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7-Ethoxy-3,4-dimethylcoumarin: a Substrate for a Cytochrome P450-mediated Mono-oxygenase Activity that is Highly Induced by Phenobarbitone and β-Naphthoflavone

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Abstract

The *O*-dealkylation of 7-ethoxy-3,4-dimethylcoumarin in rat liver microsomes was catalysed in a typical cytochrome P450-mediated reaction as judged by cofactor requirement and inhibition criteria, and displayed monophasic Michaelis–Menten kinetics. When measured at low substrate concentration, this activity was highly inducible by treatment with phenobarbitone or β-naphthoflavone (44- and 78-fold induction, respectively). These data indicate the potential usefulness of this activity as a probe for P4501A1- and P4502B-mediated activities. The *O*-dealkylation of 7-methoxy- and 7-propoxy-3,4-dimethylcoumarin were much less inducible.

The *O*-dealkylation of 7-ethoxycoumarin has become a standard marker for cytochrome P450-mediated reactions (Prough et al 1978). The fluorescent characteristics of the product, 7-hydroxycoumarin, ensures a good sensitivity, and the reaction appears to be catalysed by a number of P450 forms, particularly those of the 1A and 2B families induced by polycyclic aromatic hydrocarbons and barbiturates, respectively. 7-Methoxy- and 7-propoxycoumarin have also been used, albeit less frequently, as substrates for P450-mediated reactions, and there are some indications that *O*-dealkylations of these alkoxycoumarins may be carried out by forms of P450 different from, or in addition to, those involved in 7-ethoxycoumarin *O*-dealkylation (Matsubara et al 1983; Reen et al 1991; Fry et al 1992).

However, in any given microsomal preparation 7-ethoxycoumarin is *O*-dealkylated by at least two forms of P450, as judged by biphasic kinetics and inhibitor studies (Boobis et al 1981, 1986; Paterson et al 1984), so that 7-ethoxycoumarin is not a unique marker for a particular P450. Furthermore, in-vivo and in-vitro studies have indicated that 7-alkoxycoumarins are metabolized by routes other than *O*-dealkylation (Indahl & Scheline 1971; Jung et al 1985). In particular, the 3-hydroxylation pathway is a major contributor to the overall metabolism of these compounds, the 3-hydroxy-7-alkoxycoumarin not sharing the desirable fluorescence properties of the 7-hydroxycoumarin. Jung et al (1985) have demonstrated that activity of the 3-hydroxylation pathway is selectively increased by some P450 inducers. Induction of the 3-hydroxylation pathway, by diverting substrate to this pathway, would reduce the sensitivity of the *O*-dealkylation pathway as a marker of P450 induction. Certainly, the magnitude of induction of 7-ethoxycoumarin *O*-dealkylation by common inducers is much less than that observed with some other, more-specific, substrates, such as the alkoxyresorufins (Lubet et al 1990).

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The hepatotoxicity of coumarin in rats is believed to be mediated via a 3,4-epoxide, which is converted to other metabolites, one of which is 3-hydroxycoumarin (Lake et al 1989). We, and others, have recently demonstrated that the hepatotoxicity of coumarin is blocked by 3,4-dimethyl substitution, which we ascribe to a blocking of the epoxidation pathway (Fernyhough et al 1994; Lake et al 1994).

In an attempt to produce a better substrate for P450, we have investigated the *O*-dealkylation of 7-alkoxy-3,4-dimethylcoumarins, in particular 7-ethoxy-3,4-dimethyl-coumarin, in liver microsomes isolated from untreated rats and rats treated with one of a range of archetypal inducers. The results of this study are presented in this paper.

Materials and Methods

Chemicals

Resorcinol, ethyl 2-methylacetoacetate and the alkyl iodides were obtained from the Aldrich Chemical Co. (Gillingham, Dorset, UK). Isocitrate, isocitrate dehydrogenase, $NADP^+$, metyrapone, 1-aminobenzotriazole, and *n*-octylamine were obtained from the Sigma Chemical Co. Ltd (Poole, Dorset, UK).

Chemical synthesis

7-Hydroxy-3,4-dimethylcoumarin was prepared (35% yield) by condensation of resorcinol with ethyl 2-methylacetoacetate, according to the method of Ahluwalia et al (1988). The mp was 238–239°C (literature value: 238–240°C (Ahluwalia et al 1988)), and the compound was chromatographically pure by TLC (silica gel, solvent system chloroform:ethyl acetate, 4 : 1). The 7-alkoxy-3,4-dimethylcoumarins were prepared by alkylation of 7-hydroxy-3,4-dimethylcoumarin with the appropriate alkyl iodide, essentially as described for 7-ethoxycoumarin (Greenlee & Poland 1978). The products produced a single spot on TLC (system as described above) and had melting points of

134–135°C (methyl product), 120–121°C (ethyl product; literature value: 121–122°C (Chatteerjee et al 1987; Ahluwalia et al 1988)), and 65–66°C (propyl product).

Animals and treatment

Male Wistar rats (approx. 120 g) were obtained from the Biomedical Services Unit of the Medical School, University of Nottingham. They were housed in groups of six animals in plastic-bottomed cages at constant temperature (22°C) with a 12-h light–dark cycle (0800 h light, 2000 h dark), and allowed free access to standard laboratory diet (Diet 41B, Heygates (Pilsbury) Ltd, York, UK) and drinking fluid (water or inducer). The protocols for treatment with phenobarbitone, β-naphthoflavone, isosafrole, isoniazid, pregnenolone 16α-carbonitrile, and clofibrate have been described previously (Fry et al 1992). Control animals received no treatment.

Enzyme assay

Microsomes were isolated from the pooled livers of 4–6 rats by Ca^{2+}-aggregation as described previously (Fry et al 1992) and stored at − 80°C until use. The incubation mixture contained liver microsomes (approx. 100 μg protein unless stated otherwise), phosphate buffer (0·1 M, pH 7·4), $NADP^+$ (0·5 mM), isocitrate (5 mM), isocitrate dehydrogenase (1 unit), and magnesium sulphate (5 mM), in a total volume of 1 mL. The reaction was started by the addition of the substrate (added in 5 μL methanol), and after incubation at 37°C for up to 30 min, the reaction was terminated by the addition of 10% trichloroacetic acid (0·2 mL). A sample (0·1 mL) of the supernatant obtained after centrifugation to pellet the denatured protein was mixed with 2·5 mL 0·4 M glycine-NaOH buffer pH 10·4, and the fluorescence measured at 450 nm after excitation at 390 nm, using a Baird Fluoropoint spectrofluorimeter. The fluorescence so obtained was compared with that of standard 7-hydroxy-3,4-dimethylcoumarin added to the incubation mixture and processed as described above.

Data analysis

Comparisons between treatment groups were performed by analysis of variance followed by Dunnett's test, as run on the InStat computer program. Enzyme kinetic constants were obtained by iterative least squares analysis of the untransformed activity data (Ultrafit program).

Results

Preliminary studies indicated that the fluorescence characteristics of 7-hydroxy-3,4-dimethylcoumarin in alkaline solution were identical to those of 7-hydroxycoumarin, so that the measurement technique developed for 7-hydroxycoumarin could be used for the dimethyl-substituted derivative. Furthermore, fluorescence values obtained with 7-hydroxy-3,4-dimethylcoumarin were linear in the concentration range 5–1000 μM (data not shown).

The *O*-deethylation of 7-ethoxy-3,4-dimethylcoumarin proceeded linearly with respect to time for at least 20 min at both low (10 μM) and high (1000 μM) substrate concentrations in liver microsomes from untreated rats (Fig. 1). In addition, the rate of this reaction was linearly related to protein concentration in the range 9–450 μg protein/tube (Fig. 2). This reaction was inhibited by more than 50% by recognized inhibitors of the

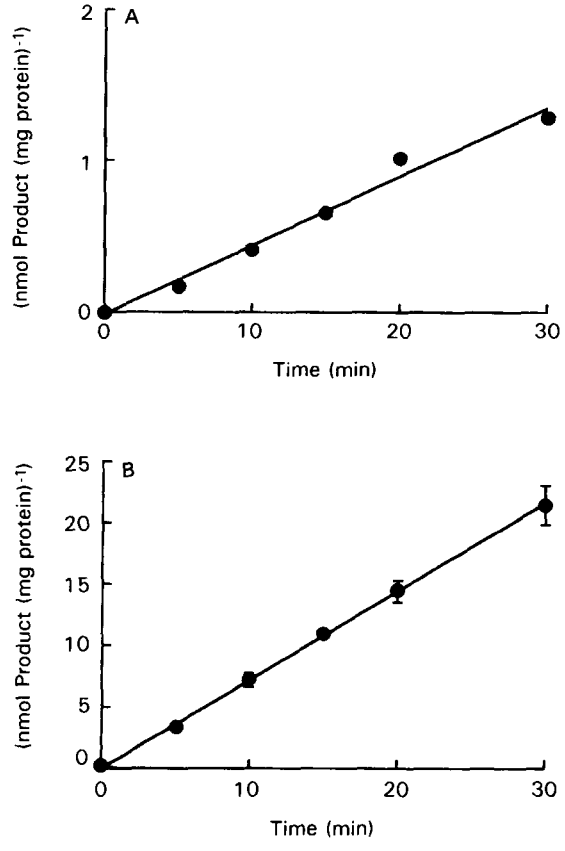

Fig. 1. Time course of 7-ethoxy-3,4-dimethylcoumarin *O*-deethylation in liver microsomes isolated from control rats at substrate concentrations of 10 (A) and 1000 μM (B) at a protein concentration of 100 μg (mL incubation)$^{-1}$. Values are mean ± s.e.m. of three experiments.

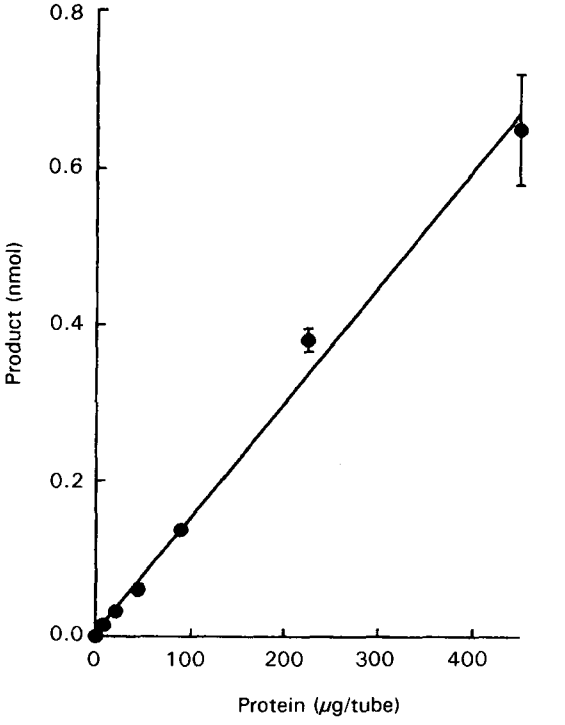

Fig. 2. Dependence of 7-ethoxy-3,4-dimethylcoumarin *O*-deethylase activity on protein concentration. Enzyme activity was measured in liver microsomes from control rats using a substrate concentration of 1000 μM and a 10 min incubation period. Values are mean ± s.e.m. of three experiments.

Table 1. Effect of inhibitors and omission of cofactors on 7-ethoxy-3,4-dimethylcoumarin *O*-deethylase activity.

Assay system	Activity (% of control)
Complete system	100
Without cofactors	0
+ CO (1 min before assay in capped tubes)	35
+ Metyrapone (1 mM)	39
+ 1-Aminobenzotriazole (0·1 mM)	38
+ *n*-Octylamine (3 mM)	6

Activity was measured in liver microsomes isolated from untreated rats at a substrate concentration of 1 mM. 100% = 1·3 ± 0·1 nmol product (mg protein)$^{-1}$ min^{-1}.

P450 system (CO, metyrapone, 1-aminobenzotriazole, octylamine), whilst omission of cofactors produced no demonstrable reaction (Table 1). Essentially identical results were obtained when 7-ethoxycoumarin was used as substrate (data not shown).

In light of the existence of biphasic kinetics for 7-ethoxycoumarin *O*-deethylation, we investigated the *O*-dealkylation of 7-methoxy-, 7-ethoxy-, and 7-propoxy-3,4-dimethylcoumarin in liver microsomes from rats treated with one of a number of prototypic inducers at two substrate concentrations, 10 and 1000 μM, following the rationale presented previously (Boobis et al 1981, 1986; Fry et al 1992). The results are presented in Table 2.

The *O*-dealkylation of 10 μM 7-methoxy-3,4-dimethylcoumarin was significantly increased following treatment with phenobarbitone, β-naphthoflavone and isosafrole, but significantly decreased following treatment with isoniazid and pregnenolone 16α-carbonitrile. Somewhat similar results were obtained with 1000 μM 7-methoxy-3,4-dimethylcoumarin, with the exception that isosafrole treatment produced a modest but significant reduction in activity. The *O*-dealkylation of 10 μM 7-ethoxy-3,4-dimethylcoumarin was markedly increased following treatment with phenobarbitone and β-naphthoflavone (44- and 78-fold, respectively), with a modest induction after isosafrole treatment (7-fold) and no induction following treatment with pregnenolone 16α-carbonitrile or clofibrate. The *O*-dealkylation of 1000 μM 7-ethoxy-3,4-dimethylcoumarin was increased following treatment with each of the prototype inducers (1·4- to 7·3-fold increase) with the exception of clofibrate for which a significant decrease was observed.

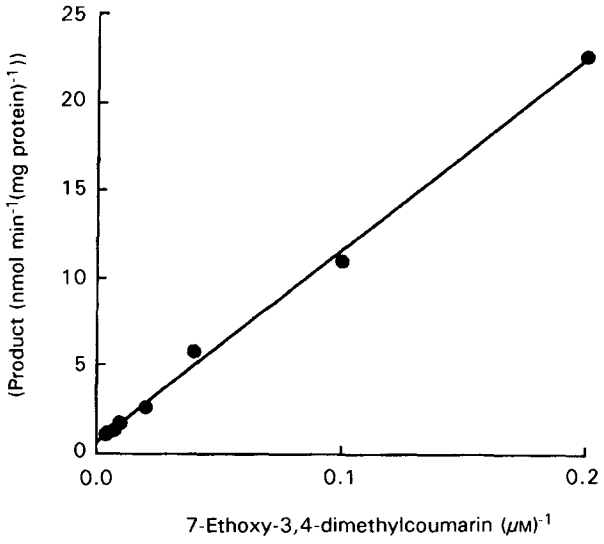

Fig. 3. Lineweaver–Burk plot of substrate concentration dependence of 7-ethoxy-3,4-dimethylcoumarin *O*-deethylase activity in liver microsomes isolated from untreated rats. Values are mean of four experiments.

The *O*-dealkylation of 10 μM 7-propoxy-3,4-dimethylcoumarin was significantly increased following treatment with phenobarbitone and β-naphthoflavone (3·8- and 16-fold, respectively) but was unaffected by isosafrole treatment. The *O*-dealkylation of 1000 μM 7-propoxy-3,4-dimethylcoumarin was again increased after treatment with phenobarbitone and β-naphthoflavone (1·5- and 4·8-fold, respectively) but significantly decreased following isosafrole treatment.

The marked inducibility of 7-ethoxy-3,4-dimethylcoumarin *O*-dealkylation by phenobarbitone and β-naphthoflavone at low substrate concentration, and the difference in magnitude of induction between low and high substrate concentration, prompted us to undertake a more detailed analysis of the kinetics of this reaction. Lineweaver–Burk analysis of these data provided good evidence for the existence of monophasic kinetics of 7-ethoxy-3,4-dimethylcoumarin *O*-dealkylation in the concentration range 5–250 μM (Fig. 3). Comparable con-

Table 2. 7-Alkoxy-3,4-dimethylcoumarin *O*-dealkylase activity in rat liver microsomes, assayed with three different substrates, each at two concentrations.

Treatment	*O*-Dealkylase activity (nmol product (mg protein)$^{-1}$ min^{-1})					
	7-Methoxy-3,4-dimethylcoumarin		7-Ethoxy-3,4-dimethylcoumarin		7-Propoxy-3,4-dimethylcoumarin	
	10 μM	1000 μM	10 μM	1000 μM	10 μM	1000 μM
Control	0·25 ± 0·01	0·75 ± 0·01	0·09 ± 0·00	1·10 ± 0·02	0·08 ± 0·00	0·40 ± 0·01
Phenobarbitone	0·70 ± 0·00*	1·30 ± 0·01*	4·00 ± 0·08*	6·50 ± 0·06*	0·30 ± 0·01*	0·60 ± 0·01*
β-Naphthoflavone	2·00 ± 0·00*	2·60 ± 0·04*	7·00 ± 0·06*	8·00 ± 0·07*	1·30 ± 0·02*	1·90 ± 0·02*
Isosafrole	0·40 ± 0·01*	0·60 ± 0·01*	0·60 ± 0·01*	2·80 ± 0·04*	0·10 ± 0·00	0·20 ± 0·03*
Isoniazid	0·12 ± 0·00*	0·40 ± 0·01*	0·15 ± 0·00	2·20 ± 0·01*	ND	ND
Pregnenolone 16α-carbonitrile	0·10 ± 0·00*	0·30 ± 0·01*	0·10 ± 0·00	1·50 ± 0·01*	ND	ND
Clofibrate	ND	ND	0·08 ± 0·02	0·70 ± 0·06*	ND	ND

Values are mean ± s.e.m. of 4–6 determinations. ND, not determined. *$P < 0·01$ compared with appropriate control.

Table 3. Kinetic constants for the *O*-deethylation of 7-ethoxy-3,4-dimethylcoumarin in rat liver microsomes.

Treatment	K_m (μM)	V_{max} (nmol product (mg protein)$^{-1}$ min^{-1})
Control	123·86 ± 12·29	1·33 ± 0·06
Phenobarbitone	8·97 ± 1·78	6·21 ± 0·25
β-Naphthoflavone	4·27 ± 0·78	7·05 ± 0·55

Values are mean ± s.e.

clusions were drawn from analysis of the concentration dependence of this reaction in liver microsomes isolated from phenobarbitone- and β-naphthoflavone-treated rats. The apparent kinetic constants obtained by nonlinear regression of the data (assuming activity of a single enzyme) are presented in Table 3. The values for the goodness-of-fit index were in the range 0·970–1000, confirming the validity of the single-enzyme model. Treatment with phenobarbitone and β-naphthoflavone produced marked increases in the number of catalytic sites, as judged by reduction in apparent K_m value, which were accompanied by approximately 5-fold increases in apparent maximal velocity. These data are consistent with the enzyme activities presented in Table 2.

Promazine (2 μM, added 2 min before substrate) inhibited 7-ethoxy-3,4-dimethylcoumarin *O*-deethylase activity by 60% (control: 4·0 ± 0·2; promazine: 1·6 ± 0·1 nmol product min^{-1} (mg protein)$^{-1}$; n = 4; $P < 0·001$) following phenobarbitone treatment, but was without effect following β-naphthoflavone treatment.

Discussion

The *O*-deethylation of 7-ethoxy-3,4-dimethylcoumarin by rat liver microsomes proceeded in a linear manner with respect to time and protein concentrations under the conditions of the assay, and was catalysed in a typical P450-mediated reaction as judged by cofactor requirement and inhibition by recognized P450 inhibitors. In these respects, 7-ethoxy-3,4-dimethylcoumarin *O*-deethylation was similar to *O*-deethylation of 7-ethoxycoumarin (Ullrich & Weber 1972) and of 7-ethoxy-4-trifluoremethyl coumarin (De Luca et al 1988). However, *O*-deethylation of 7-ethoxy-3,4-dimethylcoumarin displayed two major differences to the deethylation of 7-ethoxycoumarin.

Firstly, *O*-deethylation of 7-ethoxy-3,4-dimethylcoumarin displayed monophasic Michaelis–Menten kinetics in liver microsomes isolated from both untreated and inducer-treated rats, in contrast to the biphasic kinetics exhibited by *O*-deethylation of 7-ethoxycoumarin (Boobis et al 1981, 1986). Studies of the effect of 3-cyano-substitution (White 1988) and 4-trifluoromethyl-substitution (De Luca et al 1988; Buters et al 1993) have also indicated monophasic kinetics of the *O*-deethylation of these 3- and 4-modified substrates, and it appears that an unhindered 3,4-position is essential for *O*-deethylation of 7-ethoxycoumarin by at least one form of P450. It is pertinent that purified forms of P450 display only monophasic kinetics (Guengerich 1978).

Secondly, the magnitudes of induction of 7-ethoxy-3,4-dimethylcoumarin *O*-deethylation by phenobarbitone and β-naphthoflavone when measured at low (10 μM) substrate concentration (44- and 78-fold, respectively) are considerably greater than those determined for *O*-deethylation of 10 μM 7-ethoxycoumarin (7- and 10-fold, respectively (Fry et al 1992)). In kinetic terms, induction by phenobarbitone and β-naphthoflavone produce considerable reductions in K_m with smaller increases in V_{max} (Table 3). Further discussion will, accordingly, focus principally on rates measured at low substrate concentration. These data suggest that *O*-deethylation of 7-ethoxy-3,4-dimethylcoumarin may be a useful marker for induction by agents of the phenobarbitone- and β-naphthoflavone-type. As such, this approach would be complementary to use of highly selective substrate probes such as the alkoxyresorufins (Lubet et al 1990). The magnitude of induction of 7-ethoxy-3,4-dimethylcoumarin *O*-deethylation by phenobarbitone (44-fold) is similar to that reported for pentoxy- and benzyloxy-resorufin *O*-dealkylation (54- and 43-fold, respectively (Lubet et al 1990)), whilst the magnitude of induction of 7-ethoxy-3,4-dimethylcoumarin *O*-deethylation by β-naphthoflavone (78-fold) is similar to that reported for ethoxy- and propoxy-resorufin (82- and 92-fold, respectively (Lubet et al 1990)). In support of this proposed use of 7-ethoxy-3,4-dimethylcoumarin *O*-deethylation, promazine (a selective inhibitor of P4502B1) produced effects on this reaction in the phenobarbitone/β-naphthoflavone-induced states virtually identical to those reported for alkoxyresorufin *O*-dealkylations (Murray 1989).

However, the impressive inducibility of 7-ethoxy-3,4-dimethylcoumarin *O*-dealkylation by phenobarbitone and β-naphthoflavone is not matched by the induction of *O*-dealkylation of the 7-methoxy- and 7-propoxy-analogues. For these substrates, the magnitudes of induction are similar to those obtained with 7-methoxycoumarin and 7-propoxycoumarin as substrates (Fry et al 1992). This precludes use of a range of 7-alkoxy-3,4-dimethylcoumarin substrates to selectively identify an inducer type, as can be done with the alkoxyresorufins. This information also suggests a stringent structural requirement of the 7-alkoxy-3,4-dimethylcoumarin substrate for high inducibility.

It has been established that β-naphthoflavone increases the levels of P450 1A1 and P450 1A2, whilst isosafrole selectively increases P450 1A2 (Thomas et al 1983). The finding that isosafrole treatment produced only a 7-fold inducton of 7-ethoxy-3,4-dimethylcoumarin *O*-deethylation as opposed to the 78-fold induction achieved with β-naphthoflavone, strongly suggests that most of the activity in liver microsomes in β-naphthoflavone-treated rats can be ascribed to P450 1A1.

Whilst phenobarbitone principally induces forms of the P450 2B subfamily, it also induces forms of the P450 3A subfamily (the principal forms induced by pregnenolone 16α-carbonitrile) (Heuman et al 1982). The finding of a lack of effect of pregnenolone 16α-carbonitrile treatment on 7-ethoxy-3,4-dimethylcoumarin *O*-deethylation thus indicates that the activity in liver microsomes from phenobarbitone-treated rats is mediated by P450 2B forms.

Isoniazid treatment (which induces P450 2E1 (Koop et al 1985) and clofibrate treatment (which induces P450 4A forms (Tamburini et al 1984)) were without significant effect on 7-ethoxy-3,4-dimethylcoumarin *O*-deethylation so ruling out involvement of these P450 forms in this activity.

In conclusion, the present study has demonstrated that *O*-deethylation of 7-ethoxy-3,4-dimethylcoumarin by rat liver microsomes displays monophasic Michaelis–Menten kinetics and is highly inducible by phenobarbitone and β-naphtho-

flavone, these two properties making this reaction a convenient marker for agents which induce P450 forms 1A1 and 2B.

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