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# THE EFFECT OF TERPENOID COMPOUNDS ON CYTOCHROME P-450 LEVELS IN RAT LIVER

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**Abstract**—We have investigated the ability of camphor, menthol, pinene, limonene and myrcene to induce in rats members of a cytochrome P-450 sub-family termed PB P-450. These proteins have recently been designated as members of the P450IIB sub-family. None of these naturally occurring terpenoids significantly changed the total content of cytochromes P-450 or cytochrome $b_5$. Radioimmunoassay results showed that PB P-450 was induced 6-fold by camphor and to a lesser extent by menthol and pinene. The induction was confirmed by Western blotting. It was shown by nucleic acid hybridization that induction of PB P-450 by terpenoids was mediated by an increase in the amount of the corresponding mRNA. Analysis of the denaturation of mRNA-cDNA hybrids demonstrated that the mRNA induced by the terpenoids was encoded by a member of the P450IIB sub-family. None of the terpenoids had an effect on the amount of mRNA coding for P450IA2 (a cytochrome P-450 inducible by β-naphthoflavone and isosafrole). The results indicate that cytochromes P-450 induced by a synthetic compound, phenobarbital, may have originally evolved in response to terpenoid compounds normally present in the environment.

The cytochrome P-450-mediated mono-oxygenase system is involved in the metabolism of endogenous compounds such as steroid hormones, fatty acids and prostaglandins. It is also of central importance in the detoxification or activation of a wide range of hydrophobic foreign compounds, including many drugs, carcinogens and environmental pollutants (see, for example, Ref. 1). Many members of the cytochrome P-450 super-family are inducible by a variety of foreign chemicals, such as phenobarbital, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, β-naphthoflavone [2–4], and some naturally occurring compounds such as isosafrole [5]. The specific variant induced depends on the compound to which the organism is exposed.

The ability to induce selectively the particular cytochrome P-450 required to metabolise a potentially harmful hydrophobic compound present in the environment is clearly of great benefit to the organism. However, it is not clear how organisms have evolved an ability to respond in a specific manner to synthetic compounds such as phenobarbital. A possibility is that the synthetic chemical fortuitously elicits the synthesis of a cytochrome P-450 identical to one induced by a naturally occurring compound to which the organism is normally exposed in its environment. If this is so it should be possible to identify a natural inducer for each cytochrome P-450 variant induced by a synthetic compound.

The inducing effect has been investigated of several natural compounds in a variety of organisms including Southern army worm larva [6], housefly [7], mouse [8–10] and rat [11–13]. This early work has shown that several classes of natural products including heterocyclics [6], glycosides [6] and terpenoids [6, 8–11, 13] can affect the cytochrome P-450 mono-oxygenase system and increase the rate of their metabolism. In addition, it was found that various terpenoids increase the rate of metabolism of penta- and hexabarbital, and decrease the sleeptime induced by these barbiturates [8, 9, 11, 14], suggesting that the action of the terpenoids may be mediated through an increase in the activity of a member of the major phenobarbital-inducible cytochrome P-450 sub-family P450IIB [15].

In this paper we use specific antibodies and DNA probes to investigate the effects of five terpenoids on the expression of genes coding for components of the P450IIB sub-family (termed collectively PB P-450 [16])‖ of rat liver microsomal membranes.

## MATERIALS AND METHODS

*Animals.* Camphor, limonene, menthol, myrcene and pinene were purchased from Aldrich Chemical Co. Ltd. (Gillingham, Kent) and sodium phenobarbital from British Drug House (Poole, Dorset). All terpenoids were 97% to 99% pure. Sprague–Dawley rats were bred at the University College animal facility. After mating the females were transferred to an isolation room. For the first week after birth pups were housed in plastic cages containing

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‖ Abbreviations used: PB P-450, members of the P450IIB sub-family; SSPE, 0.18 M NaCl, 10 mM sodium phosphate (pH 7.7), 1 mM EDTA; SSC, 0.15 M NaCl, 0.015 M sodium citrate (pH 7.5).

cellulose bedding. The animals were then transferred to wire-bottomed cages with no bedding material. When weaned, animals were allowed free access to water and food (diet GR3 E.K., Dixon and Sons, Ltd., Ware, Herts., U.K.) and maintained on a controlled light cycle of 12 hr light/12 hr dark. When they had reached a weight of 180–200 g, the rats were given three consecutive daily intraperitoneal injections of either camphor, limonene, menthol, myrcene or pinene dissolved in 10% ethanol, 90% corn oil at a dose of 40 mg/kg body weight. For comparison some animals were similarly treated with an identical dose of phenobarbital dissolved in 0.9% (w/v) NaCl. Control animals were injected with vehicle (10% ethanol, 90% corn oil) alone. The vehicles have no effect on amounts of PB P-450 or NF P-450 proteins or mRNAs. The rats were starved overnight and killed by cervical dislocation 18 hr after receiving the third injection.

*Isolation and solubilization of microsomal membrane vesicles.* Liver microsomal membrane vesicles were isolated by the method of van der Hoeven and Coon [17] and solubilized with 0.7% (w/v) sodium cholate as described in [18].

*Determination of specific content of cytochromes* P-450 *and cytochrome* $b_5$. Cytochrome P-450 was assayed by CO-reduced difference spectroscopy as described by Omura and Sato [19]. Cytochrome $b_5$ was determined spectrophotometrically as in Ref. 20. Protein concentrations were determined as previously described [21].

*Preparation of P-450b and antibodies.* These were as previously described [21].

*Radioimmunoassay of P-450b.* Purified P-450b was iodinated with $^{125}$I-labelled Bolton and Hunter reagent [22] as previously described [21]. Radioimmunoassay was as described by Phillips *et al.* [21].

*Western blotting.* Microsomal membranes were solubilized at a final protein concentration of 1 mg/ml in 1% (w/v) SDS/10 mM sodium phosphate buffer (pH 7.0)/1% 2-mercaptoethanol/15% (v/v) glycerol, and electrophoresed through an SDS/polyacrylamide gel [21]. Proteins were electroblotted from the gel to a nitrocellulose filter at 30 V for 20 hr in a Transblot apparatus (Bio-Rad). The transfer buffer was 25 mM Tris base/0.192 M glycine/20% (v/v) methanol. Immunostaining was done at room temperature using anti-(P-450*b*) serum and a goat-(anti-rabbit IgG)–horse radish peroxidase conjugate (Bio-Rad Immuno-Blot (GAR-HRP) assay kit) according to the manufacturer's instructions.

*RNA hybridization.* Total rat liver RNA was isolated by the guanidinium thiocyanate method [23]. For dot hybridization RNA was denatured with 1 M glyoxal, serially diluted with water, and applied to a nitrocellulose filter [24] by means of a Hybri-dot manifold (BRL). For Northern blot hybridization RNA was denatured with glyoxal [25], electrophoresed through a 1% agarose gel and transferred to a nitrocellulose filter [24]. RNA dot blots and Northern blots were hybridized and washed as previously described [26]. The final wash was in 0.1 × SSPE, 0.1% SDS at 50°.

*Preparation and labelling of DNAs.* Plasmids purified by caesium chloride density gradient centrifugation were radiolabelled by nick translation [27] to a specific radioactivity of $\sim 1 \times 10^8$ cpm/μg with [$\alpha$-$^{32}$P]dATP (800 Ci/mmole, Amersham International Ltd.).

*Melting profile determination.* Aliquots (10 μg) of liver total RNA isolated from phenobarbital-treated or camphor-treated animals were bound to a nitrocellulose filter and hybridized with [$^{32}$P]pP450(1) [28] as described above. The filter was washed with 2 × SSC for 3 × 20 min at room temperature. The filter was then cut into individual "RNA spots", each of which was washed for 20 min in 2 × SSC [29] at a temperature between 50° and 90° as indicated in the figure legend. The filters were dried, 4 ml of Aquasol (New England Nuclear) was added and the radioactivity remaining bound to each filter was determined by liquid scintillation spectrometry.

## RESULTS

### *Effect of terpenoids on the total cytochrome P-450 content of liver microsomal membranes*

The specific content of cytochromes P-450 in control male rats reared in the absence of hardwood shaving bedding material was 0.84 nmoles/mg of liver microsomal membrane protein (Table 1). As this is within the range found previously for rats maintained on bedding material composed of hardwood shavings [21, 30] the use of such shavings results in no significant induction of the specific content of cytochromes P-450. With the exception of the 1.3-fold induction in female rats by limonene, no significant change in the specific content of cytochromes P-450 was observed following treatment of rats with naturally occurring terpenoid compounds such as camphor, menthol, pinene, limonene and myrcene. In contrast, phenobarbital treatment increased the specific content of cytochromes P-450 by almost 2-fold.

Treatment of rats with the various terpenoids caused no visible change in the pattern of liver microsomal membrane proteins, as judged by SDS–polyacrylamide gel electrophoretic analysis (data not shown), whereas phenobarbital increased the amount of a polypeptide that corresponds in molecular weight (52,000) to the major phenobarbital-inducible cytochrome P-450 (PB P-450) [30]. Neither phenobarbital nor any of the terpenoid compounds used had an effect on the amount of cytochrome $b_5$ present in liver microsomal membranes (Table 1).

### *Effect of terpenoids on the amount of PB P-450*

A previously reported radioimmunoassay procedure [21] was used to quantify PB P-450 in microsomal membrane vesicles isolated from the livers of phenobarbital-treated, terpenoid-treated or control rats (Fig. 1). The amount of PB P-450 in control male rats was 0.05 nmoles/mg of microsomal membrane protein. Phenobarbital treatment of male rats increased the amount of PB P-450 to 1.02 nmoles/mg of microsomal membrane protein, representing a 23-fold induction of this cytochrome P-450. Treatment of rats with camphor, menthol or pinene resulted in smaller but significant inductions of PB P-450 in both male and female rats, whereas treatment with limonene induced this cytochrome P-450 only

Table 1. Effect of terpenoids on PB P-450 levels in rat liver microsomal membranes

| Treatment | | Total cytochromes P-450 (nmol/mg microsomal membrane protein) | Fold induction (total cytochromes P-450) | PB P-450 (nmol/mg microsomal membrane protein) | Fold induction (PB P-450) | Cytochrome $b_5$ (nmol/mg microsomal membrane protein) |
|---|---|---|---|---|---|---|
| Control | M | 0.84 (0.11) | 1.00 | 0.05 (0.00) | 1 | 0.48 (0.04) |
| | F | 0.58 (0.03) | 1.00 | 0.06 (0.01) | 1 | 0.49 (0.07) |
| Phenobarbital | M | 1.63 (0.10)* | 1.94* | 1.02 (0.12)* | 23* | 0.48 (0.09) |
| | F | 1.00 (0.09)* | 1.72* | 0.73 (0.17)* | 12* | 0.47 (0.04) |
| Camphor | M | 0.88 (0.04) | 1.05 | 0.26 (0.05)* | 6* | 0.52 (0.06) |
| | F | 0.59 (0.01) | 1.02 | 0.23 (0.02)* | 4* | 0.45 (0.05) |
| Menthol | M | 0.96 (0.02) | 1.14 | 0.13 (0.03)* | 3* | 0.49 (0.02) |
| | F | 0.70 (0.19) | 1.20 | 0.14 (0.03)* | 2.3* | 0.36 (0.08) |
| Pinene | M | 1.11 (0.16) | 1.32 | 0.13 (0.02)* | 3* | 0.49 (0.05) |
| | F | 0.62 (0.11) | 1.07 | 0.16 (0.03)* | 2.6* | 0.54 (0.09) |
| Limonene | M | 0.95 (0.12) | 1.13 | 0.06 (0.01) | 1.2 | 0.50 (0.02) |
| | F | 0.76 (0.04)* | 1.31* | 0.12 (0.01)* | 2* | 0.46 (0.06) |
| Myrcene | M | 1.00 (0.15) | 1.19 | 0.08 (0.03) | 2 | 0.42 (0.02) |
| | F | 0.56 (0.04) | 0.97 | 0.07 (0.02) | 1.1 | 0.43 (0.05) |

Each value represents an average derived from three pools of two rat livers. Determinations on each sample were made in duplicate. Total cytochrome P-450 and cytochrome $b_5$ were determined spectrally, and PB P-450 by radioimmunoassay, as described in the Methods section.

( ) standard deviation.

* Significantly different from control with $P < 0.05$; M, male; F, female.

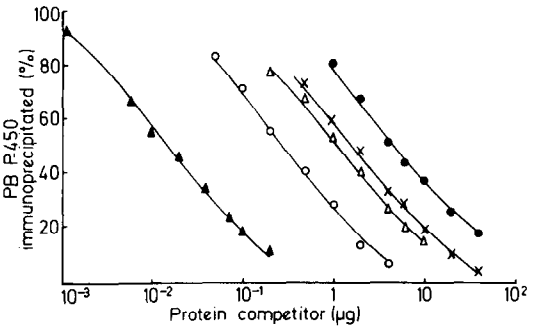


Fig. 1. Radioimmunoassay of PB P-450 in male rat liver microsomal membrane vesicles: $^{125}$I-labelled PB P-450 ($2 \times 10^4$ cpm) was mixed with the indicated amounts of unlabelled purified PB P-450 (▲), or with solubilised microsomal membranes isolated from the livers of phenobarbital-treated (○), camphor-treated (△), menthol-treated (×), or control (●) rats. Mixtures were incubated with an amount of anti-(P-450b) serum sufficient to precipitate 50% of the total immunoprecipitable radioactivity. Antibody–antigen complexes were precipitated with *Staph. aureus* cells. Radioactivity in the pellets was expressed as a percentage of that immunoprecipitated in the absence of competitor.

in females. The most effective terpenoid inducer was camphor, which increased PB P-450 to 0.26 and 0.23 nmoles/mg of microsomal membrane protein in male and female rats, respectively, an induction of 4–6-fold. Treatment with myrcene caused no significant change in the amount of PB P-450. Analysis of the proteins of rat liver microsomal membranes by Western blotting showed that anti-(P-450*b*) serum reacted with a single polypeptide band that was induced by both phenobarbital and camphor (Fig. 2). The molecular weight of the polypeptide is 52,000, the same as that of purified P-450*b*.

*Analysis of PB P-450 mRNA levels*

The above results demonstrate that PB P-450 can be induced in rat liver microsomal membranes by certain naturally occurring terpenoids. A cDNA clone (pP450(1)) [28] that codes for a microheterogeneous variant of P-450e was used as a molecular hybridization probe to determine whether the terpenoid compounds induced PB P-450 via a mechanism similar to that used by the synthetic compound phenobarbital. The effect of the compounds on the amount of PB P-450 mRNA was measured by RNA dot hybridization. Various amounts of RNA, extracted from the livers of phenobarbital-treated, terpenoid-treated or control rats, were applied to a nitrocellulose filter and hybridized to $^{32}$P-labelled pP450(1) as detailed in the Materials and Methods. For each of the RNA preparations the intensity of the hybridization signal was linear with respect to the total amount of RNA applied to the filter (Fig. 3). A comparison of the slopes of the lines generated showed that treatment of rats with phenobarbital increased the amount of PB P-450 mRNA in the liver by 25-fold. PB P-450 mRNA was also induced, but to a much lesser extent, by treatment of animals with camphor (Fig. 3), menthol or pinene (data not shown).

The order of potency of the compounds with respect to induction of PB P-450 mRNA was the same as that for induction of the protein encoded by this mRNA (Table 1). Camphor, the most effective terpenoid inducer, caused a 3.5-fold increase of PB P-450 mRNA in male rats, whereas menthol and pinene increased the mRNA by 2.5- and 2.0-fold, respectively. Analysis of rat liver RNA by Northern blot hybridization demonstrated that the mRNA induced by camphor was the same size (2100 nucleotides) as PB P-450 mRNA (Fig. 4). An mRNA of identical size was also induced by menthol and pinene (data not shown).

*Melting profile of cDNA: mRNA hybrids*

The degree of homology between the mRNA species induced by camphor and phenobarbital was

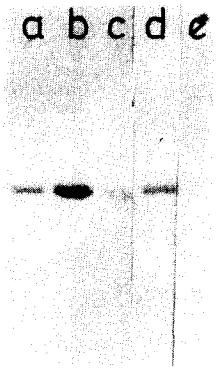


Fig. 2. Western blot analysis of PB P-450 in rat liver microsomal membranes. Liver microsomal membrane proteins (1 μg) from camphor-treated (c), phenobarbital-treated (d) and control (e) rats were electrophoresed through an SDS/polyacrylamide gel. Proteins were transferred to a nitrocellulose filter, and PB P-450 was detected as described in the Methods section. Tracks a and b contained 0.1 and 0.5 μg of purified P-450*b*, respectively.

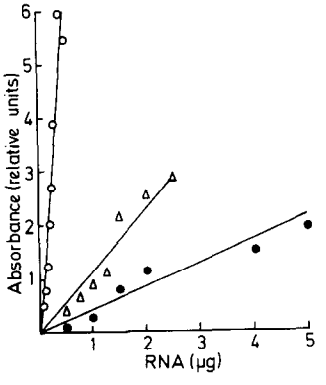


Fig. 3. Induction of PB P-450 mRNA sequences in rat liver. Total RNA was extracted from the livers of phenobarbital-treated (○), camphor-treated (△), or control (●) rats. Various amounts of RNA were applied to a nitrocellulose filter and hybridized to [$^{32}$P]pP450(1). After autoradiography the intensity of absorbance (measured by scanning densitometry) of the dots was plotted against the total amount of RNA present.


Fig. 4. Northern blot hybridization analysis of PB P-450 mRNA sequences in rat liver. Total RNA was extracted from the livers of control rats (a, b) or from rats treated with phenobarbital (c, d) or camphor (e, f). RNA was denatured with glyoxal, electrophoresed through a 1% agarose gel and blotted to a nitrocellulose filter. The filter was hybridized to [$^{32}$P]pP450(1), washed and autoradiographed. Amounts of RNA loaded were 2 $\mu$g (a, c, e) and 10 $\mu$g (b, d, f).

determined by analysing the temperature dependence of the denaturation of cDNA : mRNA hybrids. The hybrid molecule formed between the camphor induced mRNA and pP450(1), and that formed between PB P-450 mRNA and the recombinant plasmid displayed very similar melting characteristics (Fig. 5). The melting temperatures of the hybrids differed by less than 3° and was approximately 65°. Thus treatment of rats with camphor induces in liver an mRNA species that is highly homologous, if not identical, to the mRNA that codes for P-450e. The camphor-induced mRNA must therefore be a product of the P450IIB gene sub-family.

*Effect of terpenoids on the amount of P-450d mRNA*

A cloned cDNA (pP450(2)) [31] coding for P-450*d* (P450IA2) was used as a molecular hybridization probe to determine whether the abundance of the corresponding mRNA species was altered following treatment of rats with terpenoid compounds. Total RNA was isolated from the livers of phenobarbital-treated, $\beta$-naphthoflavone-treated, terpenoid-treated or control rats and analysed by Northern blot hybridization (Fig. 6). As previously reported [32], the P-450*d* cDNA clone hybridized to two mRNA species of approximately 2000 and 4000 nucleotides in RNA isolated from the livers of rats treated with $\beta$-naphthoflavone. The two mRNA size classes were also present in RNA from phenobarbital-treated rats, but only the smaller was detected in RNA from control or terpenoid-treated animals. $\beta$-naphthoflavone induces both of the mRNA species, but none of the terpenoids tested increased the amount of either of the mRNAs (Fig. 6). This was confirmed by dot blot hybridization experiments (data not shown).

## DISCUSSION

We have found that treatment of male and female rats with the terpenoid compounds camphor, menthol and pinene, and, in the case of female animals, limonene, induces a cytochrome P-450 that reacts with a polyclonal antibody raised to P-450*b*. Although the antibody appears to be highly specific

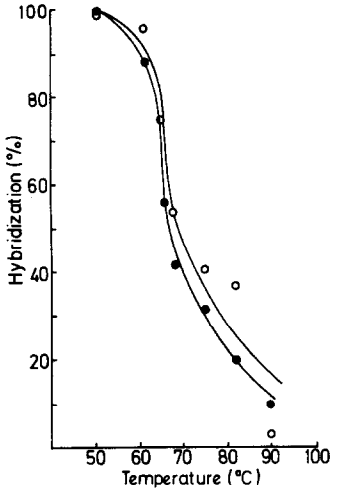


Fig. 5. Melting profile of mRNA : cDNA hybrids. Multiple 10 $\mu$g aliquots of total RNA isolated from the livers of phenobarbital-treated (○) or camphor-treated (●) animals were bound to nitrocellulose filters. After hybridization to [$^{32}$P]pP450(1) the filters were washed at various temperatures as described in the Methods section. The radioactivity that remained bound to each filter was expressed as a percentage of that bound after washing at 50°, and plotted against the wash temperature.

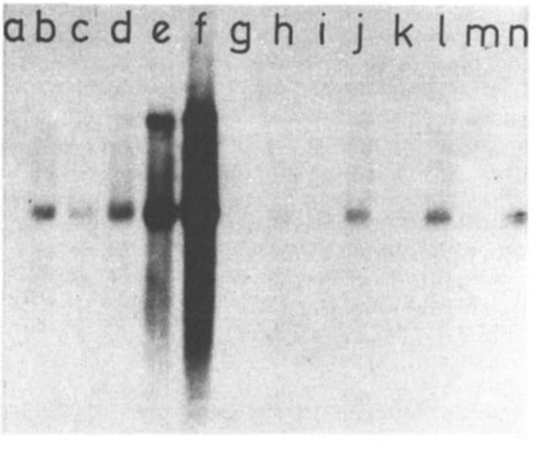


Fig. 6. Northern blot hybridization analysis of P-450*d* mRNA sequences in rat liver. Total RNA was extracted from the livers of control rats (a, b) or from rats treated with phenobarbital (c, d), $\beta$-naphthoflavone (e, f), camphor (g, h), pinene (i, j), myrcene (k, l) or limonene (m, n). RNA was denatured with glyoxal, electrophoresed through a 1% agarose gel and blotted to a nitrocelluose filter. The filter was hybridized to [$^{32}$P]P-450*d* cDNA, washed and autoradiographed. Amounts of RNA loaded were 2 $\mu$g (a, c, e, g, i, k, m) and 10 $\mu$g (b, d, f, h, j, l, n).

for P-450s *b* and *e*, and their microheterogeneous variants [21], and in Western blots reacts with a polypeptide of molecular weight 52,000, we have to consider the possibility that it may detect a different, terpenoid-inducible, cytochrome P-450 that has an epitope in common with P-450 *b/e*. A comparison of radioimmunoassay curves shows that the extent of competition obtained with microsomal membrane proteins from the livers of camphor-treated rats is the same as that observed for liver microsomal membrane proteins from phenobarbital-treated animals and for purified P-450*b*. In addition the identity of the slopes of the radioimmunoassay curves demonstrates that the corresponding antigenic determinants in the different protein preparations bind to the antibody with equal avidity. Thus all the antigenic determinants for P-450*b* that are recognised by the antibody must also be present in the cytochrome P-450 induced by camphor. Owing to its polyclonal nature, the antibody would be expected to bind to several epitopes and therefore the cytochrome P-450 induced by camphor is almost certainly P-450*b*, P-450*e* or a microheterogeneous variant of these. The ability of camphor to induce the expression of a member of the P450IIB gene sub-family of rats is confirmed by the molecular hybridization experiments. Hence it is possible that PB P-450 may have originally evolved to cope with terpenoids such as camphor, menthol or pinene that are present in the environment. Our results also indicate that the molecular basis for the terpenoid-induced increase in the rate of metabolism of drugs such as phenobarbital and hexobarbital [8, 9, 11, 14] is an induction of PB P-450 in liver microsomal membranes, and that this is mediated by an increase in the amount of the corresponding mRNA.

Phenobarbital appears to be a much more potent inducer of P-450*b/e* than are any of the terpenoids studied. This may be because the induction regime with terpenoids was not optimised with respect to dose, route of administration or duration of treatment. The rate of clearance of the compounds may also differ. Alternatively the results may reflect a more fundamental quantitative difference in response to the different compounds. A further possibility is that phenobarbital and camphor induce different members of the P450IIB sub-family. Although it is clear that phenobarbital acts at the level of the genome to increase the rate of transcription of PB P-450 gene(s) [26, 33] it remains to be established whether terpenoids act via the same mechanism.

PB P-450 represents as little as 5% of the total content of cytochromes P-450 in the microsomal membranes of control rat liver. Thus the 2–6-fold induction of this protein by some terpenoids would be expected to give rise to an apparent increase of less than 1.25-fold in the total specific content of cytochromes P-450. This small increase would be reduced or even eliminated if, in addition to inducing PB P-450, the terpenoids caused a decrease in other cytochromes P-450. We have previously shown that the cytochrome P-450 inducer β-naphthoflavone decreases PB P-450 [21, 30]; and other inducers also have been found to result in a concomitant decrease of specific cytochromes P-450 [34]. Consequently it is not surprising that camphor, menthol and pinene, while inducing PB P-450, have no significant effect on the total content of cytochromes P-450 in liver microsomal membranes. Hence the inability of a compound to increase the total content of cytochromes P-450 may not always provide a suitable criterion to judge its effectiveness as an inducer of specific cytochromes P-450.

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