, the alkoxyphenoxazones [9], which appear to be highly specific for various cytochrome P-450 isozymes, depending on the nature of the alkoxy moiety. We have directly compared some characteristics of 7-EC vs EFC, and the data are shown in Table 2A.

At very low activity levels, the calculated specific activity for both the direct and indirect 7-EC assays appeared to decrease, whereas for EFC it increased. If one assumes that the specific activity determined at high, easily measurable activity levels is an accurate reflection of the true specific activity, then significant departures from this value give an indication that the assay is no longer working reliably. The level at which the specific activity began to deviate significantly from that calculated at 150 μg/ml was approximately 1.5 μg/ml for direct 7-EC assay, 0.5 μg/ml for the indirect 7-EC assay, and 0.05 μg/ml for the EFC assay, indicating that the EFC assay was approximately ten times as sensitive as the indirect 7-EC assay and about thirty times as sensitive as the direct 7-EC assay.

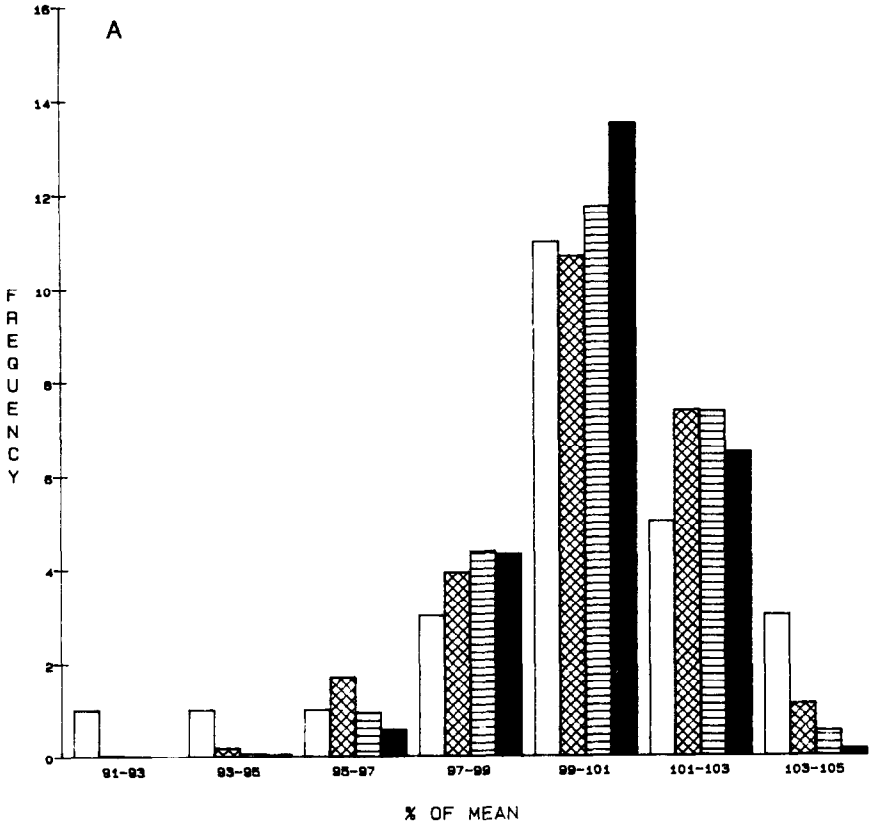
The results with different inducing agents imply that the 4-trifluoromethyl moiety of EFC alters the isozyme affinity profile of 7-EC. While 7-EC *O*-deethylase activity was most efficiently induced by MC and only moderately by PB, the reverse was true of EFC *O*-deethylation. While both substrates detected induction by DEX and ETOH, 7-EC gave a more pronounced response than EFC. The EFC response to DEX appeared sex specific, with males displaying increases in activity and females displaying decreases. A similar sex-specific response to PCN induction was seen with 7-EC but with males showing decreases and females increases, while EFC gave 12% decreases in both sexes (data not shown).

Reproducibility and statistical evaluation. Since we routinely use the EFC *O*-deethylase activity to screen unknown compounds for cytochrome P-450 induction potential, we wished to evaluate the optimal number of replicate assays to perform on each sample (inter-assay variability) as well as the optimal number of animals to use per dose group. To evaluate inter-assay variability, twenty-five replicate assays were run on the same microsomes preparation (pooled PB-induced microsomes were used as we had noted that induced microsomes tended to give somewhat more variable results than uninduced microsomes). Randomly selected groups of two, three or four separate values were generated from this data set, means were calculated, and the distributions of all four of the resulting data sets compared (Fig. 6). It can be seen that means of three or four replicates showed similar distributions and eliminated the tail seen with individual determinations.

To evaluate inter-animal variability, microsomes were prepared from two sets of rats, twelve uninduced and twelve induced with PB. Triplicate assays (as suggested by the above analysis) were run on each animal that was used. The two data sets of twelve values so generated were subjected to the same procedure outlined above (see legend to Fig. 6 for details) and are shown in Fig. 6, B and C. It can be seen, as might be expected, that inter-animal variability was much greater than inter-assay variability, particularly with induced animals. Averaging groups of three or more animals normalized the distributions, and groups of four animals gave somewhat superior distributions than did groups of three. In addition, it was noted that the occasional outlying animal could drastically skew statistical evaluation in studies with groups of only three, whereas this effect was minimized with groups of four (data not shown).

DISCUSSION

We have explored the utility of a novel coumarin analog, 7-ethoxy-4-trifluoromethylcoumarin (EFC), in the estimation of the level of uninduced and xenobiotic-induced cytochrome P-450 activities. We found that EFC had spectral characteristics which make it amenable to use in a direct fluorescence assay (Fig. 1) of high sensitivity (Table 2A). It appeared to be mediated almost exclusively by NADPH-requiring enzymes with all the hallmarks of the cytochrome P-450 mixed-function oxidase system (Table 1). The level of generation of products other than HFC seemed to be very low (Fig. 3) so substrate depletion by "invisible" metabolism was not a problem. The high fluorescence yield of HFC relative to EFC at an excitation wavelength of 410 nm combined with good linearity, a very low background drift (Fig. 4), and a high level of activity (mean of 1.5 nmol/min/mg for rat) with uninduced samples led to a very favorable degree of sensitivity in comparison to the widely used substrate 7-EC (Table 2A). EFC responded to a variety of inducing agents, as did 7-EC (Table 2B), but appeared to be more specific for PB-like inducers than 7-EC. Nevertheless, we found that our historical mean standard deviation among groups of four animals, 9.6%, allowed detection of changes in activity on the order of 10–20% at the 95% confidence level (*t*-test, equal variance) and permitted detection of a wide variety of inducing agents.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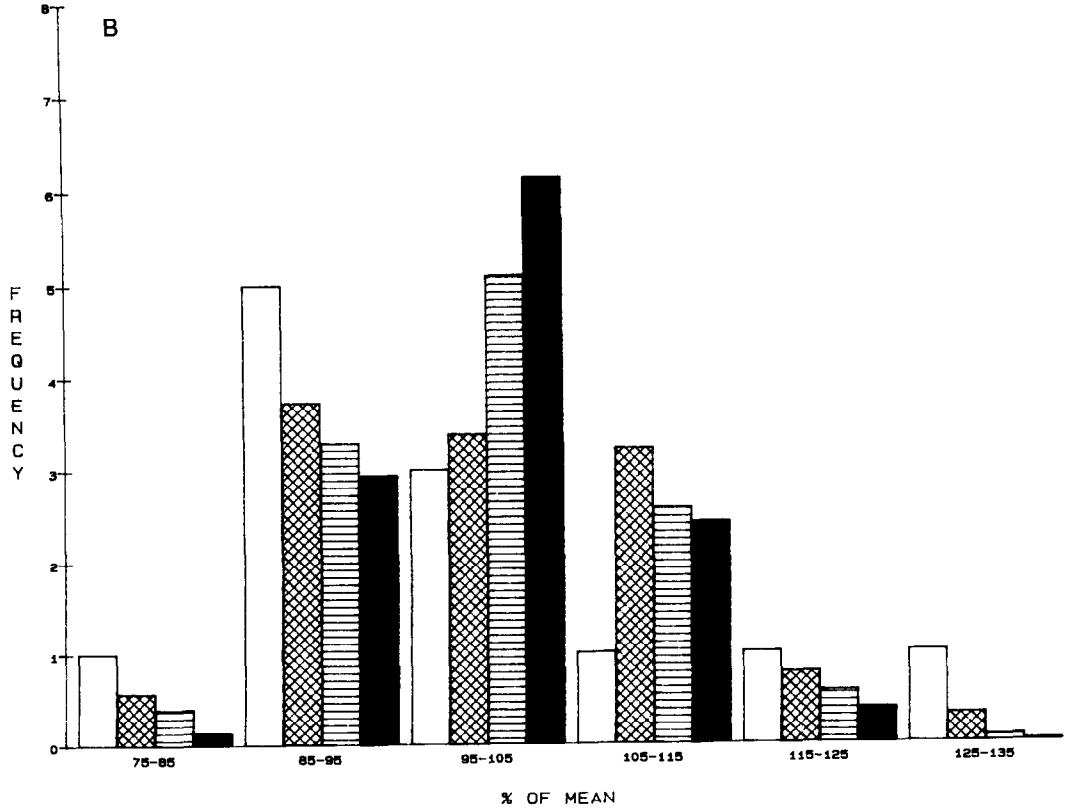
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Fig. 6. (A) and (B)

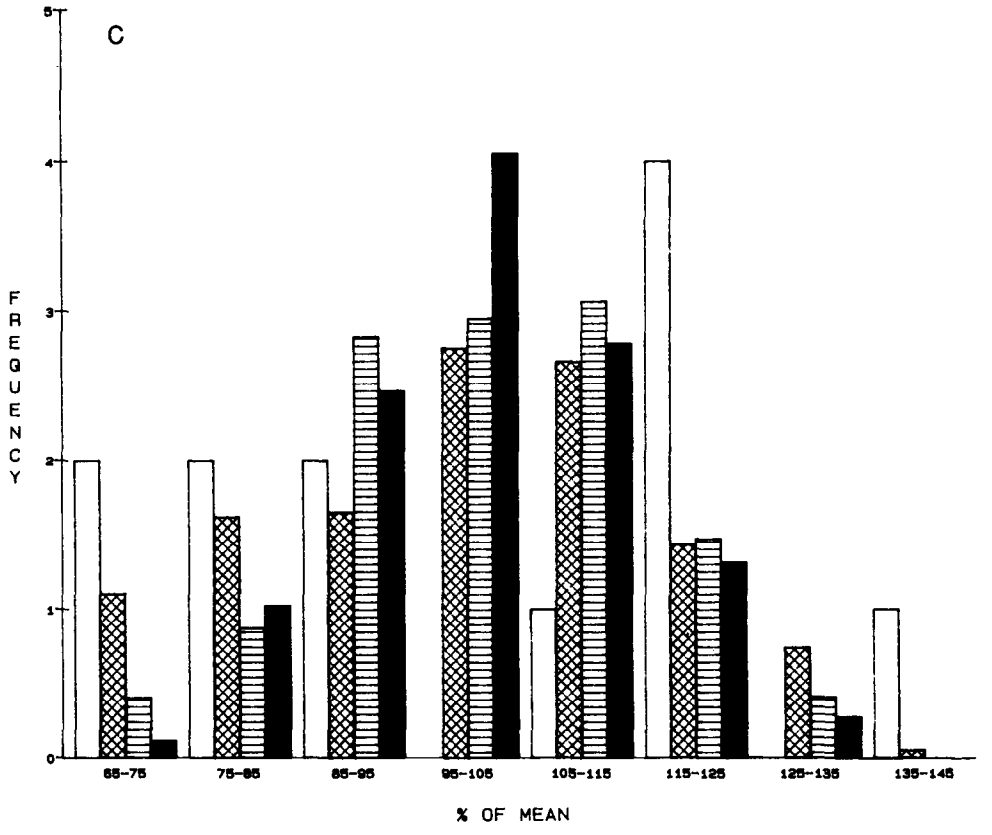

Fig. 6. Influence of replicate number on assay variability. Means of groups of two, three or four values randomly selected from the populations of twenty-five values (A) or twelve values (B and C) were calculated by a computer program. In order to eliminate bias in the selection of a particular group of means, 2500 (A) or 1200 (B and C) values were calculated, and the resultant frequencies were divided by 100. This accounts for fractional values in the frequency distributions of the mean values. Assays were performed using (A) PB-induced microsomes; (B) uninduced rats; and (C) PB-induced rats. For all graphs, (□) = individual values, (▩) = means of two (▤) = means of three, and (■) = means of four.

We are currently making use of the high sensitivity of this assay to develop a direct assay for cultured cells, allowing one to monitor cytochrome P-450 activity by medium fluorescence and avoiding the workup necessary with 7-EC [10].

In conclusion, we feel that EFC is a useful substrate for monitoring cytochrome P-450 activity in both *in vivo* and *in vitro* systems with some distinct advantages over 7-EC and that it represents a valuable addition to the tools available for investigation of this important enzyme system.

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