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Inhibition of Protein Prenylation by Metabolites of Limonene

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ABSTRACT. The monoterpenes limonene and perillyl alcohol are undergoing clinical evaluation in cancer patients. In this paper, we report the chemical synthesis, characterisation, and quantitation in patients' plasma of a novel human metabolite of limonene, which is identified as an isomer of perillic acid. The synthesis of *R*-perillic acid is also described, because previous reports on the activity of perillic acid against isoprenylation enzymes refer to the *S*-enantiomer, although it is the *R*-enantiomer which is the metabolite of *R*-limonene. The above monoterpenes, with several related compounds, were assayed for inhibitory activity towards the isoprenylation enzymes in rat brain cytosol. Although *R*- and *S*-limonene are only weak inhibitors of the isoprenylation enzymes, their major metabolites, perillic acid and perillyl alcohol, are more potent inhibitors, with IC_{50} values in the low mM range. The metabolites possess greater activity towards the geranylgeranyltransferase type I enzyme than farnesyltransferase, while the novel metabolite displays IC_{50} values similar to those of perillic acid suggesting that it may contribute to the *in vivo* activity of limonene. BIOCHEM PHARMACOL **57**;7: 801–809, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. monoterpenes; limonene; metabolism; isoprenylation; inhibitor; antitumour

R-Limonene (*p*-mentha-1,8-diene) **(1a)**, the principal component of orange peel oil, has been identified as a non-toxic agent with potential for cancer chemotherapy. In several model systems, **1a** prevents the formation of chemically induced tumours [1] and displays significant antitumour effects [1–4]. The monoterpene **1a** is extensively metabolised *in vivo*. In rat and human plasma, *R*-perillic acid ((+)-(4-*R*)-[2-propenyl]-1-cyclohexane-1-carboxylic acid) **(2a)** and dihydroperillic acid **(3)** are the most abundant circulating metabolites, other metabolites identified including limonene-1,2-diol **(4)**, limonene-8,9-diol (uroterpenol) **(5)**, and an isomer of perillic acid of proposed structure **(6)** (for formulae, see Fig. 1). The major urinary metabolites in humans and animals include glucuronide conjugates of **2a, 3,** limonene-6-ol (carveol), and limonene-7-ol (perillyl alcohol) *R*-enantiomer **(8a)** [5–8]. *S*-Perillyl alcohol **(8b)**, a natural product from cherries and other edible plants, has also been used in antitumour studies. It induces regression in rat mammary carcinomas [9] and demonstrates activity against pancreatic tumour cell lines *in vitro* and *in vivo* [10]. On this basis, *R*-limonene **(1a)** and *S*-perillyl alcohol **(8b)** have been entered in clinical trials [8, 11–13].

Ras proteins function as molecular switches in some of the signal transduction pathways that control cell growth and differentiation [14]. Mutant *ras* oncogenes encoding constitutively active proteins have been observed in approximately 30% of human cancers including ~50% of colon cancers and up to ~90% for pancreatic cancer [15]. Ras requires post-translational prenylation, usually with a farnesyl residue, prior to membrane localisation and participation in the signalling cascade [16]. FTase† is required in this process, and so the discovery of potent inhibitors has been a priority in drug discovery [17, 18].

R-Limonene **(1a)** and its metabolites have been demonstrated to selectively inhibit the isoprenylation of 21–26 kDa proteins, including the Ras protein [19, 20]. The metabolites, in particular, exert a direct inhibitory effect on FTase and GGTase type I and II [21, 22]. The monoterpenes interfere with other aspects of the isoprenoid pathway, including the activity of hepatic β-hydroxy-β-methylglutaryl-CoA reductase [23] and the conversion of lathosterol to cholesterol [24]. Recently, perillic acid has been shown to deplete levels of farnesylated Ras protein in human T lymphocytes, but by a mechanism independent of FTase [25]. Other mechanisms of action for the monoterpenes have been suggested that are independent of the Ras pathway, including induction of apoptosis [26, 27].

Limonene may be seen as a prodrug for its more potent metabolites; therefore, accurate quantitation of all the

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† *Abbreviations:* FPP, farnesylpyrophosphate; GGPP, geranylgeranylpyrophosphate; FTase, farensyltransferase; GGTase, geranylgeranyltransferase; and LC–MS, liquid chromatography-mass spectrometry.

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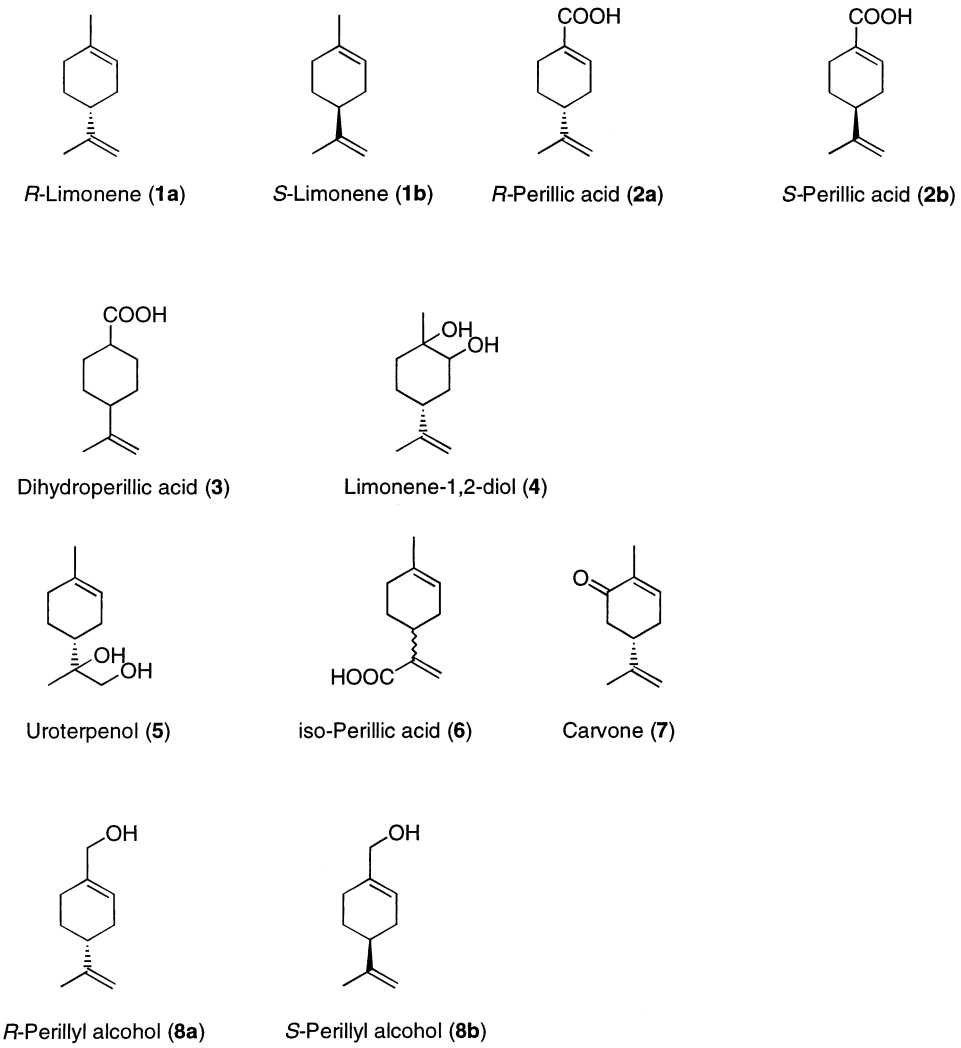


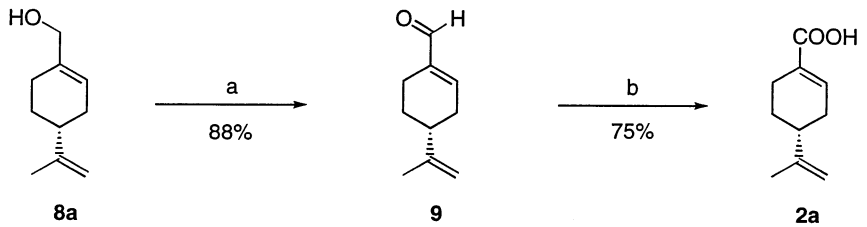
FIG. 1. Formulae of the monoterpene limonene and its metabolites.

human metabolites and knowledge of their *in vitro* potencies against the prenylation enzymes acting on Ras are vital to the understanding of its mechanism of action. In this paper, we report the chemical synthesis, characterisation, and quantitation in patients' plasma of the novel human metabolite **6** which appeared to be an isomer of perillic acid. We also describe the synthesis of *R*-perillic acid **(2a)**, because previous reports on the activity of perillic acid against isoprenylation enzymes refer to the *S*-enantiomer, although it is the *R*-form which is the metabolite of *R*-limonene. The above monoterpenes with several related compounds were assayed for inhibitory activity towards FTase and GGTase type I.

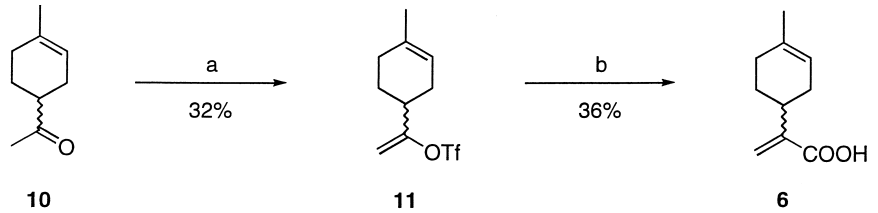
MATERIALS AND METHODS

Materials

(*R*)-Perillic acid **(2a)** and *p*-menth-1,8-dien-10-oic acid **(6)** were synthesised as described below (Schemes 1 and 2). (*S*)-Perillic acid **(2b)** was purchased from Sigma Chemical Co., and all other monoterpenes were from Aldrich Chemical Co. Chaetomellic acid A and α-hydroxyfarnesylphos-



SCHEME 1. Synthesis of *R*-perillic acid (2a). Reagents and conditions: (a) PDC, CH_2Cl_2; (b) 2-methylbut-2-ene, *t*-BuOH, $NaClO_2$, KH_2PO_4 H_2O.



SCHEME 2. Synthesis of *p*-Menth-1,8-dien-10-oic acid (6). Reagents and conditions: (a) Et_3N,2,6-di-*t*-butyl-4-methylpyridine, Tf_2O, CH_2Cl_2; (bi)Et_3N, MeOH, bis(diphenylphosphino)propane, DMSO, Pd(II)OAc, CO(1 atm); (bii) NaOH, H_2O, MeOH.

phonic acid were obtained from Calbiochem-Novabiochem. L744832 was a kind gift from Merck, Sharp and Dohme. [1-^{3}H] FPP and [1-^{3}H] GGPP were from NEN Life Science Products. Recombinant human H-Ras (WT) and H-Ras (CVLL) were obtained from Panvera Corporation. Whatman GF/C glass microfibre filters were purchased from Fisher Scientific. HPLC grade solvents were purchased from Laserchrom Analytical Ltd.

Plasma Sample Collection from Patients

The clinical trial was performed at Charing Cross Hospital, London. All patients gave written informed consent to participate in the study, in a form approved by the Research Ethics Committee of Charing Cross Hospital [8, 13]. Patients were given an oral dose of either 6, 8, 10, or 12 g/m^2/day of *R*-limonene. Plasma samples were obtained at intervals for up to 12 hr after dosing. All samples were stored at −20° prior to analysis.

Isolation of Limonene Metabolites in Human Plasma

Stock solutions of *S*-perillic acid **(2b)** and the isomer **(6)** were prepared at concentrations of 1 mg/mL in methanol. A standard curve was prepared by spiking blank human plasma with known concentrations of the stock solutions, giving a series of calibration standards from 1 to 10 mg/mL plasma. α-Terpinene was used as the internal standard.

Each patient plasma sample (1 mL) was spiked with α-terpinene (2 μg) and the sample was adsorbed onto a C18 Bond Elut cartridge (100 mg capacity; Thames Chromatography). The cartridge was conditioned with metha-

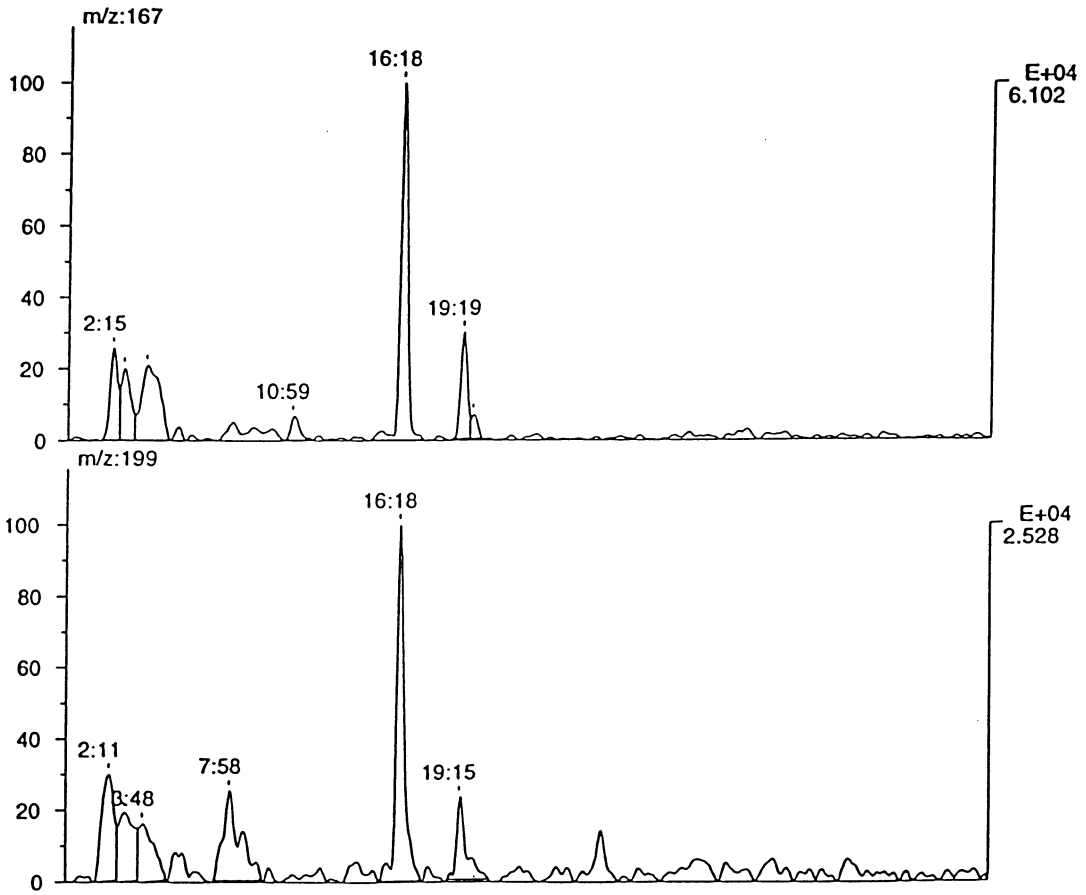


FIG. 2. HPLC analysis of patients' plasma extract with selected ion monitoring at A: m/z 167 and B: m/z 199.

nol (1 mL) and water (1 mL). After the sample was transferred to the cartridge, each cartridge was washed with 10 mM ammonium acetate (4 × 1 mL), and the compounds of interest were eluted with methanol (300 μL), of which 100 μL was analysed by LC–MS.

LC–MS Analysis of Limonene Metabolites

The HPLC system consisted of a Waters 600 MS gradient controller and a Waters 717 autosampler (Millipore Ltd.). Separation of the plasma extracts was achieved on a 5 μm ODS Apex I column (150 × 4.6 mm; Jones Chromatography) at a flow rate of 1 mL/min. The mobile phase consisted of 0.075% trifluoroacetic acid (A) and methanol (B). A linear gradient elution program was used: 45% B for 5 min; 45–70% B for 5 min; 70% B for 5 min; 70–80% B for 10 min; and 80% B for 10 min. The eluate was introduced into a Finnigan TSQ 700 mass spectrometer equipped with an APCI source. The corona discharge voltage was 5kV, and the vapouriser temperature and heated capillary temperatures were 450° and 220°, respectively. Selected ion monitoring was achieved using a DEC 2100 station running Finnigan ICIS and ICL software.

Chemical Synthesis

(*R*)-(+)-PERILLALDEHYDE (9). To a well-stirred suspension of pyridinium dichromate (0.90 g, 2.41 mmol) in anhydrous dichloromethane (7 mL) was added (*R*)-(+)-perillyl alcohol **(8a)** (0.25 g, 1.64 mmol) at room temperature and stirring continued for 4 hr. The mixture was filtered through a pad of Celite® and washed with dichloromethane (50 mL). The filtrate and the washings were combined, dried ($MgSO_4$) and concentrated under reduced pressure. Flash column chromatography (silica, Merck 15111), eluting with hexane/ethyl acetate (9:1), afforded **9** as a colourless oil (0.217 g, 88%); ^{1}H-NMR ($CDCl_3$) δ 1.37–2.50 (m, 7H, CH_2), 1.76 (s, 3H, CH_3) 4.12 (m, 1H, vinylic H-8), 4.77 (m, 1H, vinylic H-8), 6.83 (m, 1H-2), 9.44 (s, 1H, CHO); MS m/z 250 [$(M\text{-}H)^-$, 95%].

To a mixture of **9** (0.2 g, 1.33 mmol) and methylbutene (6 mL) and *t*-butanol (25 mL) was added dropwise over 10 min a solution of sodium chlorite (1.0 g, 11.1 mmol) and potassium dihydroorthophosphate (1.13 g, 8.3 mmol) in H_2O (10 mL). The resulting mixture was stirred overnight then concentrated under reduced pressure. The residue was dissolved in water (30 mL) and extracted with hexane (2 × 15 mL). The aqueous layer was acidified to pH ~ 3 with 1 M HCl and extracted with ether (3 × 20 mL). The combined organic layers were washed with cold water (50 mL), dried ($MgSO_4$), and concentrated under reduced pressure. Flash column chromatography (silica, Merck 15111), eluting with hexane/ethyl acetate (8:2), afforded **2a** as a white solid (0.166 g, 75%); mp 131–133° (ex hexane), $[\alpha]_D^{24}$ + 120.70° (c 0.86, $CHCl_3$); ^{1}H-NMR (DMSO) δ 1.36–1.41 and 1.76–2.35 (m, 7H, CH_2), 1.71 (s, 3H, CH_3) 4.72 (pseudo d, 2H, vinylic H_8), 6.87 (m, 1H, vinylic H_2), 12.14 (s, 1H, COOH); MS m/z 165 [$(M\text{-}H)^-$, 100%], 331[$(2M\text{-}H)^-$, 50%]. Anal. Calcd for $C_{10}H_{14}O_2$: C, 72.27; H 8.48. Found: C 72.03, H 8.36.

1-METHYL-4-(1-TRIFLUOROMETHYLSULPHONYLOXY)VINYL-1-CYCLOHEXENE (11). To a stirred solution of 4-acetyl-1-methylcyclohexene **(10)** (2 mL, 13.66 mmol) and 2,6-di-*tert*-butyl-4-methylpyridine (3.225 g, 15.71 mmol) in dry dichloromethane (90 mL) was added trifluoromethanesulfonic anhydride (2.91 mL, 17.30 mmol). The mixture was stirred for 12 hr, then filtered and concentrated under reduced pressure. Flash chromatography (silica, Merck 15111) of the brown–black residue, eluting with hexane, afforded **11** as a yellow oil (1.185 g, 32%) that proved unstable and was used in the next step without further purification; ^{1}H-NMR ($CDCl_3$) δ 1.55–2.90 (m, 7H, CH_2), 1.66 (s, 3H, CH_3) 4.94 (dd, J = 3.8 Hz and J = 1, of H-9), 5.12 (d, J = 3.8, other of H-9), 5.35 (m, 1H-2).

***P*-MENTH-1,8-DIEN-10-OIC ACID** (6). A solution of **11** (0.5 g, 1.85 mmol), triethylamine (0.57 mL, 4.10 mmol), methanol (1.13 mL, 27.93 mmol), 1,3-bis(diphenylphosphino)propane (9 mg, 0.02 mmol) in 10 mL DMSO was degassed repeatedly. Then, carbon monoxide (CAUTION: TOXIC) was bubbled through for a period of 15 min before palladium (II) acetate (4.6 mg, 0.02 mmol) was added. The reaction mixture was slowly heated to 75° and stirred overnight. The mixture was concentrated under reduced pressure, diluted with water (50 mL), and extracted with ethyl acetate (2 × 50 mL). The organic extracts were concentrated under reduced pressure, and the yellow residue added to a solution of NaOH (0.40 g) in methanol (25 mL) and the mixture refluxed for 1 hr. The mixture was concentrated under reduced pressure, acidified to pH 3–4 with conc HCl and extracted with ethyl acetate (2 × 50 mL). The extracts were dried ($MgSO_4$) and concentrated under reduced pressure. Flash column chromatography (silica, Merck 15111), eluting with hexane/ethyl acetate (9:1), followed by crystallisation (hexane) afforded **6** as a white solid (0.110 g, 36%); mp 71–73° (hexane); ^{1}H-NMR (DMSO) δ 1.40–2.15 (m, 7H, CH_2), 1.62 (s, 3H, CH_3), 5.38 (m, 1H-2), 5.53 (app s, 1H-10), 6.06 (d, J = 0.9 Hz, 1H-10), 12.44 (s, 1H, COOH); MS m/z 167 [$(M + H)^+$, 58%], 208 [$(M + MeCN + H)^+$, 100%]. Anal. Calcd for $C_{10}H_{14}O_2$: C, 72.27; H 8.48. Found: C 72.18, H 8.45.

Protein Isoprenylation Assays

The rat brain cytosol, which was used as a source of the enzyme activity, was prepared as described by Harwood [28]. Homogenates were first centrifuged at 10,000 *g* for 20 min at 4°, and the resultant supernatants were centrifuged at 178,000 *g* for 90 min at 4°. The cytosol (protein concentration of 10 to 15 mg/mL) was removed, divided into 0.5 mL portions, and stored under liquid nitrogen. There was no appreciable loss of enzyme activity after six months' storage. Activity of the enzymes was determined by

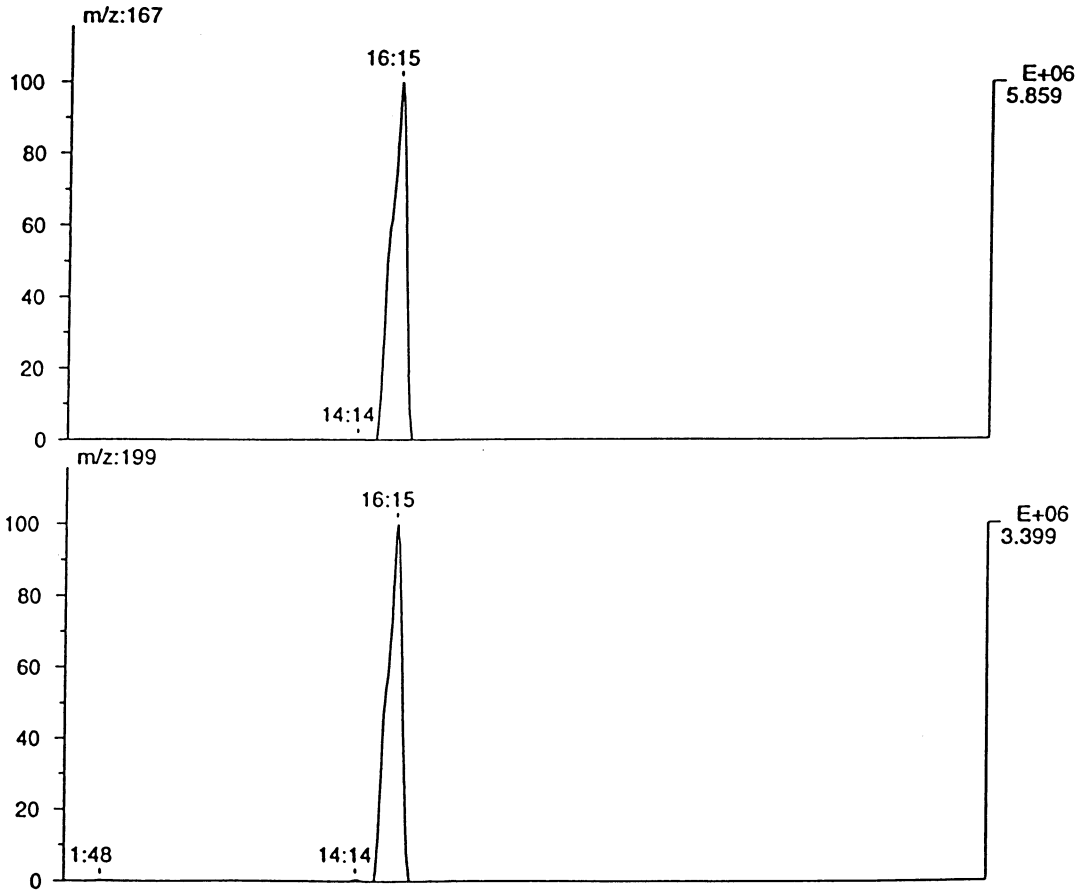


FIG. 3. HPLC analysis of a sample of *iso*-perillic acid (6) with selected ion monitoring at A: m/z 167 and B: m/z 199.

quantifying the amount of tritium transferred from [1-^{3}H]-GGPP or [1-^{3}H]-FPP into the appropriate acceptor protein, H-Ras(CVLL) for GGTase type I or H-Ras(WT) for FTase, by acid precipitation and filtration through glass fibre filters [29–31]. The standard reaction mixture for FTase contained the following components in an Eppendorf: 50 mM Tris–HCl pH 7.5, 4mM $MgCl_2$, 20 mM KCl, 5 mM dithiothreitol, 20 μM $ZnCl_2$, 0.5 μM [1-^{3}H] FPP (0.15 μCi), 5 μM H-Ras(WT) and 1 μL of inhibitor stock solution in DMSO. An aliquot of rat brain cytosol was added to start the incubation at 37° and give a final volume of 25 μL. The reaction was terminated by the addition of 40 μL of 10% HCl in ethanol and left to stand for 2 hr. Each sample was spotted onto a 2 cm × 2 cm square of Whatman GF/C filter paper and the Eppendorf rinsed with 25 μL of ethanol which was spotted onto the square. The filter paper was dried and washed with four 100 mL portions of ethanol on a Büchner funnel. After drying, each square was added to 10 mL of scintillant for counting. The GGTase type I was assayed as described above, except that 0.04% (w/v) *p*-octyl-β-D-glucopyranoside was added to the assay buffer to overcome problems with the solubility of GGPP. The background radioactivity (typically 5–10% of control values) was measured in tubes with the protein substrate omitted and subtracted from the test assays. Dose–response curves for inhibitors used triplicate determinations at each drug concentration, and the IC_{50} values were made from enzyme activity versus log drug concentration plots at the stated substrate concentrations.

RESULTS

Identification and Quantitation of Novel Metabolite in Patients' Plasma

Patients' plasma was analysed by LC–MS. Selected ion monitoring at m/z = 167 [M + H] for perillic acid and m/z = 199 [M + CH_3OH] for the methanol adduct of perillic acid (Fig. 2) showed the presence of two distinct compounds with the same molecular mass. The smaller fraction with a retention time of 19.2 min corresponded to perillic acid **(2)**. The larger fraction with a retention time of 16.2 min was compared with the HPLC analysis of an authentic sample of the perillic acid isomer **(6)** (Fig. 3) and found to have an identical retention time. A comparison of the mass spectra of the patients' plasma fraction and the authentic isomer **6** (Fig. 4) confirms the identity of the novel metabolite as **6**. The peak plasma concentrations of the metabolite **6** which were detected in the plasma of patients receiving *R*-limonene at doses of 6, 8, 10, and 12 g/m^2/day are shown in Table 1 and compared with those of perillic acid, one of the major metabolites of limonene.

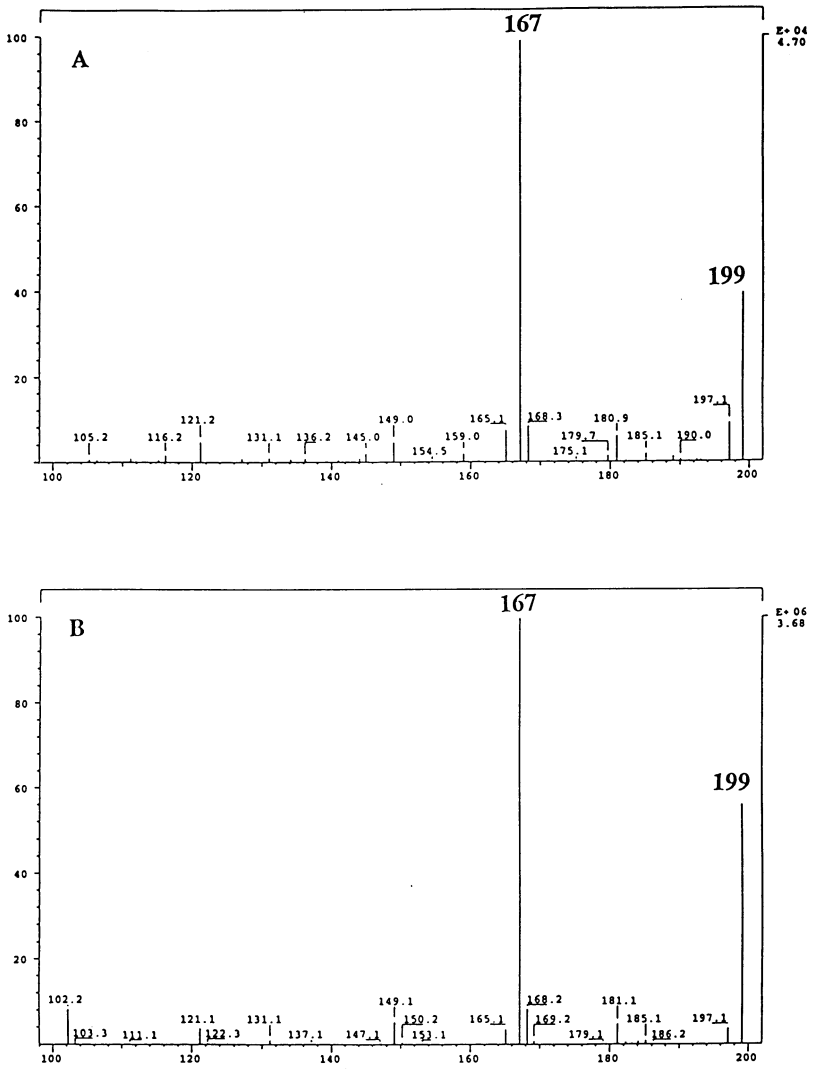


FIG. 4. (A) Mass spectrum of the 16.2-min fraction from patients' plasma; (B) Mass spectrum of *iso*-perillic acid (6).

Inhibition of Protein Prenylation

Kinetic analysis of the FTase activity in the rat brain cytosol, using the Eadie–Hofstee plot of initial velocity against initial velocity divided by the substrate concentration, gave K_m values of 3.5 ± 1.1 μM for H-Ras(WT) and 0.048 ± 0.008 μM for FPP with a maximal velocity of 1.75 ± 0.6 pmol product formed/min/mg protein. Similarly for the GGTase type I activity, K_m values of 4.3 ± 0.9 μM for H-Ras (CVLL) and 0.066 ± 0.007 μM for GGPP with

TABLE 1. Peak plasma concentrations of *iso*-perillic acid (6) and perillic acid (2)

Dose (g/m²/day)	*iso*-Perillic Acid (6)	Perillic acid (2) (μM)
12*	24.5 ± 15.8	65.8 ± 4.7
10*	16.4 ± 1.9	74.3 ± 43.5
8†	9.6 ± 2.2	40.5 ± 22.6
6†	7.9 ± 0.2	30.1 ± 8.7

Values are means ± SD.
*: three patients; †: two patients.

TABLE 2. IC_{50} values for the inhibition of the isoprenylation enzyme activities by limonene, its metabolites, and standard compounds

Compound		FTase (mM)	GGTase I (mM)
R-Limonene	**1a**	>40	>40
S-Limonene	**1b**	>40	>40
R-Perillic acid	**2a**	8.1 ± 1.0	3.4 ± 0.3
S-Perillic acid	**2b**	10.7 ± 0.9	4.1 ± 0.5
p-Menth-1,8-dien-10-oic acid	**6**	5.0 ± 0.8	2.6 ± 0.4
R-Carvone	**7**	1.5 ± 0.4	2.3 ± 0.5
S-Carvone		1.4 ± 0.2	7.0 ± 2.0
R-Perillyl alcohol	**8a**	10.4 ± 1.5	2.1 ± 0.4
S-Perillyl alcohol	**8b**	10.2 ± 2.0	1.9 ± 0.5
Positive controls (μM)			
L 744832		0.1 ± 0.004	25 ± 8
α-hydroxyfarnseylphosphonic acid		2.6 ± 0.24	25 ± 5
Chaetomellic acid		2.5 ± 0.5	40 ± 7

Each IC_{50} value is the mean of three independent experiments ± SD.

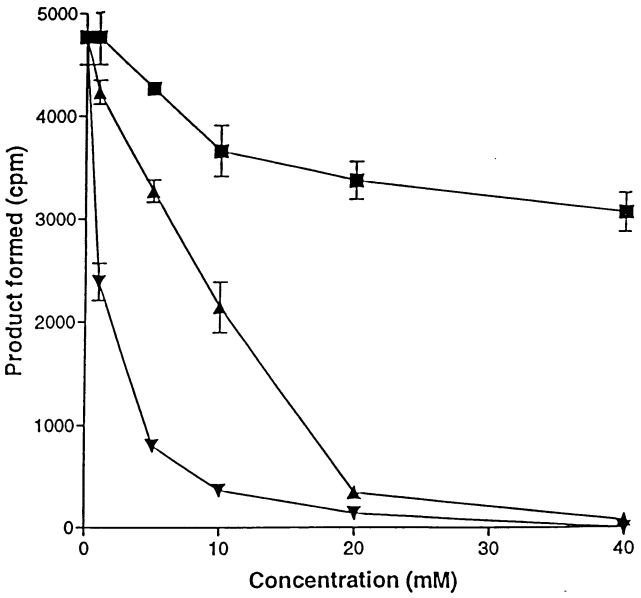


FIG. 5. Inhibition of the FTase activity in rat brain cytosol by the monoterpenes *R*-limonene (■), *R*-perillic acid (▲), and *R*-carvone (▼). Each point represents the mean of triplicate determinations ± SD.

a maximum velocity of 0.5 ± 0.1 pmol product formed/min/mg protein were obtained. These values are similar to the published values [28–31]. Therefore, the final substrate concentrations used in the assays were 5 μM for the protein acceptor and 0.5 μM for the isoprenoid cofactor. Under these conditions, enzyme activity was linear with time up to 1 hr and protein concentration 100 μg/25 μL assay volume. Validation was carried out by determination of the IC_{50} values for the standard inhibitors, namely L744832, α-hydroxyfarnesylphosphonic acid, and chaetomellic acid A and the results are shown in Table 2. The published IC_{50} values for chaetomellic acid A and α-hydroxyfarnesylphosphonic acid against the Ftase enzyme are 55 and 30 nM, respectively [32], these having been obtained under non-saturating substrate conditions with FPP at a concentration of 0.10 μM and H-ras(CVLS) at 0.65 μM. In the same paper, both compounds were identified as competitive inhibitors of FTase activity with respect to FPP, the IC_{50} values for such compounds being dependent on the substrate concentration used. Therefore, because we have employed higher substrate concentrations (0.5 μM for FPP and 5 μM for H-ras(CVLS), our IC_{50} values are correspondingly higher. All the standard compounds were potent inhibitors of the FTase enzyme, with IC_{50}s in the low μM range but possessing only weak activity against GGTase type I as previously shown [32–34]. For the monoterpenes, Fig. 5 illustrates a representative experiment demonstrating the dose-dependent decrease in FTase activity in the presence of **1**, **2**, and *R*-carvone. The resulting IC_{50} values are listed in Table 2 together with the results from the remaining monoterpenes and the effects of the compounds on GGTase activity. Overall, the results show that rat brain cytosol is a good source of enzyme and that the assays can differentiate between the two isoprenylation pathways.

DISCUSSION

The isomer of perillic acid **(6)** was previously detected as a novel metabolite of (*R*)-limonene in human plasma by LC–MS and its structure was tentatively assigned from the mass spectrum [8]. Previous studies employing GC–MS did not detect the presence of **6** [6, 7]. We have synthesised an authentic sample of **6** which displays identical chromatographic and mass spectral characteristics to the metabolite, thus confiming the initial assignment. Although *R*- and *S*-limonene are only weak inhibitors of the prenylation enzymes (Fig. 5 and Table 2), their major metabolites, perillic acid **(2)** and perillyl alcohol **(8)**, are more potent inhibitors of isoprenylation with IC_{50} values in the low mM range. The metabolites possess somewhat greater activity against the GGTase type I enzyme than FTase. These results are consistent with the published values [7, 19–22]. The other metabolites of limonene, namely dihydroperillic acid **(3)**, limonene-1,2-diol **(4)**, and uroterpenol **(5)**, were not tested in the present study. However, previous work has demonstrated that these monoterpenes are far weaker inhibitors of isoprenylation than either perillic acid or perillyl alcohol [20]. Carvone, which is a possible metabolite of limonene but has never been detected in humans, is the most potent inhibitor of FTase (IC_{50} = 1.5 mM). Although the individual enantiomers of **6** may differ in their activity, the racemate of novel metabolite **6** displays IC_{50} values similar to those of *R*-perillic acid, suggesting that **6** may contribute to the *in vivo* activity of limonene. When one compares the low mM values for the inhibition of isoprenylation with the peak plasma concentrations of **6** and **2**, which are in the range 8 to 74 μM (Table 1), it appears unlikely that the mechanism of action of the monoterpenes *in vivo* is due to the blockade of prenylation by a single metabolite. However, an additive effect is possible, and as these are lipophilic compounds accumulation may occur within the tumour tissues [13]. This combined with a determination of the pharmacokinetics of the other active metabolites of limonene may account for the discrepancy. Alternatively, there is increasing evidence that monoterpenes have other mechanisms of action which may account for their antitumour activity [22–27].

Interestingly, there is little significant difference in the *in vitro* activity between the newly synthesised *R*-perillic acid **(2)**, the metabolite of *R*-limonene **(1)**, and the commercially available *S*-enantiomer. This is fortutitous, as in many cases enantiomers display markedly different activities, and the commercially available *S*-perillic acid has been used in many studies without any comment on its chirality [7, 20].

The structure–activity relationships for the monoterpenes suggest that they are acting as mimics of FPP. The recent X-ray crystal structure of FTase reveals a large hydrophobic region in the β-subunit lined with aromatic

residues which is responsible for binding the FPP molecule [35]. The diphosphate residue chelates the zinc atom found in the pocket. It seems likely that the carboxylic acid or alcohol functionality of **2**, **6**, or **8** would occupy a position close to the zinc, while the hydrophobic terpene ring would bind in the hydrophobic pocket. The relatively small size of the monoterpenes, compared with the large natural substrates FPP and GGPP, and the large area of the binding pocket would result in a low stringency of binding to the enzyme, thus explaining the lack of discrimination between enantiomers and structural isomers and their low potency.

In conclusion, we have identified and characterised a novel human plasma metabolite **6** of *R*-limonene **(1a)**, which is a structural isomer of perillic acid. The metabolite **6** is present in significant levels in patients' plasma. The metabolite **6** shows similar inhibitory potency against FTase and GGTase type I to perillic acid **(2)** and so may contribute to the *in vivo* antitumour activity of limonene **(1)**.

REFERENCES

1. Crowell PL and Gould MN, Chemoprevention and therapy of cancer by *d*-limonene. *Crit Rev Oncol* **5:** 1–22, 1994.
2. Elegbede JA, Elson CE, Tanner MA, Qureshi A and Gould MN, Regression of rat primary mammary tumors following dietary *d*-limonene. *J Natl Cancer Inst* **76:** 323–325, 1986.
3. Haag JD, Lindstrom MJ and Gould MN, Limonene-induced regression of mammary carcinomas. *Cancer Res* **52:** 4021–4026, 1992.
4. Chander SK, Lansdown AGB, Luqmani YA, Gomm JJ, Coope RC, Gould N and Coombes RC, Effectiveness of combined limonene and 4-hydroxyandrostenedione in the treatment of NMU-induced rat mammary cancers. *Br J Cancer* **69:** 879–882, 1994.
5. Igimi H, Nishimura R, Kodama R and Ide H, Studies on the metabolism of *d*-limonene (*p*-mentha-1,8-diene). I. The absorption, distribution and excretion of *d*-limonene in rats. *Xenobiotica* **4:** 77–82, 1974.
6. Crowell PL, Elson CE, Bailey HH, Elegbede A, Haag JD and Gould MN, Human metabolism of the experimental cancer therapeutic agent *d*-limonene. *Cancer Chemother Pharmacol* **35:** 31–37, 1994.
7. Crowell PL, Lin S, Vedejs E and Gould ML, Identification of metabolites of the antitumor agent *d*-limonene capable of inhibiting protein isoprenylation and cell growth. *Cancer Chemother Pharmacol* **31:** 205–212, 1992.
8. Poon GK, Vigushin D, Griggs LJ, Rowlands MG, Coombes RC and Jarman M, Identification and characterization of limonene metabolites in patients with advanced cancer by liquid chromatography/mass spectrometry. *Drug Metab Dispos* **24:** 565–571, 1996.
9. Haag JD and Gould MN, Mammary carcinoma regression induced by perillyl alcohol a hydroxylated analog of limonene. *Cancer Chemother Pharmacol* **34:** 477–483, 1994.
10. Stark MJ, Burke YD, McKinzie JH, Ayoubi AS and Crowell PL, Chemotherapy of pancreatic cancer with the monoterpene perillyl alcohol. *Cancer Lett* **96:** 15–21, 1995.
11. Ripple G, Gould M, Stewart J, Tutsch K, Alberti D, Feierabend C, Simon K, Arzoomanian R, Pomplun M, Wilding G and Bailey H, Phase I trial of perillyl alcohol administered daily. *Proc Natl Acad Sci USA* **37:** 221, 1997.
12. Committee NDCBaAD, Clinical development plan: *l*-perillyl alcohol. *J Cell Biochem* **26S:** 137–148, 1996.
13. Vigushin DM, Poon GK, Boddy A, English J, Halbert GW, Pagonis C, Jarman M, Coombes RC and Committee CRC-PIICT, Phase I and pharmacokinetic study of *d*-limonene in patients with advanced cancer. *Cancer Chemother Pharmacol* **42:** 111–117, 1998.
14. Hall A, A biochemical function for Ras—at last. *Science* **264:** 1413–1414, 1994.
15. Rodenhuis S, Ras and human tumors. *Semin Cancer Biol* **3:** 241–247, 1992.
16. Gelb MH, Protein prenylation, et cetera: signal transduction in two dimensions. *Science* **275:** 1750–1751, 1997.
17. Graham SL, Inhibitors of protein farnesylation: a new approach to cancer chemotherapy. *Exp Opin Ther Patents* **5:** 1269–1285, 1995.
18. Leonard DM, Ras Farnesyltransferase: a new therapeutic target. *J Med Chem* **40:** 2971–2990, 1997.
19. Crowell PL, Chang RR, Ren Z, Elson CE and Gould MN, Selective inhibition of isoprenylation of 21-26-kDa proteins by the anticarcinogen *d*-limonene and its metabolites. *J Biol Chem* **26:** 17679–17685, 1991.
20. Crowell PL, Ren Z, Lin S, Vedejs E and Gould M, Structure–activity relationships among monoterpene inhibitors of protein isoprenylation and cell proliferation. *Biochem Pharmacol* **47:** 1405–1415, 1994.
21. Gelb MH, Tamanoi F, Yokoyama K, Ghomashchi F and Esson K, The inhibition of protein prenyltransferases by oxygenated metabolites of limonene and perillyl alcohol. *Cancer Lett* **91:** 169–175, 1995.
22. Ren Z, Elson CE and Gould MN, Inhibition of type I and type II geranylgeranyl-protein transferase by the monoterpene perillyl alcohol in NIH3T3 cells. *Biochem Pharmacol* **54:** 113–120, 1997.
23. Clegg RJ, Middleton B, Bell GD and White DA, Inhibition of hepatic cholesterol synthesis and *S*-3-hydroxy-3-methylglutaryl-CoA reductase by mono- and bicyclic monoterpenes administered *in vivo*. *Biochem Pharmacol* **29:** 2125–2127, 1980.
24. Ren Z and Gould MN, Inhibition of ubiquinone and cholesterol synthesis by the monoterpene perillyl alcohol. *Cancer Lett* **76:** 185–190, 1994.
25. Schulz S, Reinhold D, Schmidt H, Ansorge S and Holt V, Perillic acid inhibits Ras/MAP kinase-driven IL-2 production in human T lymphocytes. *Biochem Biophys Res Commun* **241:** 720–725, 1997.
26. Ruch RJ and Sigler K, Growth inhibition of rat liver epithelial tumor cells by monoterpenes does not involve Ras plasma membrane association. *Carcinogenesis* **15:** 787–789, 1994.
27. Mills JJ, Chari RS, Boyer IJ, Gould MN and Jirtle RL, Induction of apoptosis in liver tumors by the monoterpene perillyl alcohol. *Cancer Res* **55:** 979–983, 1995.
28. Harwood HJ, Protein farnesyltransferase: measurement of enzymatic activity in 96-well format using Topcount microplate scintillation counting technology. *Anal Biochem* **226:** 268–278, 1995.
29. Moores SL, Schaber MD, Mosser SD, Rands E, O'Hara MB, Garsky VM, Marshall MS, Pompaliano DL and Gibbs JB, Sequence dependency of protein isoprenylation. *J Biol Chem* **266:** 14603–14610, 1991.
30. Seabra MC, Reis Y, Casey PJ, Brown MS and Goldstein JL, Protein farnesyl transferase and gerenylgeranyltransferase share a common α subunit. *Cell* **65:** 429–434, 1991.
31. Pompliano DL, Rands E, Schaber MD, Mosser SD, Anthony NJ and Gibbs JB, Steady-state kinetic mechanism of ras farnesyl protein transferase. *Biochemistry* **31:** 3800–3807, 1992.
32. Gibbs JB, Pompliano DL, Mosser SD, Rands E, Lingham RB,

Singh SB, Scolnick EM, Kohl NE and Oliff A, Selective inhibition of farnesyl-protein transferase blocks Ras processing *in vivo*. *J Biol Chem* **269:** 7617–7620, 1993.
33. Tamanoi F, Inhibitors of ras farnesyltransferases. *TIBS* **18:** 349–353, 1993.
34. Kohl NE, Wilson FR, Mosser SD, Giuliani E, DeSolms SJ, Conner MW, Anthony NJ, Holtz WJ, Gomez RP, Lee T-J, Smith RL, Graham SL, Hartman GD, Gibbs JB and Oliff A, Protein farnesyltransferase inhibitors block the growth of ras-dependent tumors in nude mice. *Proc Natl Acad Sci USA* **91:** 9141–9145, 1994.
35. Park HW, Boduluri SR, Moomaw JF, Casey PJ and Beese LS, Crystal structure of protein farnesyl transferase at 2.25 Angstrom resolution. *Science* **275:** 1800–1804, 1997.